TAXONOMIC AND GENETIC STUDIES OF

BACILLUS THERMOPHILES

by

RICHARD JAMES SHARP

A thesis submitted to the C.N.A.A. in partial fulfilment for the award of Doctor of Philosophy

Microbial Technology Laboratory
PHLS Centre for Applied Microbiology and Research
Porton, Salisbury, Wiltshire.

Department of Life Sciences
Trent Polytechnic
Burton Street
Nottingham

July 1982

DECLARATION

I declare that while registered as a candidate for the degree of Dr. of Philosophy, I have not been a registered candidate for another award of the C.N.A.A., or of a University, during this research programme. I acknowledge the collaboration of Dr. A. Bingham in the work on bacterial plasmids presented in Section 4. This work was also submitted by A.H.A. Bingham for the award of Doctor of Philosophy to Imperial College, London (1980).

The course of advance studies included:-

1. Attendance 3 hours weekly for 1 year at evening course leading to Fellowship of the Institute of Virology, Salisbury Technical College (1978-1979).

2. Attendance at SGM symposium; 'Relations between structure and function in the procaryote cell' Southampton (1979).

3. Attendance of SGM symposium; "Functional Analysis of Virus genomes", Leeds (1980).

4. 1 day SGM (systematics group) symposium; "Critical surveys of some methods for generating taxonomic data". Q.E.C., London (1980).

5. Attendance at SGM symposium; "Genetics as a Tool in Microbiology". Cambridge (1981).

6. 10 week evening course; 'Computer programming in BASIC'. Salisbury Technical College (1982).

7. 1 day SGM (genetics group) symposium; "DNA recombination and repair". Cambridge (1982).

R. J. Sharp

ABSTRACT

Gibson and Gordon (1974) in the 8th edition of Bergey's manual considered all obligate thermophilic strains of Bacillus belonged to one species, B. stearothermophilus. In this study the biochemical and physiological data from over 100 strains of thermophilic Bacillus was examined by numerical taxonomy. The strains were divided into a total of ten groups, seven of which contained obligately thermophilic strains. The results show general similarity to those reported by Walker and Wolf (1971) and Klaushofer and Hollaus (1970), but also display some significant differences. The three strains, B. caldotenax, B. caldovelox and B. caldolyticus reported by Heinen and Heinen (1972), do not appear to merit the rank of species, and closely resemble the earlier isolate of B. kaustophilus (Prickett, 1928). A new group of thermophilic Bacillus was ;identified in this study and were tentatively assigned the name B. thermotyrovorans. The taxonomic groups established following single linkage analysis were supported by data from bacteriocin typing, pyrolysis mass spectrometry and mole % G+C. The mole % G+C of a number of strains previously considered to be B. stearothermophilus were found to extend over a range of almost 30% and clearly could not be regarded as members of one species.

Thermophilic bacteriophages were isolated by various techniques including lysogenic induction. They were characterised by a number of techniques including, buoyant density estimations, restriction enzyme cleavage of the DNA and examination by electron microscopy. Phage JS 017, which was unusual in having a cylindrical shaped head, was able to cotransduce a methionine and thymine auxotroph of B. caldotenax. Transduction within obligately thermophilic strains of Bacillus has not previously been described.

Dedicated to Sandie, Rebecca and James

CONTENTS

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank my two supervisors, Professor Tony Atkinson and Dr. S. Ahmad for their help, guidance and encouragement through this project.

It is also a pleasure to thank many of my colleagues in CAMR for their help and advice on various aspects of this work, including, Mr. B. Dowsett for preparation of the electron micrographs; Dr. J. R. Woodrow for preparation of computer software; Dr. A. Bingham and Mr. M.J. Munster for many helpful discussions; Mr. C.G.T. Evans for proof reading much of the text; Dr. K. Sargeant for providing time for the work on this project while Head of the Laboratory; Mr. Len Pavey, Mrs B.C. Ling and Miss A.P. Steadman, for carrying out their work with so little help or supervision from me over the past three years.

I would also like to express my gratitude to, Dr. J. Wolf for his encouragement and helpful criticisms, Dr. C. Gutteridge for supplying the pyrolysis mass spectrometry data and Dr. Elizabeth Wellington for her helpful comments on the numerical taxonomy data presented in this thesis.

I am extremely grateful to Miss Val Bowden for the considerable time and trouble she has taken in the typing of this thesis.

Lastly, but by no means least, I would like to thank my wife Sandie for her support and seemingly endless patience over the past five years.

ABSTRACT

Gibson and Gordon (1974) in the 8th edition of Bergey's manual considered all obligate thermophilic strains of Bacillus belonged to one species, B. stearothermophilus. In this study the biochemical and physiological data from over 100 strains of thermophilic Bacillus was examined by numerical taxonomy. The strains were divided into a total of ten groups, seven of which contained obligately thermophilic strains. The results show general similarity to those reported by Walker and Wolf (1971) and Klaushofer and Hollaus (1970), but also display some significant differences. The three strains, B. caldotenax, B. caldovelox and B. caldolyticus reported by Heinen and Heinen (1972), do not appear to merit the rank of species, and closely resemble the earlier isolate of B. kaustophilus (Prickett, 1928). A new group of thermophilic Bacillus was ;identified in this study and were tentatively assigned the name B. thermotyrovorans. The taxonomic groups established following single linkage analysis were supported by data from bacteriocin typing, pyrolysis mass spectrometry and mole % G+C. The mole % G+C of a number of strains previously considered to be B. stearothermophilus were found to extend over a range of almost 30% and clearly could not be regarded as members of one species.

Thermophilic bacteriophages were isolated by various techniques including lysogenic induction. They were characterised by a number of techniques including, buoyant density estimations, restriction enzyme cleavage of the DNA and examination by electron microscopy. Phage JS 017, which was unusual in having a cylindrical shaped head, was able to cotransduce a methionine and thymine auxotroph of B. caldotenax. Transduction within obligately thermophilic strains of Bacillus has not previously been described.

ABBREVIATIONS

ade	adenine marker
arg	arginine marker
Arg	arginine
Asp	aspartate
Asn	asparagine
ATCC	American Type Culture Collection
BBL	Baltimore Biological Labs
BHI	Brain Heart Infusion
DSM	Deutsche Sammlung von Mikroorgnismen
EDTA	ethylenediaminetetraacetic acid
EMS	ethyl methane sulfonate
EP	Experimental Plant (CAMR, Porton)
citK	citric acid cycle marker
deoC	deoxyriboaldolase marker
deoB	deoxyribomutase marker
deoR	thymine metabolism marker
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
d.w.	distilled water
e.o.p.	Efficiency of plating
furF	5-fluorouracil marker
Gln	glutamine
glnA	glutamine marker
Glu	glutamate
h	hours
His	histidine
his	histidine marker
ilv	isoleucine/valine marker
J	Joules
KauA	keto acid uptake marker
LDH	lactate dehydrogenase
lexA	DNA repair marker
Lys	lysine
met	methionine marker
min	minutes
MR	methyl red

NCA	National Canners Association
NCIB	National Collection of Industrial Bacteria
NCTC	National Collection of Type Cultures
OD	Optical Density
OTU	Operational taxonomic units
p.f.u.	plaque forming units
PMFS	phenylmethyl sulfonyl fluoride
PNT	programme for numerical taxonomy
rec	recombination marker
RNA	Ribonucleic acid
RNase	Ribonuclease
r.p.m.	revolutions per minute
sec	seconds
SMS	Stearothermophilus minimal salts
Str^r	Streptomycin resistant
Str^s	Streptomycin sensitive
SSC	Standard saline citrate
TSB	Tryptone Soya Broth
TSBA	Tryptone Soya Broth Agar
thy	thymidylate synthetase marker
Tc^r	Tetracycline resistant
Tlr	low thymine requiring mutant
tmp	trimethoprim resistance marker
u.v.	ultra-violet radiation
uvr	ultra-violet repair marker
VP	Voges-Proskauer
v/v	volume to volume
w/v	weight to volume
YSI	Yellowstone Instruments

CONTENTS

TABLES

FIGURES

PLATES

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1 DISTRIBUTION, ORIGIN AND CLASSIFICATION OF THERMOPHILIC MICROORGANISMS

1.1.1 Distribution of thermophilic microorganisms

The occurrence of heat resistant spores in the mesophile B. subtilis was first reported by Cohn in 1876. Twelve years later Miquel (1888) isolated a thermophilic aerobic sporeformer, able to grow at 73 °C, from the River Seine in France. In the following 60 years numerous thermophilic microorganisms were isolated from samples of water, mud and soil. The majority of the isolates reported during this period were members of the genus Bacillus. They were isolated as spoilage organisms during sugar refining, being able to grow in hot (60 °C) sucrose solutions (Laxa, 1900), in milk pasteurisation during which they were reported to be actually growing (Jacobsen, 1918), and as heat resistant spores resistant to sterilisation in the canning industry. It is from this latter source that many thermophilic strains of Bacillus, held by culture collections, originated. These aerobic thermophilic sporeformers were often given the name "flat sour bacteria" (e.g. ATCC 12016) since, being unable to produce gas from carbohydrates, they did not cause infected cans to swell. Cameron and Esty (1926) at the National Canners Association (NCA), Washington DC, USA, isolated over 200 strains of Bacillus cultures from spoilt cans, of which 150 strains proved to be facultative or obligate thermophiles.

Since these early isolations of thermophilic strains of Bacillus many other species and strains of thermophilic microorganisms have been isolated from a variety of natural and man made environments. These include areas of volcanic activity, boiling and superheated springs, hot pools, glaciers, freshly fallen snow, solar heated soils and ground litter, self heating organic rich materials such as compost heaps, seaweed piles, hay and straw, coal refuse piles, and hot water systems, cooling water systems, steam lines and steam condensate lines.

One of the most closely studied regions and one which has yielded the greatest diversity of thermophilic microorganisms is the Yellowstone National Park, Wyoming, USA. Brock (1978) and Tansey and Brock (1978) reviewed the numerous strains and species isolated at that time. Some examples of these strains, together with others reported more recently, are listed in Table 1.1. Bacteria isolated from

Table 1.1

Thermophilic species of microorganisms

Group, genus or species	Optimum temperature	Maximum temperature	Comments	Ref
Bacillus stearothermophilus	50 - 65	65 - 75		1
Bacillus coagulans	50 - 55	50 - 60	acidophile	1
Bacillus acidocaldarius	60 - 65	65 - 70	acidophile	2
Bacillus caldotenax	60 - 65	85		3
Bacillus caldovelox	60 - 65	76		3
Bacillus caldolyticus	60 - 65	82		3
Bacillus thermodenitrificans	60	-		2
Bacillus schlegelii	70	-		4
Bacillus thermocatenulatus	-	78		5
Bacillus sphaericus	65	65 - 70		6
Bacillus licheniformis	45 - 55	50 - 55		1
Bacillus brevis		40 - 60		1
Chloroflexus aurantiacus	55	70 - 73	photosynthetic	
Chromatium species		57 - 60	photosynthetic	
Clostridium thermo-saccharolyticum	55	67		2
Clostridium thermohydro-sulfuricum		74 - 76	reduces sulphate	2
Clostridium tartarivorum		67		2
Clostridium thermocellum	60	68	cellulose digester	2
Clostridium thermoaceticum	55 - 60	65		2
Desulfovibrio thermophilus	65	85		2
Hydrogenomonas thermophilus	50	60		2
Lactobacillus thermophilus	50 - 63	65		2
Leptospira biflexa		54		2
Methanobacterium thermo-autotrophicum	65 - 70	75		2
Micropolyspora rectivirgula	45 - 55	65		2
Micropolyspora rubrobrunea	45 - 55	65		2

Table 1.1 (Cont'd)

Group, genus or species	Optimum temperature	Maximum temperature	Comments	Ref
Streptococcus thermophiles	40 - 45	50		2
Streptomyces fragmentosporus	50 - 60			2
Sulfolobus acidocaldarius	70 - 75	85 - 90	acidophile	2
Thermoplasma acidophilum	59	65	acidophile	2
Thermomicrobium roseum	70 - 75	85		2
Thermoanaerobacter ethanolicus		78	ethanol producer	
Thermus aquaticus	70	79		2
Thermus thermophilus	70	85		2
Thermus XI	69 - 71			2
Thermoactinomycetes vulgaris	60	70		2
Thermomonospora citrina	55 - 60	70 - 75		2
Thermothrix thiparus	70 - 73	77 - 80	filamentous	2
Thiobacillus thermophilica	55 - 60	80		2

Table 1.1 (Cont'd)

Group, genus or species	Optimum temperature	Maximum temperature	Comments	Ref
CYANOBACTERIA				
Synechococcus lividus	63 - 67	74		2
Synechococcus elongatus		66 - 70		2
Oscillatoria okenii		>60		2
Phormidium laminosum		57 - 60		2
Mastigocladus laminosus		63 - 64		2
EUCARYOTIC ALGAE				
Cyanidum caldarium		55 - 60		2
PROTOZOA				
Cercosulcifer hamathensis	-	57 - 58		2
Vahlkampfia reichi	-	57 - 58		2
Cyclidium citrullus	-	50 - 58		2
FUNGI				
Zygomycetes				
Mucor pusillus	35 - 55	55 - 60		2
Rhizomucor sp.	45 - 53	60 - 61		2
Ascomycetes				
Chaetomium thermophile	50	58 - 61		2
Thermoascus aurantiacus	40 - 46	55 - 62		2
Basidiomycetes				
Coprinus sp.	45	55		2
Deuteromycetes				
Aspergillus candidus	45 - 50	50 - 55		2
Humicol lanuginosa	45 - 50	60		2
Paecilomyces spp. group b	45 - 50	55 - 60		2
Penicillium sp. A	42	55		2
Sporotrichum thermophile	40 - 50	55		2
Thermomyces ibadanensis	42 - 47	60 - 61		2

Legend

References 1, Gibson and Gordon (1974); 2, Brock (1978); 3, Heinen and Heinen (1972); 4, Schenk and Aragno (1979); 5, Golovacheva et al. (1975); 6, Klaushofer and Hollaus (1970).

thermophilic environments include aerobic and anaerobic sporeformers, Gram negative rods, cocci and filamentous strains, photosynthetic bacteria, methane producers, sulphur oxidisers, Mycoplasma, Spirochetes and Actinomycetes. Cyanobacteria are represented by strains of Synechoccus and Oscillatoria. Approximately 67 different varieties of fungi are able to grow at temperatures above 56 °C representing the Zygomycetes, Ascomycetes, Basidiomycetes and Deuteromycetes (Tansey & Brock, 1978). Cyanidium caldarium is the only representative of the eucaryotic algae reported to grow above 50 °C (Doemel & Brock, 1970). It has a maximum growth temperature of 55 - 60 °C and has been classified as a member of the red algae (Chapman, 1974). There are numerous reports of the isolation of protozoa from hot pools and springs, although it is not clear if they complete their life cycle under these conditions or just grow vegetatively. Kahan (1969) isolated four species of protozoa from hot springs able to reproduce between 55 °C and 60 °C. Hindle (1932) isolated an amoeba from a hot spring and successfully cultured it for one year at 53 - 54 °C. One of the more surprising reports of the survival of protozoa at high temperature was made by Dallinger (1887). He claimed to have adapted three species of flagellates to grow at 70 °C by slowly increasing their incubation temperature over the course of seven years.

The presence of many thermophilic strains of aerobic and anaerobic sporeformers in non-thermal environments, e.g. soil and water in temperate regions, is not explained solely on the basis of solar heating or on self heating organic material. Solar heating has been proved sufficient to warm shallow puddles and top soil sufficiently for the growth of thermophiles, but this would be restricted to a few hours daily during the summer months. The maximum temperatures recorded within piles of composting vegetation were between 60 °C and 90 °C (Gaughran, 1947), although this must be regarded as an exceptional environmental condition.

Isolation of thermophiles from soils using complex media at 55 - 60 °C is relatively easy. When these strains are transferred back to ambient temperatures (15 - 25 °C) found in their normal environment growth is not observed (Sharp, this thesis). It appears that generation times at these temperatures must be measured in days and colony formation does not become apparent. Alternatively, laboratory media may not sufficiently simulate normal environmental conditions to

permit growth at low temperature. Golikowa (1926) considered that in the natural environment heat liberated by mesophiles might support the growth of thermophiles in a symbiotic relationship.

1.1.2 Origin of thermophilic microorganisms

The origin of thermophiles has been the source of considerable speculation. Arrhenius (1927) considered the natural habitat of thermophilic bacteria to be the planet Venus "where the average temperature is 47 - 50 ^{o}C". The organisms, or their spores, were believed to have travelled to earth within a few days, propelled by the radiation pressure of the sun. Studies of molecular structure and protein sequencing however, suggest a common origin for mesophiles and thermophiles. Kluyer and Baars (1932) considered thermophiles to originate as a result of spontaneous mutation in mesophilic microorganisms. This seems highly improbable, since a single mutation event may alter the thermal stability of one protein, but it would require numerous mutations to convert most, if not all, of the cell proteins to thermostability. Conversion of a strain by a single mutation from mesophily to thermophily implies the presence of a thermophilic factor converting or stabilising most enzymes and cell structures. Such a factor has not been isolated (Section 1.2.7).

The theory receiving greatest support (Golikowa, 1926; Gaughran, 1947; Brock, 1967) considers mesophilic microorganisms evolving from thermophilic microorganisms. Assuming an anaerobic and hot environment (Ljungdahl, 1979), it appears that when life forms began to develop, anaerobes and thermophiles preceded mesophiles and aerobes. Brock (1967) notes the resemblance between fossil microorganisms in ancient rocks (2×10^{9} years old) and some of the Flexibacteriales common in hot water pools today.

1.1.3 Classification of thermophilic microorganisms

Various schemes have been proposed to classify thermophilic microorganisms based on the maximum, minimum and optimum growth temperatures, a number of which are outlined in Table 1.2. Any classification based merely on growth temperatures is incomplete and is largely prescribed by the interests or experience of the individual worker. Related strains may fall on the border line of two groups. There have been reports of adaptation of microorganisms to grow at higher maximum temperatures (Haberstich & Zuber, 1974) thus resulting in a strain from one category moving to another. In this study the term "thermophilic" has been used to describe any strain with a maximum growth temperature of 60 ^{o}C or above.

Table 1.2

Classification schemes devised to define microorganisms based on their maximum, minimum and optimum growth temperatures

Reference	Designation of Group	Definition
Miehe (1907)	Thermophile	Tmin >25 °C
	Orthothermophile	Tmax 50 - 70 °C
	Thermotolerants	Tmax 50 - 55 °C
Cameron and Esty (1926)	Facultative thermophiles	Growth at 37 °C and 55 °C
	Obligate thermophiles	Growth at 55 °C but not at 37 °C
Robertson (1927)	Thermoduric mesophiles	Growth between 20 °C and 57 °C and survive pasteurisation
Imsenechi and Solnzeva (1945)	True thermophiles	Topt 55 - 60 °C
	Steno thermophiles	Tmin >28 °C Tmax >60 °C
	Eurithermal thermophiles	Growth at 60 °C and at 28 - 30 °C
Gyllenberg (1951)	Mesophiles	Topt 20 - 50 °C growth at 28 - 30 °C no growth at 50 °C
	Thermotolerant mesophiles	Topt 20 - 50 °C growth at 28 - 30 °C and 50 °C
	Eurithermal thermophiles	Topt >50 °C growth at 28 - 30 °C and 50 °C
	Stenothermal thermophiles	Topt >50 °C no growth at 28 - 30 °C, growth at 50 °C
Farrell and Campbell (1969)	Strict or obligate thermophiles	Topt 65 - 70 °C Tmin >40 - 42 °C
	Facultative thermophiles	Tmax 50 - 65 °C, Tmin, room temperature
	Thermotolerant organisms	Tmax 45 - 50 °C
Williams (1975)	Caldoactive bacteria	Tmax >90 °C, Topt 65 °C, Tmin >40 °C
	Thermophilic bacteria	Tmax >60 °C, Topt 50 °C, Tmin 30 °C
Ljungdahl (1979)	Extreme thermophile	Tmax >90 °C, Topt 65 °C, Tmin 40 °C

1.2 THE BIOCHEMICAL, PHYSIOLOGICAL AND GENETIC BASIS OF THERMOPHILY

1.2.1 Introduction

The ability of microorganisms to not only survive, but actively grow and multiply at temperatures which normally denature proteins and particularly enzymes, has led to considerable interest in the molecular basis of thermophily.

Several recent reviews discussed the stability of proteins and other cellular constituents at elevated temperatures (Singleton & Amelunxen, 1973; Williams, 1975; Ljungdahl & Sherod, 1976; Singleton, 1976; Welker, 1976; Amelunxen & Murdock, 1978; Zuber, 1978; Shilo, 1979; Zeikus, 1979).

Much of the work which has been carried out has involved the comparison of proteins, either from related species, from mesophilic or thermophilic strains of the same species, or from one strain which is able, either inherently or by adaptation to grow at both mesophilic and thermophilic temperatures.

1.2.2 Stability of thermophilic proteins

Militzer et al. (1949) examined the thermal stability of malate dehydrogenase in extracts from an obligate Bacillus thermophile grown at 65 °C and from the mesophile, B. subtilis. The enzyme from the thermophile was stable for 120 min at 65 °C, whereas that from B. subtilis was rapidly inactivated. Amelunxen and Lins (1968) compared the thermostability of 11 enzymes in extracts from B. stearothermophilus and from the mesophile B. cereus. Nine of the enzymes from the former were more thermostable than the corresponding enzymes from B. cereus. However, pyruvate kinase and glutamate oxaloacetate transaminase from thermophilic and mesophilic strains showed similar rates of inactivation at 70 °C.

Singleton and Amelunxen (1973) considered three distinct mechanisms to account for the activity of thermophilic microorganisms:

i. Thermophiles may contain factors which increase the thermal stability of enzymes.

ii. Conversely, mesophiles may contain factors which increase the thermolability of their enzymes.

iii. Cellular components of thermophiles may have inherent stability independent of exogenous factors.

Attempts to transfer heat stability and heat lability by mixing cell-free extracts from thermophiles and mesophiles failed to change the original properties of individual enzymes (Amelunxen & Lins, 1968; Koffler & Gale, 1957; Koffler, 1957). These studies did not, however, take into account the possible presence of stabilising factors tightly bound to the enzyme and not readily transferable. Amelunxen and Murdock (1978) point out that thermal stability persists even after extensive purification and recrystallization of certain enzymes. Also, since molecular weights of thermostable enzymes and their homologous enzymes from mesophiles are essentially identical, any stabilising factors present would be of very low molecular weight.

Some enzymes from thermophiles proved unstable at the optimum growth temperature of the organism. These enzymes may be stabilised by normal cell constituents such as membranes, cofactors and substrates. Welker (1976) reported the loss of thermal stability of alkaline phosphatase from B. stearothermophilus when released from protoplasts, and attributed the stabilisation of the enzyme to the cell membrane. Glutamine synthetase from B. stearothermophilus is stabilised by:

i. chemical binding of L-glutamate and NH_4^+, or L-glutamate, Mn^{2+} and ATP;

ii. changes in protein structure by increases in polar amino acids and probably by formation of disulphide bridges;

iii. enzyme aggregation driven by energy released from hydrophobic interactions (Wedler, 1978).

Calcium ions confer stability on several extracellular enzymes such as α-amylase (Yutani, 1976), thermolysin (Tajima et al., 1976) and proteinases (Sidler & Zuber, 1977). Cobalt ions are involved in the thermal stability of aminopeptidase 1 from B. stearothermophilus NCIB 8924 (Deranleau & Zuber, 1977).

The stabilisation of proteins by aggregation has been suggested by Wedler and Hoffman (1974). Amelunxen and Singleton (1976) demonstrated thermolabile glycerophosphate dehydrogenase in the facultative thermophile B. coagulans. The enzyme was stabilised only at a critical ionic strength by the addition of salt. They attributed the stabilisation to a highly charged intracellular macromolecular environment.

The rapid resynthesis of macromolecules by thermophiles to counteract thermal inactivation has been suggested, but evidence for

this is insufficient. Allen (1953) reviews some of the earlier work relating to this hypothesis and Koffler (1957) has pointed out that, assuming a doubling in the rate of enzyme reactions for each 10 °C rise in temperature, thermophiles should exhibit 16 times more activity at 70 °C than at 30 °C.

The only thermostable enzyme reported to differ considerably from its mesophilic counterpart was an α-amylase from a strain of B. stearothermophilus (Manning & Campbell, 1961). It exhibited a semi-random coil configuration existing in a semi-denatured state. The enzyme had a molecular weight of 15,600 (well below that of other amylases), contained four cysteine residues and was stabilised by one disulphide bridge. This enzyme appears to be an exception and doubts on the validity of these findings have been expressed by one of the authors (Campbell, pers. comm.). Other α-amylases isolated from B. stearothermophilus have molecular weights in the region of 50,000 and contain fewer cysteine residues (Pfueller & Elliot, 1969; Isono, 1970).

Small conformational changes occur without denaturation in some thermophile proteins when heated from room temperature to 55 °C or 60 °C. This is in contrast to most mesophile proteins which are denatured at this temperature. Magsanaga and Nosoh (1974) demonstrated a conformational change in the glutamine synthetase of B. stearothermophilus at well below the temperature of enzyme inactivation; at 55 °C the enzyme became susceptible to thermolysin digestion

Almost all in vitro studies on the thermal stability of proteins, suggest some inherent stability of the protein structure which is dependent upon the amino acid sequence and composition. Several proteins from thermophiles and mesophiles have proved similar in molecular weight and in structure and differences were associated with a very small number of amino acids. Alterations in a small number of amino acids in the polypeptide chain may result in changes affecting the stability of the secondary and tertiary structures of the molecule. These changes may involve formation of disulphide bridges, increased hydrogen bonding, increases in hydrophobic bonds and ionised group interactions.

The number of disulphide bridges does not correlate well with enzyme thermal stability. The high thermal stability of ribonuclease has been attributed to the presence of four disulphide bonds

(Spackman et al., 1960; Smith et al., 1963); however, data from most thermophilic enzymes suggests the occurrence of fewer cysteine residues than in their mesophilic counterparts.

Several apparently homologous proteins have now been isolated from a variety of organisms and their amino acid sequence determined enabling comparative studies of the amino acid sequences of enzymes from thermophiles and mesophiles and also higher organisms.

Examination of ferredoxins from the thermophiles Clostridium tartarivorum and C. thermosaccharolyticum and the mesophiles C. pasteurianum, C. acidurici and C. butyricum (Tanaka et al., 1971; Devanathan et al., 1969) indicated similarities in their molecular weights, iron, inorganic sulphide and cysteine content, adsorption spectra and number of amino acid residues. The proteins from the thermophiles, however, showed greater thermal stability. Since ferredoxin is a small protein (molecular weight 6,000) having no secondary or tertiary structure, the increased stability is presumably associated with changes in the amino acid composition. Comparison of sequence data indicated that the thermophile ferredoxins were the only ones to contain histidine which replaced either serine or tyrosine (Table 1.3). These may therefore serve as ligands for the tighter binding of atoms of iron or the differences in charge may increase the opportunities for hydrogen bonding. There is also some evidence for a greater number of basic amino acids in protein from thermophiles. Perutz and Raidt (1975) suggested this might permit the formation of additional ionic bonds. In the thermophile ferredoxins a maximum number of four ionic bonds are formed in C. thermosaccharolyticum and only one in the mesophile C. acidurici. Increased thermal stability also correlates with an increase in the number of glutamic acid residues; since C. pasteurianum and C. acidurici contain a total of two each, while the thermophile C. tartarivorum contains five and C. thermosaccharolyticum contains seven residues.

Hase et al. (1976) reported a total of six glutamic acid residues in ferredoxins from B. stearothermophilus. Robson and Pain (1971) pointed out that glutamic acid residues are the best α-helix promoting amino acid. The ferredoxin from B. stearothermophilus also had a lower number of cysteine residues than other ferredoxins examined.

Arginine content, particularly in lactate dehydrogenase (Frank et al., 1975), appears to increase in thermophile proteins (Ljungdahl et al., 1976; O'Brien et al., 1976) at the expense of lysine. In

Table 1.3

Mesophilic and thermophilic ferredoxins, differences in basic and acidic amino acid residues and possible ionic bond formation

	Basic residues			Acidic residues				Possible salt bridges*		
	His	Lys	Arg	Glu	Gln	Asp	Asn	(1)	(2)	(3)
C. pasteurianum	0	1	0	2	2	5	3	1	0	0
C. acidurici	0	0	1	2	2	6	1	1	1	1
C. tartarivorum	2	2	0	5	2	4	0	4	2	2
C. thermosaccharolyticum	2	2	0	7	0	4	0	4	4	4

*Salt bridges (1) Based on amino acid composition.

(2) Based on amino acid sequence data.

(3) Based on three dimensional structure data.

(Data from Zuber, 1978)

contrast to the ferredoxins, the overall number of basic amino acids in lactate dehydrogenase remains constant, although the incorporation of arginine at specific points at the expense of lysine in other positions, results in greater thermal stability.

Examination of rhodanase from Thiobacillus denitrificans (molecular weight 15,000) indicated the half-life of the enzyme to be 0.5 min at 65 °C; from B. subtilis the half-life was 4.5 min and from B. stearothermophilus 36 min. The only significant changes in amino acid composition were in the aspartate: glutamate ratio, from 8:2 in T. denitrificans, 5:5 in B. subtilis and 1:10 in B. stearothermophilus (Atkinson, 1976). No primary sequence data are available on this enzyme, although it is interesting to note that a change from aspartate to glutamate requires only a single base change in the third base of the triplet code.

Frank et al. (1975) studied the production of thermostable enzymes from B. stearothermophilus (NCA 1503) and B. caldotenax at mesophilic and thermophilic temperatures. Both "obligate" thermophiles were adapted to grow at 30 °C following cultivation via an intermediate temperature (46 °C). At temperatures between 37 - 50 °C thermolabile glucose-6-phosphate isomerase was produced, whereas above 60 °C the enzyme became thermostable. They postulated three mechanisms by which the enzyme variants might arise.

i. The two enzymes were derived from two different structural genes which were repressed or de-repressed as a function of the growth temperature.

ii. Enzyme adaptation occurred in a special phase of protein synthesis.

iii. The newly synthesised enzymes were modified by enzymatically catalysed reactions.

Frank et al. (1975) also studied the properties and structure of lactate dehydrogenase (LDH) from cells grown at 37 °C and 55 - 70 °C. The former mesophilic enzyme from B. caldotenax was heat labile and could not be isolated in the same form; however, the mesophilic enzyme from B. stearothermophilus and the two thermophilic enzymes from B. stearothermophilus and B. caldotenax were studied. The first 41 amino acid residues from the N-terminal end of the protein indicated differences in seven amino acid residues. The first 41 amino acid residues from the N-terminal end of the protein were examined. The two thermophilic enzymes were 86% homologous, indicating a close

phylogenetic relationship between B. stearothermophilus NCA 1503 and B. caldotenax (comparative studies with LDH from dogfish indicated only 32% sequence homology in the first 41 amino acid residues). Comparative studies between the mesophilic and thermophilic LDH from B. stearothermophilus showed 55% sequence homology. They also observed methionine at the end of the N-terminal sequence of many thermophilic enzymes.

To increase the melting temperature of a polypeptide chain (molecular weight 35,000 Daltons) from 35 °C - 45 °C requires only a 3% increase in hydrogen bonding and up to 10 kcal/mole differences in activation energy can be obtained by changes in one or two amino acid residues (Atkinson, 1976). A thirty fold difference in thermal stability at 60 °C of triosephosphate isomerase from rabbit muscle and B. stearothermophilus can, therefore, be accounted for by a difference in activation energy of only 2.2 kcal/mole (Hocking & Harris, 1976); similarly, the differences in the thermal stability of the ferredoxins could be due to differences of only 4.5 to 8.5 kcal/mole. Studies on the inactivation of Haemoglobin A and an abnormal Haemoglobin A2 indicated that the latter had a six fold greater thermal stability at 45 °C. The abnormal protein had only ten amino acid changes out of a total of 670 amino acid residues. Three-dimensional studies indicated that only three of these contributed to increased interactions in the form of two non-polar interactions and one hydrogen bond (Perutz & Raidt, 1975).

Present evidence indicates and emphasizes the close structural similarity of proteins from mesophiles and thermophiles. Differences are subtle and appear to involve a small but significant number of amino acid residues. These amino acid substitutions are responsible for changes in hydrogen bonding, hydrophobic bonding and ionic bonding. Slight changes in bonding within the protein molecule appear to account substantially, if not entirely, for increases in the thermal stability of proteins in thermophiles.

1.2.3 The contribution of the cell wall to thermal stability of the organism

There is little evidence that the cell wall plays any significant role in thermal stability. Forrester and Wicken (1966) studied the walls of B. coagulans and B. stearothermophilus grown at 37 °C and 55 °C. At the higher temperature there was an increase in the proportion of mucopeptide and a decrease in the proportion of teichoic acid in the walls of both organisms. Each had a higher wall lipid content than is

usually found in Gram-positive organisms.

Novitsky et al. (1974) compared the amino acid and amino sugar composition of the wall peptidoglycan of a facultative thermophilic strain of B. coagulans grown at 37 °C and 55 °C. At 55 °C, all of the wall components, except alanine, were present in a higher proportion, the cells also showed a lower level of amylolytic activity, bound more Mg^{++} and contained less peptide cross bridging.

1.2.4 The contribution of the cell membrane to thermal stability of the organism

Heilbrun (1924) and Belehradek (1931) observed that membrane lipids of thermophiles had higher melting points than those of mesophiles. They suggested that the melting temperature of cell lipids might represent the upper temperature limit for cell growth. Examination of mesophilic and thermophilic strains of Bacillus sp. indicated that the cell membranes of thermophiles generally contained a higher content of saturated and branched chain fatty acids (Cho & Salton, 1964; Shen et al., 1970). Examination of facultative and obligate thermophilic strains (Ray et al., 1971; Weerkamp & MacElroy, 1972) indicated that increasing the growth temperature resulted in a change in membrane lipid composition to those with a higher melting point. An increase in growth temperature normally results in the production of lipids containing a relatively lower proportion of straight-chain saturated fatty acids with greater than average chain length (McElhany, 1976).

Daron (1970) reported a three to four fold increase in branched chain fatty acids when the growth temperature of B. stearothermophilus (NRS 1511) was increased from 40 - 60 °C. Heinen et al. (1970) found the level of the iso-C_{17} branched chain fatty acid, in the membrane of Thermus aquaticus, increased from 30 - 50% when the growth temperature was raised from 50 - 80 °C. Conversely the level of the iso-C16 component of the total fatty acid fraction was reduced from 30 - 10%.

Comparison of wild type cells of B. stearothermophilus grown at 42 °C and 65 °C indicated an increase of 27% in fatty acids at the latter temperature, with melting points above 55 °C (Souza et al., 1974). A temperature sensitive mutant failed to make similar changes in its fatty acid composition above 52 °C. The authors proposed that the lowest and highest boundary temperatures for the growth of thermophiles were dependent on specific lipid mixtures synthesised by

the organism.

Most cell lipids are known to undergo phase transitions and phase separations (Lee, 1977). The temperature at which these occur depends upon the chemical composition of the phospholipids, such as the length of fatty acid chains, the degree of unsaturation, the presence of methyl or cyclopropyl sidechains and the charge and size of the head group; the presence of neutral lipids such as cholesterol, and the presence of proteins and ions would also have an effect.

McElhany (1976) found that cells of Acholeplasma laidlawii could grow within, as well as above, the temperature range of phase transition. Esser (1978) considered that phase separations and phase transitions might serve as a means of amplifying small signals into having a large effect on the membrane and may be determining factors in the outcome of immunological reactions expressed at the cell surface. Esser (1978), using freeze etching and fluorescent label spectroscopy, concluded that B. stearothermophilus was able to grow at a temperature when most, if not all, of the lipid hydrocarbon chains were in the fluid state. A further increase in temperature, above the lipid melting point, resulted in the weakening of the various physical interactive forces still present (Van der Waal's forces, for example), resulting in randomization of the membrane components and inability to function.

The regulatory mechanism for this control in bacteria has not been fully elucidated although studies on Tetrahymena pyriformis suggested that 'fatty acid desaturase', a membrane bound enzyme may be functioning as a thermometric regulator (Thompson & Nozawa, 1977).

A study of the protein content of the cell membrane reveals an increase in the level of membrane protein with an increase in growth temperature (Wisdon & Welker, 1973); thus, protoplasts of B. stearothermophilus showed an increase in the ratio of protein to lipid as the growth temperature was increased. This may in part explain the thermal stabilisation of certain enzymes by association with the cell membrane.

1.2.5 DNA from thermophilic microorganisms

Comparisons of melting temperatures (Tm) of DNA isolated from thermophilic strains of Bacillus and from mesophiles, for example E. coli, indicate no apparent correlation between Tm values and the ability to grow at higher temperatures (Marmur, 1960; Welker &

Campbell, 1965; Saunders & Campbell, 1966). Stenesh et al. (1968) found that DNA from the thermophiles had a consistently higher guanine and cytosine (G+C) content (53% compared with 45% for the mesophiles). DNA with a higher G+C content is more stable at higher temperatures, since guanine and cytosine co-bond via three hydrogen bonds, while adenine and thymine co-bond via only two. The extreme thermophiles B. caldovelox and B. caldotenax (Sharp et al., 1980) and B. acidocaldarius (Gibson & Gordon, 1974) have G+C contents in the region of 60 - 65%. Different strains of B. stearothermophilus have a G+C content that varies between 49 - 53% and 44 - 46% whilst B. coagulans is in the range of 47 - 48% (Gibson & Gordon, 1974). Table 1.4 compares the optimum growth temperature and the moles % G+C content in various Bacillus species. Although in general this indicates higher G+C values with increasing growth temperature, there is no direct evidence for a relationship between increased DNA thermostability and the ability to grow at higher temperatures.

Stenesh (1976) reported studies on the fidelity of DNA replication in B. licheniformis and B. stearothermophilus at a range of temperatures between 37 ^{o}C and 72 ^{o}C. Using nearest neighbour frequency analysis, Stenesh and Roe (1972) found that the differences were related to increase in temperature. These were more pronounced in B. stearothermophilus and particularly in adenine-thymine dinucleotides where bases are bonded by only two hydrogen bonds.

Atkinson (pers. comm.) found that raising the growth temperature of B. stearothermophilus NCA 1503 increased significantly the mutation frequency of several genes. The terminal methionyl transferase involved in methionine biosynthesis is a temperature sensitive protein such that B. stearothermophilus NCA 1503 becomes auxotrophic for methionine only above 53 ^{o}C. Segregation and reversion frequencies at higher growth temperatures are in the region of 10^{-4}. This is one order of magnitude higher than that found for "hot spots" in genes from mesophilic organisms.

1.2.6 RNA from thermophilic microorganisms

Ribosomal RNA from thermophiles appears to have a higher G+C content than that from mesophiles. Pace and Campbell (1967) studied ribosomes from 19 different microorganisms whose optimum growth temperatures ranged from 20 - 70 ^{o}C. There was a general increase in G+C content with increasing growth temperature. Irwin et al. (1973) investigated the ribosomes and ribosomal RNA from psychrophilic,

Table 1.4

A comparison of the % G+C content and optimum growth temperatures in representatives of some Bacillus species

Species	Maximum growth temperature (°C)	G+C content	References
B. thermocatenulatus	-	69	Golovacheva et al. (1975)
B. thermoruber	-	67	Guicciardi et al. (1968)
B. schlegelii	70 opt.	67 - 68	Schenk and Aragno (1979)
B. caldovelox	76	65.1	Sharp et al. (1980)
B. caldotenax	85	64.8	Sharp et al. (1980)
B. acidocaldarius	65 - 70	61 - 62	Darland and Brock (1971)
B. stearothermophilus	65 - 75	44 - 46	Gibson and Gordon (1974)
		49 - 53	
B. coagulans	50 - 60	37 - 48	Belly and Brock (1974)
		47 - 48	Gibson and Gordon (1974)
B. macerans	40 - 50	49 - 51)	
B. licheniformis	50 - 55	43 - 47)	
B. polymyxa	35 - 45	43 - 46)	
B. circulans	35 - 50	35, 47)	
B. subtilis	45 - 55	42 - 43)	
B. pasteurii	33 - 42	42)	Gibson and Gordon (1974)
B. firmus	40 - 45	41)	
B. sphaericus	30 - 45	37, 43)	
B. megaterium	35 - 45	36 - 38)	
B. cereus	35 - 45	32 - 33)	
		33 - 37)	
B. globisporus	25 - 30	36.9 - 39.5	Ruger and Richter (1979)
B. insolitus	25	41.0	Ruger and Richter (1979)

mesophilic and thermophilic Clostridium spp. The thermal melting temperatures (Tm) of ribosomes were 64.4°C, 63.8°C and 68.7 - 59.6 °C respectively, and Tm values of ribosomal RNA were similar for all three groups, 66.2 - 67.9 °C. Stenesh et al. (1968) reported that the total RNA from thermophiles had a G+C content of 61.4% compared with 56.9% from mesophilic strains. These values are higher than those for ribosomal RNA, indicating transfer RNA (tRNA) to be more stable than ribosomal RNA.

Studies by Friedman (1978) on the heat stability of ribosomes suggested a more orderly conformation in ribosomes from B. stearothermophilus than from E. coli, which may contribute to increased thermal stability. Agris et al. (1973) reported that tRNA from B. stearothermophilus grown at 70 °C had 1.4 times more methyl groups than the tRNA from cells grown at 50 °C. This did not result in any increase in bonding since the thermal melting profiles were similar. Saunders and Campbell (1966) confirmed that the base composition of messenger RNA from B. stearothermophilus was almost identical to that of its DNA.

Studies on translation of the genetic code (Arca et al., 1965) showed the mischarging of isoleucyl tRNA with valine by isoleucyl tRNA synthetase from B. stearothermophilus to occur at temperatures of 70 - 75 °C but not at 50 - 60 °C.

The incorporation of one amino acid in response to the codon for an alternative has been studied by Friedman and Weinsten (1964) in B. stearothermophilus. They found that this type of error in translation (termed 'ambiguity') is affected by increased temperature or ionic composition during amino acid incorporation into the growing polypeptide chain. In most cases the amino acids involved were those in which the codons contained at least two bases that were present in the copolymer used as a template. The results of Stenesh (1976) implicate the ribosome as the main cause of ambiguity in B. stearothermophilus. Schlanger and Friedman (1973) and Gorini (1971) suggest the accuracy of translation is controlled by the ribosome, involving the presence of a tRNA screening site on the ribosome, which interacts with a portion of the tRNA molecule preventing the incorporation of particular amino acids into the polypeptide.

Studies by Stenesh (1976) into the degeneracy of the genetic code and the possibility that thermophiles and mesophiles differ in their relative utilisation of synonym codons suggested a preference for

certain synonym codons to be a function of incubation temperature.

Current work in the UK by Barker (pers. comm.) (Laboratory of Molecular Biology, Cambridge) may enable comparative studies of codon utilisation in E. coli and B. stearothermophilus using the tyrosyl-tRNA synthetase gene. The two enzymes show greater than 70% amino acid homology. Barker has cloned the tyrosyl-tRNA synthetase gene from B. stearothermophilus into a temperature sensitive E. coli mutant and is currently sequencing the DNA. Winter (pers. comm.) has sequenced the E. coli tyrosyl-tRNA synthetase gene. Examination of the respective DNA sequences will enable the examination of codon utilisation for amino acid incorporation into the two homologous enzymes.

1.2.7 The genetic basis of thermophily

Since genetic transfer in thermophilic species of Bacillus is not yet established, studies into the genetic basis of thermophily have been restricted to the use of the B. subtilis transformation system developed by Spizizen (1958).

McDonald and Matney (1963) reported the transformation of streptomycin resistant B. subtilis 168 unable to grow at 55 °C. The transformants, which arose at a frequency of 10^{-4}, grew at 55 °C, but only 10 - 20% of these high temperature transformants still retained their high level resistance to streptomycin. Lindsay and Creaser (1975) using DNA from B. caldolyticus transformed B. subtilis to grow at 70 °C. They also observed the cotransference of genes mapping close to those coding for ribosomal and tRNA functions. Studies of the enzyme L-histidinol dehydrogenase indicated that the transformed cells possessed a thermostable enzyme similar to that present in B. caldolyticus. They suggested that changes in the synthesis of proteins by thermophiles at the ribosomal or tRNA level give rise to translationally altered enzymes which can function at higher temperatures. Friedman and Mojica-a (1978) similarly reported the transformation of B. subtilis with DNA from streptomycin resistant B. caldolyticus. Their work implies the transfer of genes coding for ribosomal proteins from donor to recipient during transformation to thermophily.

Studies on homologous enzymes from mesophiles and thermophiles show small changes in amino acid sequences which can result in increases in thermal stability through hydrogen bonding, hydrophobic bonding and ionic bonding. It yet remains to be seen if these small, but

significant, amino acid differences are determined by the genetic code or by changes at the level of translation; or whether the genes for all thermophile proteins differ from those of complementary mesophile proteins in having a small number of different base triplets in the code.

The work of McDonald and Matney (1963), Lindsay and Creaser (1975) and Friedman (1978) suggests that only a small number of genes are involved in determining the thermophily of an organism, and these operate at the level of translation. This would involve the controlled mischarging of tRNA molecules resulting in selection of particular amino acids in preference to others. Alternatively, the ribosome may possess a site which enables selective screening of incoming tRNA molecules, or might fail to recognise certain tRNA anticodons.

Further comparative studies into the role of ribosomal proteins, hybridisation of DNA and messenger RNA from thermophiles and mesophiles, together with studies of genetic transfer between thermophiles and mesophiles should throw additional light on the genetic basis of thermophily.

1.3 TAXONOMY OF THE BACILLUS THERMOPHILES

1.3.1 Introduction

The taxonomy of Bacillus thermophiles has recently been extensively reviewed by Wolf and Sharp (1981), highlighting the major contributions of a number of researchers (Gyllenberg, 1951; Stark & Tetrault, 1952; Smith et al., 1952; Allen, 1953; Golovacheva, 1965; Klaushofer & Hollaus, 1970; Walker & Wolf, 1971) to the development of an orderly taxonomic system for this group of organisms.

Numerous isolations of thermophilic strains of Bacillus have been reported over the past 70 years. The 6th edition of Bergey's Manual (Breed et al., 1948) characterises 18 species of Bacillus able to grow at or above 60 °C, and lists more than 30 others which were not individually characterised due to the lack of published data. The earlier reports described the isolation of Denitrobacterium thermophilum (Ambroz, 1913), B. coagulans (Hammer, 1915), B. stearothermophilus (Donk, 1920), B. kaustophilus (Prickett, 1928), B. thermomylolyticus (Coolhass, 1928) B. calidolactis (Hussong & Hammer, 1928), B. thermoacidurans (Berry, 1933) and B. thermoacidificans (Renco, 1942) and many others.

The first systematic study of aerobic thermophilic sporeformers was made by Gordon and Smith (1949) in which they examined 216 cultures from a variety of sources. Fifty-six were considered to belong to the mesophilic species of B. subtilis, B. circulans, B. macerans, B. pumilus, B. cereus, B. brevis and B. sphaericus; the remaining strains were considered to be strains of B. stearothermophilus (87 strains) or B. coagulans (73). B. kaustophilus was considered to be similar to B. stearothermophilus and did not merit species status. The position of five strains of B. calidolactis was left in doubt since, of the five cultures examined, two closely resembled B. stearothermophilus and three resembled B. coagulans. Isolates previously named B. thermoacidificans and B. thermoacidurans were considered to be strains of B. coagulans. They concluded that some of the major characteristics of B. stearothermophilus were, good growth at 50 - 65 °C, no growth at 28 °C and variable growth at 37 °C and 70 °C. Starch hydrolysis was positive, growth in 3% (w/v) NaCl broth was scant or negative, hydrolysis of gelatin was positive (weakly positive in seven strains), hydrolysis of casein was variable and reduction of nitrate to nitrite was positive (negative in ten strains). Spores were variable in size, oval,

terminal to sub-terminal and the spore walls were thick and stainable.

Gordon and Smith (1949) considered that new species might be found following the examination of more strains in which case modifications to their classification would be required.

The extensive investigation by Gordon and Smith (1949) and Smith et al. (1952) resulted in the 7th edition of Bergey's manual (Breed et al., 1957) listing only two strains with optimum growth at 55 °C or above. These strains were B. coagulans and B. stearothermophilus the type strain of the latter being the organism isolated by Donk (1920).

Gyllenberg (1951) studied 22 Bacillus thermophiles, 15 of which grew at 65 °C. Eleven of these produced swollen sporangia with oval to cylindrical spores. Gelatin and starch hydrolysis, acetoin production and nitrate production were variable.

Stark and Tetrault (1952) examined 34 Bacillus thermophiles isolated from soil. They carried out a number of tests at 55 °C and 65 °C and compared their results with those of Gordon and Smith (1949), Prickett (1928) and Donk (1920). They considered their strains most resembled the description of B. kaustophilus by Prickett (1928).

Allen (1953) classified 105 thermophilic isolates of Bacillus into four groups, all of which grew at 65 °C:

Group 1. The sporangia were swollen with oval, terminal spores. The minimum growth temperature was 35 °C and the maximum 65 - 70 °C. Growth was present at pH 6 but not at pH 5 and starch, casein and gelatin were all hydrolysed by most strains. Some strains reduced nitrate to nitrite, acetoin was not produced. Four strains in this group grew anaerobically in glucose media and utilised citrate. Growth on plates was described as thin, translucent and spreading. Comparative studies with a strain of B. stearothermophilus from the collection of Gordon and Smith led Allen to conclude that the cultures in this group resembled B. stearothermophilus (Donk, 1920).

Group 2. Spores were oval, terminal or sub-terminal with slightly swollen sporangia. The minimum growth temperature was 35 °C and the maximum 60 - 65 °C. All strains grew at pH 5.0 and most produced acetoin. Starch was hydrolysed although gelatin and casein were not, and citrate was not utilised. Many of the isolates were considered to be typical strains of B. coagulans (Hammer, 1915). A minimum growth temperature of 35 °C however is atypical for B. coagulans; Gordon et al. (1973) reported a minimum temperature between 15 °C and 25 °C.

Group 3. Spores were terminal, oval to cylindrical but did not swell the sporangia. Starch, gelatin and casein were hydrolysed, acetoin was produced and citrate utilised. Nitrate was reduced to nitrite and in some instances, to gas. The growth temperature appeared to vary with the temperature of isolation; strains isolated at 55 °C grew from 55 - 60 °C down to room temperature; strains isolated at 65 °C often had a minimum growth temperature of 40 - 45 °C and a maximum of 65 - 70 °C. Following several transfers at 55 °C the latter group was also found to grow at room temperature. This group was considered to resemble B. subtilis.

Group 4. Spores were round, terminal or sub-terminal with swollen sporangia. Nitrate was not reduced and acetoin was not produced. Some strains utilised citrate; gelatin and casein were hydrolysed although starch was not. Growth appeared to be best in slightly alkaline media. With the exception of the temperature range these strains most closely resembled the description of B. sphaericus.

Grinsted and Clegg (1955) reported the isolation of 36 thermophilic Bacillus isolates from milk. Three of their strains did not hydrolyse starch and two others were considered doubtful. None of the strains produced acetoin or utilised citrate, casein hydrolysis was variable. In litmus milk all the cultures produced a firm acid clot. None of the isolates grew below 37 °C and all but one grew at 65 °C. They did not consider their isolates to resemble either B. coagulans or B. stearothermophilus as defined by Gordon and Smith (1949) and classified them as strains of B. calidolactis (Hussong & Hammer, 1928). B. coagulans differs from B. calidolactis in the Voges-Proskauer reaction and growth temperature; B. stearothermophilus differed in gelatin hydrolysis and in the swelling of sporangia during spore formation.

Galesloot and Labots (1959) described five strains isolated from milk products which were unable to hydrolyse starch and appeared to resemble B. thermoliquefaciens as described by Bergey (Bergey et al., 1923). Subsequently, Gordon et al. (1973) and Walker and Wolf (1971) reported that these five strains did hydrolyse starch at 55 °C. The reason for the apparent change of starch negative strains to starch positive is not clear, but may have been due to differences in technique, media and temperature when carrying out tests. Allen (1953) discussed the effect of continuous sub-culture on various characteristics of thermophilic species of Bacillus and demonstrated how five strains

previously considered starch negative became starch positive after repeated sub-culture.

Daron (1967) and Epstein and Grossowicz (1969) described the isolation of starch negative strains of B. stearothermophilus; Gordon et al. (1973) in their study of 31 strains confirmed these isolates as starch negative strains of B. stearothermophilus (29 strains were starch positive). They reported the maximum temperature for growth of B. stearothermophilus was 65 - 75 °C and the minimum was 30 - 45 °C. Growth in 5% (w/v) saline was variable (negative in all strains at 7%), hydrolysis of casein and reduction of nitrate to nitrite was also variable. All strains examined were resistant to lysozyme and to 0.02% (w/v) azide. Spores were ellipsoidal, sub-terminal or terminal, variable in size and usually with swelling sporangia.

In the 8th edition of Bergey, (Gibson & Gordon, 1974) the distinguishing features of B. stearothermophilus are considered to be a maximum growth temperature of 65 - 75 °C, a minimum growth temperature of 30 - 45 °C, and the inability to grow on sabouraud dextrose agar and in 0.02% (w/v) sodium azide; starch hydrolysis was considered to be variable. Gibson and Gordon (1974) discuss the problem of classifying all strains of aerobic sporeformers with the ability to grow at 65 °C as B. stearothermophilus. This resulted in the formation of an extremely heterogeneous group which excluded organisms indistinguishable from B. stearothermophilus except by their inability to grow at 65 °C.

Golovacheva et al. (1965) studied the microflora of two localities in the USSR renowned for their thermal springs; they assembled a collection of 61 thermophiles able to grow at 65 - 70°C. They allocated their isolates to seven species: B. stearothermophilus, B. thermodenitrificans, B. coagulans, B. brevis, B. circulans, B. lentus and B. megaterium. One of the strains, initially identified as a strain of B. megaterium, was later reclassified and proposed as a new species, B. thermocatenulatus (Golovacheva et al., 1975).

Klaushofer and Hollaus (1970) reported a taxonomic study of 84 thermophilic strains of Bacillus isolated during sugar beet extraction. In addition, for reference purposes, they included B. stearothermophilus NCA 26 (the original strain of Donk), NCA 1503 and ATCC 8005 (the Prickett strain of B. kaustophilus). The cultures were examined using the tests of Smith et al. (1952) and when 68 characters were examined by numerical taxonomy using the simple matching coefficient (Sokal &

Sneath, 1963), four taxonomic groups were apparent:

Group I. This group comprised 14 strains with distinctly swollen sporangia. The strains failed to grow anaerobically, caused no visible change in litmus milk, and did not hydrolyse starch, casein or gelatin. They did not grow anaerobically nor ferment a variety of sugars, but growth did occur in 3% (w/v) NaCl broth. The group was sub-divided into groups 1A and 1B based essentially on differences in the growth temperatures (Group 1A <28 - 64°C; Group 1B 37 - 68 °C).

Group II. The eight strains in this group exhibited slightly swollen sporangia. They grew anaerobically in glucose broth, hydrolysed starch and fermented several sugars (arabinose, xylose, glucose, mannose, fructose, maltose, trehalose and raffinose). All strains were able to reduce nitrate to gas and were designated as strains of B. thermodenitrificans.

Group III. Fifty-six isolates were included in this group together with the three B. stearothermophilus reference strains. The strains were allocated to two sub-groups; sub-group IIIA comprised 50 strains including NCA 26 and NCA 1503. These strains had distinctly swollen sporangia, grew anaerobically, were sensitive to 3% (w/v) saline, hydrolysed starch and did not produce gas from nitrate. Sub-group IIIB comprised nine strains including ATCC 8005.

Group IV. These five strains resembled B. coagulans, but were atypical in having maximum growth temperatures in the range of 66 - 68°C. These strains may well resemble the highly thermophilic strains of B. coagulans described by Allen (1953).

1.3.2 Recent biochemical and physiological studies of B. stearothermophilus

Walker and Wolf (1971) reported the result of an extensive study of the physiological, biochemical and serological properties of 230 strains of B. stearothermophilus including, the 75 strains of Smith et al. (1952), 16 strains from Galesloot and Labots (1959) previously described as B. calidolactis or B. thermoliquefaciens, five strains from Grinsted and Clegg (1955), and a number of strains isolated from soil and milk in the Leeds area. Using essentially the methods of Smith et al. (1952) they were able to divide the strains into three distinct major groups with minor sub-groups (Table 1.5):

Group 1. This was characterised by the ability to form gas from nitrate under anaerobic conditions (except three strains in group 1b4) and the ability to weakly hydrolyse starch within the immediate vicinity of the colony. Strains in this group did not grow, or grew

weakly in anaerobic glucose broth, sporangia were either slightly or definitely swollen with oval to cylindrical spores. Group 1, which comprised the largest group of organisms, was sub-divided into five sub-groups based on the maximum and minimum temperature for growth (organisms in group 1a did not grow at 70 °C), reduction of nitrate to nitrogen and fermentation ability. The strain B. kaustophilus (Prickett, 1928) was found to be a member of group 1b4.

Group 2. Organisms in this group were relatively inert, they did not hydrolyse starch, gelatin, or casein and did not reduce nitrate. This was the only group to show growth in 3% (w/v) NaCl. In general the temperature range for growth was lower: none grew at 70 °C and out of the 40 strains in this group all but five grew at 30 °C. The starch negative strains isolated by Daron (1967), Epstein and Grossowicz (1969) and Sharp et al. (1979) all appear to belong to this group since they all showed growth in 5% (w/v) NaCl and no growth at 70 °C. The sporangia were swollen by oval spores central to sub-terminal.

Group 3. This showed less variation than group 1 and was composed of 63 strains which divided into four sub-groups. The characteristic reactions of this group were: the strong production of amylase giving a zone of hydrolysis around the colony, the inability to reduce nitrate under anaerobic conditions, and the ability to grow on glucose under anaerobic conditions. Spores were oval to cylindrical producing definite swelling of the sporangium. Group 3a (48 strains) was mainly distinguished from groups 3b1, 3b2 and 3c by its inability to change litmus milk or to ferment lactose. Groups 3b1 and 3b2 correspond to the ten strains of B. calidolactis isolated by Grinsted and Clegg (1955) and Galesloot and Labots (1959) respectively. They showed close similarity to the five strains included in group 3c, being the only organisms in the study to ferment lactose and to produce an acid clot and reduction in litmus milk; these five strains comprising group 3c were the organsims identified as B. thermoliquefaciens by Galesloot and Labots (1959). They differed from groups 3b1 and 3b2 in showing growth at 37 °C (three strains grew at 30 °C) and were the exception in group 3 in showing only restricted starch hydrolysis.

1.3.3 Serological typing

Following their biochemical and physiological study, Walker and Wolf (1971) studied the agglutination reactions of their isolates using

Table 1.5

The biochemical reactions of the taxonomic groups of B. stearothermophilus (from Walker & Wolf, 1971)

	1a	1b1	1b2	1b3	1b4	2	3a	3b1	3b2	3c
Number of strains	39	70	10	5	3	40	48	5	5	5
Spore morphology	cylindrical	← oval to cylindrical →				oval	oval	oval	oval	oval
Swelling of sporangia	slight or absent	← definite →								
Growth in 3% (w/v) saline	0	0	0	0	0	40	0	0	0	0
Starch hydrolysis	39R	70R	9R(1+)	5+	3R	0	48+	5+	4+(1R)	5R
Gelatin hydrolysis	39	35	7	5	2	0	42	2	2	3
Casein hydrolysis	0	5	0	2	0	0	32	1	0	0
$NO_3 - NO_2$	0	0	0	0	0	0	40	2	2	4
$NO_3 - N_2$	39	70	10	5	0	0	0	0	0	0
Growth in anaer. glu.	5W	11W	10W	0	0	0	48	5	5	5
Arabinose	39	2	10	0	0	0	0	0	0	0
Mannitol	39	60	10	0	2	40	0	0	0	0
Lactose	0	0	0	0	0	0	0	5	5	5
Sucrose	39	63	10	3	3	40	48	5	5	5
Rhamnose	0	2	10	0	0	40	NT	NT	NT	NT
Cellobiose	39	7	10	0	0	40	6	0	4	0

Legend

Numbers refer to the strains giving a positive reaction. R, restricted zone of hydrolysis; W, weak growth; NT, not tested or no result.

the methods established by Norris and Wolf (1961). Antisera was prepared against spores from representatives of the major and minor groups which they had identified. Antisera prepared against a representative of group 1a, showed positive agglutination with the other 39 strains comprising group 1a. Cross reaction was found with one strain from the 70 strains included in group 1b1 and with three strains from group 1b4. No agglutination was found with any strains from groups 1b2, 1b3 or groups 2 and 3. Antisera prepared against spores from two strains in group 1b1 showed varied reactions with other strains in this group and also cross reacted with strains from group 1b2. No cross reaction was found with groups 1a, 1b3 (one strain only agglutinated), 1b4 or groups 2 and 3. Agglutination with antisera prepared from a member of group 2 showed positive agglutination with all strains of group 2, but no agglutination with members of groups 1 and 3. Three strains from group 3 used to prepare spore agglutinins failed to produce an antigenic response.

Attempts by Walker and Wolf (1971) to use 0 antigens of the vegetative cell for classification proved to be of limited value since the reactions were relatively strain specific or specific to a small number of related strains. Antisera prepared against the vegetative cells of a representative of group 2 showed positive agglutination with 29 of the 40 strains within this group.

Although the inability to elicit spore agglutinins against the strains in group 3 limited the value of agglutination reactions for classification, the fact that no cross reactions were observed between the three major groups, using either spore or vegetative agglutinins adds considerable weight to the stability of these major sub-groups based on biochemical and physiological characterisation.

1.3.4 Esterase analysis

Examination of esterase enzymes has provided valuable information for classifying a wide range of microorganisms including Corynebacterium spp. (Robinson, 1966), Streptococcus (Lund, 1965) and B. thuringiensis (Norris & Burgess, 1963; Norris, 1964). Baillie and Walker (1968) applied this technique to study the collection of strains assembled by Walker and Wolf (1971). Using starch gel electrophoresis they examined the esterase patterns of 217 strains and found they could be divided into groups very close to those already established.

In group 1 they established six sub-groups. Sub-group 1a (38 cultures) all had identical esterase patterns, strains in sub-group 1b1 (67

strains) exhibited two distinct esterase patterns and could be further divided into two groups 1b1(A) and 1b1(B). Of the ten groups in sub-group 1b2, nine had characteristic esterase patterns and the tenth identified with sub-group 1b1(A). Four cultures examined from group 1b3 showed a more divergent pattern, two had esterase patterns identical with sub-group 3a, one corresponded to sub-group 1b1(A) and one to sub-group 1b1(B). Sub-group 1b4 comprising three cultures produced identical esterase patterns. All the 38 strains examined from group 2 had identical esterase patterns, although the presence of esterase enzymes in these strains proved difficult to detect, illustrating again the biochemical inertness of this group of strains.

All 46 cultures comprising sub-group 3a shared a common esterase pattern. The two strains of B. calidolactis (Grinsted & Clegg, 1955) from group 3b1 differed in their esterase patterns to the three strains of B. calidolactis (Galesloot & Labots, 1959) from group 3b2. This latter group had an identical esterase pattern to sub-group 3c which comprised four of the strains of B. thermoliquefaciens described by Galesloot and Labots (1959).

Examination of proteins from these strains by acrylamide gel electrophoresis (Baillie & Walker, 1968) again indicated the same three main groups found by esterase analysis, but sub-divisions within groups 1 and 3 were less evident.

Since the work of Walker and Wolf (1971) and Baillie and Walker (1968) several new strains of thermophilic Bacillus have been isolated, and five new species proposed.

1.3.5 The caldoactive bacteria and other recently isolated thermophilic species of Bacillus

Following the isolation of the Gram-negative, non-sporing thermophile, Thermus aquaticus (Brock & Freeze, 1969) from a super-heated pool in Yellowstone National Park, a sporing thermophile was isolated from the same pool (Heinen, 1971). The temperature at the sampling site was a constant 86 ^{o}C and the pH 8.2. The new strain, designated YT-G, was reported to be a Gram-negative rod, which produced short filaments, distinctively composed of a number of single cells and producing dull yellowish grey colonies. Two more thermophilic strains, YT-F and YT-P, were subsequently isolated from Yellowstone National Park and compared with the original strain YT-G (Heinen & Heinen, 1972). All three strains were found to be Gram-variable though young cells of strains YT-F and YT-P appeared Gram-

Table 1.6

Characteristics of the caldoactive strains*

	B. caldotenax (YT-G)	B. caldolyticus (YT-P)	B. caldovelox (YT-F)
Optimum pH range	7.5 - 8.5	6.0 - 8.0	6.3 - 8.5
Optimum temperature for growth	80 °C	72 °C	60 - 70 °C
Maximum temperature for growth	85 °C	82 °C	76 °C
Cell diameter	0.48 µm	0.7 µm	0.62 µm
Position of spores	terminal	terminal	terminal
Swelling of sporangia	definite	definite	definite
Shape of spores	oval	cylindrical	oval

*Data compiled from Heinen and Heinen (1972)

negative. The physiological and morphological properties of the strains are shown in Table 1.6.

Strain YT-G utilises a variety of carbon sources such as glucose, succinate, pyruvate and acetate when grown at 70 - 75 °C, but only pyruvate is used at 80 °C. Growth does not occur, however, unless the basal medium is supplemented with Brain Heart Infusion (BHI) for which there is an increased requirement when the growth temperature is increased from 70 °C - 80 °C. Strain YT-P utilises glucose, sucrose and pyruvate and grows without the addition of BHI while strain YT-F grows best with succinate as a carbon source.

Heinen and Heinen (1972) considered the production of extracellular proteases and amylases to be a distinguishing character of strain YT-P, but Sharp et al. (1980) reported strong amylase production from YT-P and restricted production from strains YT-G and YT-F. The abundance of branched chain fatty acids was typical of all three strains although the amount of individual branched and straight chain fatty acids changed with growth temperature (Weerkamp & Heinen, 1972). The fatty acid composition of the individual strains is characteristic under standard conditions. Logarithmic phase cells of YT-G are resistant to rupture by ultrasonics while strains YT-F and YT-P are relatively sensitive. Strain YT-F was motile with many flagella.

Heinen and Heinen (1972) differentiated the three caldoactive strains from B. stearothermophilus on the basis of their temperature optima, fatty acid pattern and sub-microscopical structure. The three strains can be distinguished by their readiness to spore, their different temperature optima, and the morphological differences in their cell walls and membranes. Heinen and Heinen (1972) proposed the names, B. caldotenax, B. caldolyticus and B. caldovelox for strains YT-G, YT-P and YT-F, respectively, and used the term "caldoactive" to describe extremely thermophilic bacteria which show no active metabolism at lower temperatures.

Studies by Golovacheva et al. (1975) of a strain initially considered to be a thermophilic strain of B. megaterium, revealed some unusual characteristics and they proposed a new species, Bacillus thermocatenulatus. The isolate produced yellowish colonies, reduced nitrate to gas, had a % G+C content of 69% and maximum growth temperature of 78 °C.

The most recent of the obligate thermophiles to be described is Bacillus schlegelii (Schenk & Aragno, 1979). The strain is strictly

aerobic, oxidises hydrogen in the presence of O_2 and CO_2, also grows heterotrophically and produces spherical spores within a swollen sporangium. Its optimum growth temperature is 70 °C and its % G+C content is 67 - 68%.

1.3.6 Taxonomic methods for the classification and identification of micro-organisms

The classical method for studying the taxonomy and classification of microorganisms involves the examination of a large number of biochemical, physiological and morphological characters. The demand for more rapid methods for identifying microorganisms has led to the introduction of miniaturised testing systems, such as the Enterotube (Roche) and the API 20E system (API, Basingstoke), etc. for the rapid identification of Enterobacteriaceae. Similar systems have been introduced to examine other groups of microorganisms; Logan and Berkeley (1981) described extensive studies of the taxonomy of the genus Bacillus based on the API tests. These systems, while saving considerable time and labour, and often backed up by manufacturers with extensive data bases, still require subjective decisions to be made by the technician or microbiologist.

The data generated from extensive biochemical, physiological and morphological studies on groups of microorganisms can now be examined by computer using the techniques of numerical taxonomy (Sneath & Sokal, 1973; Sneath, 1978; Jones & Sackin, 1980). The technique has gained momentum over the past 20 years following greater access to computing facilities; the recent introduction of the microcomputer has made the technique even more widely available. The procedure basically involves the numerical coding of the raw data, calculation of similarities between groups of organisms and their ordering into clusters based on these similarity values.

Other novel systems have been examined and developed to aid rapid identification and studies of taxonomy including, gel electrophoresis of bacterial proteins (Kersters & De Ley, 1980), examination of electrophoretic enzyme patterns such as esterases (Baillie & Walker, 1968; Williams and Shah, 1980) or cytochrome patterns (Jones, 1980), examination of lipid composition (Minnikin & Goodfellow, 1980, 1981), and examination of protein sequences (Frank, et al., 1975). Longer established methods include serological typing (Walker & Wolf, 1971), bacteriophage typing (Parker, 1972; Billing & Garrett, 1980) and bacteriocin typing (Govan & Gillies, 1969).

All of these methods require considerable time for sample preparation, processing, and analysis of the data. Two of the more recent developments are the use of pyrolysis gas liquid chromatography (PGLC) (O'Donnell & Norris, 1981) and pyrolysis mass spectrometry (PMS) (Gutteridge & Norris, 1979) as diagnostic tools for the identification of microorganisms. The techniques require the culturing of the organisms under closely standardised conditions. A sample of culture is heated in a chemically inert environment at 500 - 900 ^{o}C to yield a number of fragments of organic material. These fragments are then examined by GLC or MS and a spectrum of the various fragments produced. The spectrum, or pyrogram, consists of a series of peaks and troughs corresponding to the presence or absence of various organic fragments; it can be considered as a profile or fingerprint of an organism. Discrimination between microorganisms can then be made using the qualitative differences in the pyrograms. Statistical techniques and more sophisticated data processing techniques have been developed to extend the resolution of the system.

The validity of taxonomic relationships between strains, species and genera, established by phenotypic characterisation, depends ultimately on the genotypes of the organisms. The genetic affinity of strains, or closely related species, can be observed from studies of conjugation, transduction and transformation. In many groups of organisms such systems of genetic transfer have not been demonstrated, although examination of in vitro DNA hybridisation (Bradley, 1980), and chemical and physical determinations of DNA base composition provide an indication of genetic relatedness. DNA base composition may be determined directly by hydrolysis of the DNA followed by separation of the bases by electrophoresis or chromatography (Bendich, 1957). Indirect methods include, calculation of the mole % guanine + cytosine (G+C) by adsorption differences at two wavelengths following acid depurination (Huang & Rosenberg, 1966; Marmur et al., 1963), buoyant density estimations in caesium chloride (Schildkraut et al., 1962), or observation of the hyperchromic shift accompanying thermal denaturation of DNA (Marmur & Doty, 1962). During heating of DNA in aqueous solution the complementary strands separate to give single stranded DNA. This is accompanied by an increase in absorbance at 260 nm. The thermal denaturation temperature (Tm) is defined as that temperature corresponding to 50% of the change in hyperchromicity.

The mole % G+C provides a criterion to assess the homogeneity of a taxonomic cluster or group established by phenetic characterisation. When organisms assigned to a particular species have significantly dissimilar mole % G+C, their classification warrants closer re-examination.

1.4 GENETICS OF THERMOPHILIC MICROORGANISMS

Considerable study into the genetics of thermophilic micro-organisms over a period of some 20 years has yielded little information regarding the production and isolation of mutants, the presence of extrachromosomal DNA and methods of genetic exchange. Several early studies into the nutritional requirements of B. stearothermophilus and B. coagulans (Baker et al., 1953, 1960) indicated that most strains have a requirement for methionine. Efforts to obtain mutants as genetic markers have been made by several groups. A mutant, unable to produce pyruvate decarboxylase, was isolated by Sundaram (1973) from the starch negative thermophile of Epstein and Grossowicz (1969), following mutagenesis in a mineral salts medium using ethyl methane sulfonate (EMS). Rowe et al. (1973) reported the isolation of catabolic mutants of B. stearothermophilus NCA 1503 defective in aconitase, fumarase and alcohol dehydrogenase, following mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) in physiological saline. Rowe et al. (1975) later reported the further development of a defined medium for the growth of B. stearothermophilus NCA 1503 and the isolation of auxotrophic mutants for arginine, threonine and tryptophan.

Plasmids have been detected in numerous species including several mesophilic groups of Bacillus, such as B. pumilus, B. subtilis and B. licheniformis, but only recently evidence of plasmids in thermophilic Bacillus species has been reported (Bingham et al., 1979, 1980; Sharp et al., 1980). Bingham et al. (1977) and Hishinuma et al. (1978) reported the isolation of plasmids from the Gram negative genus, Thermus.

A number of thermophilic bacteriophages, able to infect strains of B. stearothermophilus, have been isolated and characterised including; $\phi\mu 4$ (Thompson & Shafia, 1962), TP 1C (Welker & Campbell, 1965), TP 84 (Saunders & Campbell, 1965), Tϕ3 (Egbert & Mitchell, 1967) and the earlier isolates of White et al. (1955) and Koser (1926). However, none have yet been reported to act as a general or specialised transducing phage.

Transformation in B. stearothermophilus was first reported by Isono (1970); an amylase deficient mutant of a facultative thermophilic strain of B. stearothermophilus was transformed at 37 °C to the wild type. This report has not been followed by further reports from this group.

Attempts over a number of years by Welker and his group to establish a system of genetic transfer in B. stearothermophilus have resulted in the ability to transfect B. stearothermophilus strain 4S (Streips & Welker, 1969). This was accomplished with the help of intact phage TP 12 which appears to act in a manner similar to a competence factor. Phage TP 12 was found to be a lysogen of strain 4S. Efforts to transform Str^s isolates of strain 4S to Str^r were unsuccessful although later work using strains cured of phage TP 12 were transformed to rifampicin resistance using chromosomal DNA.

Lindsay and Creaser (1975) reported the transformation of a B. subtilis auxotroph with DNA from a prototrophic B. caldolyticus strain using the method of Spizizen (1958). They selected for transformants of Str^r, ade, his, leu, trp and met prototrophy and ability to grow at 70 °C. They were successful in obtaining transformants able to grow at 70 °C and inheriting Str^r or adenine prototrophy.

Attempts to determine genetic determinants of thermophily (described in 1.2.7) have been based mainly on the B. subtilis transformation system using DNA isolated from thermophilic micro-organisms. Stahl and Olsson (1977) reported the isolation of a range of temperature variants of B. megaterium able to grow at 55 - 70 °C but not at 37 °C. The ability to grow at these elevated temperatures was lost following treatment with acridine orange, suggesting some genetic "curing" was occurring although the presence of plasmid DNA was not reported.

1.5 COMMERCIAL, INDUSTRIAL AND MEDICAL SIGNIFICANCE OF THERMOPHILIC MICROORGANISMS

1.5.1 Enzymes and industrial feed stocks

The genus Bacillus produces a wide range of commercially important extracellular enzymes (Priest, 1977). Many strains of B. stearothermophilus are strong producers of thermostable amylase and protease enzymes (Table 1.7).

Amylases have been employed in the brewing industry to reduce the requirement for malt: during the mashing process, ground malt and barley are mixed with amylase to convert the starch within the barley and malt to sugar. These enzymes are also utilised in the paper and textile industries.

The commercial exploitation of protease enzymes was given considerable impetus in 1963, when the first biological washing powder was introduced in Europe. The leather industry utilises protease enzymes for the removal of unwanted hair and interfibrillar material. The use of a protease/amylase mixture from B. subtilis as a mouthwash has been demonstrated to be effective in the reduction of dental plaque (Shaver & Schiff, 1969).

Many of the commercially available amylase and proteinase enzymes are well established and are produced from B. subtilis, B. licheniformis and other mesophiles, including a number of fungi. The increased stability of similar enzymes from thermophilic microorganisms will no doubt eventually, increase their application in industrial processes.

Enzymes which are involved in carbohydrate metabolism have been utilised for a number of industrially important bioconversions. Enzymes available from B. stearothermophilus include glucose isomerase which converts glucose to fructose for use as a sweetener in the food industry; and β-galactosidase which hydrolyses lactose to glucose and galactose. The largest source of lactose is milk whey which contains 8% (w/w) lactose. Whey, which also contains protein, is used commercially as an animal food supplement following hydrolysis of lactose by β-galactosidase.

Enzymes are also widely used as diagnostic tools in clinical laboratories for the assay of various compounds in body fluids. The "shelf life" of these reagents is dependent upon the inherent stability of the enzyme. Glycerokinase, used for the assay of serum triglycerides,

Table 1.7

Examples of the industrial and commercially important aspects of thermophilic microorganisms

	Organisms	Comments	References
Extracellular enzymes			
α-Amylase	B. stearothermophilus, B. coagulans,* B. caldolyticus, B. licheniformis*	Endohydrolysis of α-1-4 glucosidic linkages. Utilised in the brewing, paper and textile industry.	Ogasahara et al. (1970) Grootegoed et al. (1973) *Medda and Chandra (1980)
Pectate lyase	B. stearothermophilus	Endocleavage of polygalacturonic acid. Used in preparation of fruit juices, etc.	Karbassi and Vaughn (1974)
Metal protease	B. thermoproteolyticus		Endo (1962) Levy et al. (1975)
Neutral protease	B. stearothermophilus NCIB 8924, B. caldolyticus	Stable to 65 °C. Stable to 80 °C.	Sidler and Zuber (1980) Sidler and Zuber (1980)
Enzymes for Bioconversions			
Glucose isomerase	B. coagulans, B. stearothermophilus	Converts glucose to fructose. Fructose is regarded as sweeter than glucose, however, glucose is readily available and inexpensive.	Outtrup (1976) Suekane et al. (1974)
Cyclodextrin glycosyl-transferase	B. stearothermophilus	Converts starch to cyclodextrin.	Shiosaka (1976)
β-galactosidase	B. coagulans	Converts lactose to glucose and galactose. Reduces lactose levels in milk products. Lactose deficiencies due to ageing or inheritance lead to intestinal disorders.	Long and Lee (1979)
α-galactosidase	B. stearothermophilus	Aids carbohydrate utilisation. Used as animal feed supplement.	Delente et al. (1974)

Table 1.7 (Cont'd)

	Organisms	Comments	References
Diagnostic enzymes			
Glycerokinase*	B. stearothermophilus NCA 1503	Used for the titration of serum triglycerides. *Half life at 60 °C is 80 x that of the enzyme isolated from E. coli.	Comer et al. (1979)
Glyceroldehydrogenase	B. stearothermophilus RS 93 (NCIB 11401)		Atkinson et al. (1979)
Production of fuels			
Ethanol	Thermoanaerobacter ethanolicus	Hexoses to ethanol plus CO_2.	Weigel and Ljungdahl (1981)
Ethanol	Clostridium thermocellum, Methanobacterium thermoautotrophican	Cellulose to ethanol plus acetic acid plus H_2 plus CO_2.	Weimer and Zeikus (1977)
Methane	Mixed culture of anaerobes at 60 °C	CO_2 plus $4H_2O$ to CH_4 plus $2H_2O$.	Wise et al. (1978)
Methane	Methanobacterium thermoautotrophicus	H_2 plus CO_2 $\xrightarrow{\text{methyl cobalamin}}$ CH_4.	Zeikus and Wolf (1972)
Methane	Anaerobic thermophilic population at 60 °C (organisms from cattle waste)	Cattle waste to CH_4.	Varel et al. (1977)
Methane	Methanobacterium thermoautotrophicum	Carbon monoxide CH_4 plus CO_2.	Daniels et al. (1977)
Spores			
Sterilisation indicators	B. stearothermophilus NCTC 10003	Steam sterilisation control.	Kelsey (1961)
Tracers	B. stearothermophilus RS 91	Marine Tracer for use in esteurine and coastal regions.	Evans and Yeo (unpublished studies)
Tracers	B. stearothermophilus	Airbourne tracer studies.	Sattar et al. (1972)

Table 1.7 (Cont'd)

	Organisms	Comments	References
Spoilage			
Canning industry	B. stearothermophilus (Flat sour aerobes)	One of the major causes of spoilage within the canning industry, utilises sucrose and causes deterioration in the quality of sugar.	Ito (1981)
Sugar beet extraction	Thermophilic species of Bacillus		Klaushofer et al. (1971)
Others			
Single cell protein	Mixed population at 65 °C	Methanol to Biomass.	Snedecore and Cooney (1974)
Degradation of phenol and cresols	B. stearothermophilus PH 24		Buswell and Twomey (1975)

and isolated from a constitutive mutant of B. stearothermophilus NCA 1503, has been demonstrated to have a half life of 310 min at 60 °C compared with 4.5 min for a commercial preparation extracted from E. coli (Comer et al., 1979). At 20 °C the E. coli enzyme had a half life of 8.6 days while the enzyme from B. stearothermophilus showed no loss in activity after one year at this temperature. Similar stability has been demonstrated for glycerol dehydrogenase (also used for assay of triglycerides) isolated from B. stearothermophilus RS 93 (Atkinson et al., 1979).

Thermophilic enzymes have potential in the development of immobilised processes due to their high enzyme stability and kinetic activity. At present there has been little study into the immobilisation of enzymes or whole cells from thermophilic species. De Rosa et al. (1981) reported the immobilisation of cells of Caldariella acidophila in crude egg white and glutaraldehyde. They examined the activity of β-galactosidase within the trapped cells under varying conditions of temperature and pH.

The production of ethanol and methanol using a range of substrates and a number of different thermophilic microorganisms has been demonstrated (Table 1.7) although aerobic species of Bacillus have not been involved.

The production of single cell protein from thermophiles appears limited due to the lower cell yields of thermophiles compared to mesophiles. This may be balanced against less expensive cooling costs, faster growth rate and the destruction of the most potential animal pathogens at these higher growth temperatures.

1.5.2 Spores

B. stearothermophilus spores are available as commercial preparations for the monitoring of steam sterilisation processes (Oxoid Ltd., Wade Rd., Basingstoke). Use of the spores of B. stearothermophilus RS 91 to follow water movements is being investigated (Evans & Yeo, pers. comm.). Water authorities and other investigators currently use a range of bacteriophages or spores of B. subtilis var. niger. as tracers. The availability of a range of tracers permits a number of trials to be performed in close proximity or within a short time period and avoids the need to wait for previous tracer material to disperse. Sattar et al. (1972) considered B. stearothermophilus spores ideal as airborne tracers.

1.5.3 Spoilage organisms

The problem of B. stearothermophilus as a spoilage organism in the canning industry has already been discussed in Section 1.1.1 Klaushofer et al. (1971) discussed the problems of thermophilic species of Bacillus in the refining of sugar beet. The extraction process for the beet pulp is generally completed at temperatures between 65 °C and 73 °C and the organisms isolated at this stage included, B. stearothermophilus, B. thermodenitrificans, B. coagulans, and B. sphaericus. The consequence of bacterial activity during the extraction of beet pulp was loss of sucrose through:

i. conversion into acids,
ii. increase in the production of molasses,
iii. decrease in extraction yield.

Deterioration in the quality of the final product occurred through:

i. contamination with microorganisms,
ii. increase in ash content.

Reduction of nitrate to nitrite may result in the production of imidosulphonations through the reaction of nitrite and sulphite in factories which sulphurise dilute juices. Imidosulphonates crystallize with the sucrose and thereby increase the ash content which is regarded as a criterion of quality.

1.5.4 Process considerations

Growth at higher temperatures has a marked effect on process design. The solubility of oxygen in water at 70 °C is 43% of that found at 20 °C. When thermophilic anaerobes are involved this may present a distinct advantage but aerobes will require greater mixing or aeration rates which results in greater media evaporation. Due however, to the lower viscosity and surface tension in thermophilic cultures less energy will be required for mixing and product recovery.

Atkinson (pers. comm.) observed the presence of high levels of glycolytic enzymes in B. stearothermophilus NCA 1503 when grown under apparently aerobic conditions at 65 °C and considered a change to anaerobic metabolism to occur at high growth temperatures. Cooling costs which may be considerable when growing mesophiles are negligible when growing thermophiles. Increased solubility of substrate at higher temperatures may be an advantage where substrate solubility is a rate limiting parameter, but the half life of heat labile medium constituents will be reduced.

The growth of cultures at high temperatures reduces the number

of potential contaminants, but it is not uncommon to find a thermophilic spore forming contaminant which can readily outgrow cultures of Thermus aquaticus grown at 70 °C.

1.5.5 Pathogenicity

B. stearothermophilus was considered by Breed et al. (1929) to be non-pathogenic due to its inability to grow at body temperature. Sattar et al. (1972) challenged mice to a spore suspension given intraperitoneally or as an aerosol. Mice were challenged with 0.2 ml of spores at concentrations of 10^3, 10^5 and 10^7 spores per 0.2 ml. After three weeks they concluded that B. stearothermophilus was unable to produce active infection in three week old (25 g), male, Swiss, albino mice.

1.6 RESEARCH AIMS AND OBJECTIVES

It is remarkable that such a widely studied group of micro-organisms has yielded so much information in the field of enzyme structure and function, yet so little information is available on their genetic systems. Despite the considerable commercial potential of enzymes from thermophiles, at present enzyme production can only be developed by improvements in culturing techniques and by strain selection and adaptation. Genetic techniques, such as mutagenisis to provide constitutive enzyme production, gene duplication to increase enzyme levels and gene cloning to transfer genes to more suitable hosts or linkage to other commercially exploitable genes are not readily available.

Gene cloning has now become a widely used technique exploited in many laboratories. Since the genetics and physiology of E. coli have been so widely studied over many years compared with other micro-organisms, E. coli and its associated plasmids and bacteriophages have been developed as the major system for genetic manipulation. E. coli is a normal gut commensal organism of man and domestic animals and many strains produce endotoxins. The occurrence of endotoxin production, the high risk of gut infection and the possible subsequent transfer of the plasmid to other members of the Enterobacteriaceae led to concern over the wisdom of using E. coli as the basis of such work (Berg et al., 1974). Crippled strains of E. coli have been produced which are sensitive to bile acids and have several strict growth requirements (Curtiss, 1976). Although the risk of infection has been reduced by the use of these strains, the problem of endotoxin production remains. An alternative appears to be the use of a different type of host microorganism having no association with man, animals or plants and producing no endotoxins or exotoxins.

Thermophilic microorganisms with a minimum growth temperature of 40 - 42 ^{o}C are unlikely to be invasive to humans, animals and plants and appear to present a possible alternative safe host vector system. The physiology of B. stearothermophilus has been extensively studied, although knowledge of its genetics is limited. The genetics of other species of Bacillus are more widely understood. B. subtilis has a well studied transformation system and established transducing phages (Thorne, 1968; Bramucci & Lovett, 1977); several species of Bacillus including B. subtilis, B. pumilus, (Lovett &

Bramucci, 1975), B. cereus (Bernhard et al., 1978), B. thuringiensis (Fisher et al., 1980) and B. brevis (Marahiel et al., 1981) have been found to contain plasmid DNA. It seemed that the techniques developed with mesophilic strains of Bacillus might well be used to study the genetics of thermophilic strains.

Against this background, research was initiated into the genetics of thermophilic microorganisms, in particular the Bacillus thermophiles with the aim of developing a system of genetic transfer and understanding more of the genetics of these organisms. Initial studies involved an investigation of several strains of thermophilic microorganisms for the presence of plasmid DNA. Three strains examined were Thermus sp. and although two of these indicated the presence of extrachromosomal DNA they were not further studied since they did not grow easily on solid media and presented problems for further genetic study.

A number of bacteriophages were isolated which readily infected strains of B. stearothermophilus, B. caldotenax, B. caldovelox and B. caldolyticus. These phages were investigated for their transducing abilities. Many thermophilic strains of Bacillus were isolated from a wide variety of sources in the search for new phages and plasmids and it soon became apparent that these thermophilic strains, isolated at 60 ^{o}C, represented a diverse taxonomic group. Apart from strains of B. coagulans and B. licheniformis, the obligate thermophiles isolated and considered to be strains of B. stearothermophilus comprised a metabolically and morphologically heterogeneous group. Some strains appeared to resemble the caldoactive bacteria described by Heinen (1971) and the obligate thermophile B. thermocatenulatus isolated by Golovacheva et al. (1975). At this stage it appeared essential to clarify that I was working with a group of homogeneous strains comprising one species or genetic group. Taxonomic studies were carried out to determine if the caldoactive strains (Heinen & Heinen, 1972) and B. thermocatenulatus could be classified using the system of Walker and Wolf (1971). Comparative studies were carried out on a number of clearly identified reference strains of Bacillus thermophiles and on approximately 80 unknown isolates, using biochemical and physiological tests, morphological observations, DNA analysis and phage and bacteriocin typing. The data was further evaluated using the techniques of numerical taxonomy.

CHAPTER 2

MATERIALS AND METHODS

2.1 MEDIA BUFFERS AND CHEMICALS

2.1.1 General growth media

All media were sterilised by autoclaving at 121 °C for 15 minutes (min) and their pH adjusted with 2 N HCl or 2 N NaOH unless otherwise indicated.

Tryptone soya broth (TSB) (Oxoid CM 131). This medium was used for routine culturing of mesophiles and thermophiles.

	$g\ l^{-1}$
Pancreatic digest of casein	17.0
Papaic digest of soybean meal	3.0
Sodium chloride	5.0
Potassium dihydrogen phosphate	2.5
Glucose	2.5
Distilled water to	1,000 ml

The pH was adjusted to 7.3.

Tryptone soya agar (TSA). This medium was used for the routine culturing of mesophiles and thermophiles. TSB was supplemented with 1.5% (w/v) agar.

BS broth (Sargeant et al., 1971). This medium was used for the growth of thermophilic strains.

	$g\ l^{-1}$
Bacto tryptone (Difco)	20
Yeast extract (Oxoid)	10
$FeCl_3.6H_2O$	0.007
$MnCl_2.4H_20$	0.0015
$CaSO_4.2H_20$	1.3
$MgSO_4.7H_20$	0.27
Citric acid	0.315
$NaH_2PO_4.2H_2O$	3.2
Sucrose	10
Distilled water to	1,000 ml

The pH was adjusted to 7.1.

TYF broth (Welker & Campbell, 1965). This medium was used for the culturing of phage hosts and subsequent phage infection.

	$g\ l^{-1}$
Trypticase (BBL)	20
Yeast extract (Difco)	5
$FeCl_3.6H_2O$	0.007
$MnCl_2.4H_2O$	0.001
$MgSO_4.7H_2O$	0.015
Distilled water to	1,000 ml

The pH was adjusted to 7.3 with sterile KOH after autoclaving. Fructose or glucose was used as carbon source (TYF or TYG) to give a conc. of 0.5% (w/v).

TYF soft agar (Welker & Campbell, 1965). This medium was used in the assay of bacteriophage. TYF broth was supplemented with 0.6% (w/v) purified agar (Oxoid L28).

L broth (single and double strength). This medium was used for the general culturing of thermophiles where a complex medium was required.

	$g\ l^{-1}$
Tryptone (Difco)	15
Yeast extract (Difco)	5
NaCl	5
Distilled water	1,000 ml

The pH of the medium was adjusted to 7.2 with NaOH. This medium was also used at double strength by doubling the quantities of the individual constituents.

DSM thermophile medium (DSM culture collection catalogue, 1977). This medium is described by the DSM for the growth of B. caldovelox (DSM 411), B. caldolyticus (DSM 405), B. caldotenax (DSM 406) and B. thermocatenulatus (DSM 730).

	$g\ l^{-1}$
Casitone	2
Starch	0.5
KH_2PO_4	0.05
$MgSO_4.7H_2O$	0.25
$FeSO_4.7H_2O$	0.01
$MnCl_2.4H_2O$	0.001
Agar	12
Distilled water to	1,000 ml

The pH was adjusted to 7.0.

Basal mineral medium for Bacillus schlegelii (DSM 2000, DSM 2001) (Schenk & Aragno, 1979).

	$g\ l^{-1}$
$Na_2HPO_4.2H_2O$	4.5
KH_2PO_4	1.5
NH_4Cl	1.0
$MgSO_4.7H_2O$	0.2
$CaCl_2.2H_2O$	0.01
Ferric ammonium citrate	0.005
Trace element sol'n (SL-6)	3 ml
Pyruvate (sodium salt)	1.5
Distilled water	1,000 ml

The pH was adjusted to 7.1.

Trace element sol'n SL-6

	$g\ l^{-1}$
$ZnSO_4.7H_2O$	0.1
$MnCl_2.4H_2O$	0.03
H_3BO_3	0.3
$CoCl_2.6H_2O$	0.2
$CuCl_2.2H_2O$	0.01
$NiCl_2.6H_2O$	0.02
$Na_2MoO_4.2H_2O$	0.03
Distilled water	1,000 ml

Ramaley and Hixon medium. This medium was used for the growth of Thermus aquaticus strains.

Castenholtz basal salts

	$g\ l^{-1}$
Nitrilotriacetic acid	1.0
$CaSO_4.2H_2O$	0.6
$MgSO_4.7H_2O$	1.0
NaCl	0.08
KNO_3	1.03
$NaNO_3$	6.89
Na_2HPO_4 (anhydrous)	1.11
$FeCl_3$ (0.28 g l^{-1} in d.w.)	10 ml
Nitches trace element sol'n	10 ml
Distilled water	1,000 ml

Before the following additions were made the pH was adjusted to 8.2 with NaOH.

	$g\ l^{-1}$
Tryptone (Difco)	1.0
Yeast extract (Difco)	1.0

Nitches trace element solution

	$g\ l^{-1}$
H_2SO_4 conc.	0.5 ml
$MnSO_4.4H_2O$	2.2
$ZnSO_4.7H_2O$	0.5
H_3BO_3	0.5
$CuSO_4.5H_2O$	0.016
$Na_2MoO_4.2H_2O$	0.025
$CoCl_2.6H_2O$	0.046
Distilled water	1,000 ml

Low phosphate medium. This medium was used for the growth of thermophiles in DNA hybridisation studies.

	$g\ l^{-1}$
Tris	6
Glucose	10
KCl	5
$MgSO_4.7H_2O$	1
NaCl	4
Glutamic acid	5
Casamino acids (Difco)	1
Yeast extract (Oxoid)	0.1
Tryptophan	0.025
Distilled water	1,000 ml

2.1.2 Minimal and defined media

Cal II minimal medium. This medium was used for the growth of B. caldotenax, B. caldovelox, B. caldolyticus and various other thermophiles where a minimal or defined medium was required.

	$g\ l^{-1}$
NH_4NO_3	0.5
KH_2PO_4	0.5
NaCl	0.3
$MgSO_4.7H_2O$	0.25
$FeSO_4.7H_2O$	0.05
$CaCl_2.2H_2O$	0.05
Sucrose	30
Na_2HPO_4 (anhydrous)	0.05
Nitches trace elements	0.15 ml
Distilled water	1,000 ml

The pH was adjusted to 7.5 with sodium silicate solution. (Agar was added at 1.5% (w/v) for solid media.) Sucrose was deleted from media as required and replaced with 2% (w/v) glucose.

Stearothermophilus minimal salts (SMS). This medium was used for the growth of B. stearothermophilus strain RS 93 and other thermophiles

where a minimal or defined medium was required.

	$g\ l^{-1}$
$(NH_4)_2SO_4$	10
K_2HPO_4	70
KH_2PO_4	30
Sodium citrate ($2H_2O$)	5
$MgSO_4.7H_2O$	1
Nitches trace elements	8 ml
Distilled water	1,000 ml

The pH was adjusted to 7.2. The salts were prepared at 5 x conc. and dispensed as 40 ml aliquots. For use 40 ml of salts solution were added to 160 ml of sterile water, or 160 ml of 2% (w/v) agar.

Vitamins (thiamine, biotin and nicotinic acid) were added to minimal medium as required at a level of 0.0001% (w/v) and amino acids at 0.01% (w/v).

2.1.3 Characterisation media for taxonomic studies

Starch agar (Cowan & Steel, 1974).

Potato starch (BDH)	10 g
Distilled water	50 ml
Nutrient agar	1,000 ml

The starch was mixed to a smooth cream with the distilled water and added to the molten nutrient agar. After mixing, the medium was autoclaved at 115 °C for 10 min and then distributed to petri dishes.

Attempts to prepare batches of agar in 8 oz bottles for stock, and remelting when required, were generally unsuccessful due to precipitation or clumping of the starch.

Casein agar (modified from Cowan & Steel, 1974).

Skimmed milk powder (Oxoid L31)	100 ml
Agar (3%, w/v)	100 ml

The skimmed milk was sterilised at 115 °C for 10 min and the agar at 121 °C for 15 min. When required, the agar was remelted by steaming for 30 min and the skimmed milk warmed to 55 °C. After allowing the agar to cool to approx 50 °C, the skimmed milk was added

and the two gently mixed. The medium was then used as an overlayer (6 ml) on TSBA plates.

Gelatin stabs.

	$g\ l^{-1}$
Beef extract (Difco)	3
Peptone (Difco)	5
Gelatin (Gibco)	120
Distilled water	1,000 ml

The gelatin was dissolved by heating the medium to 50 °C and the pH was adjusted to 7. The medium was distributed as 12 ml aliquots and autoclaved at 121 °C for 15 min.

Tyrosine stabs (modified from Gordon et al., 1973).

L-Tyrosine	0.5 g
Distilled water	10 ml
Nutrient agar	100 ml

Tyrosine crystals were suspended in 10 ml of distilled water, autoclaved and mixed thoroughly with 100 ml of sterile nutrient agar. The agar was distributed (12 ml) in sterile 1 oz bottles to prepare agar stabs.

Phenylalanine slopes (Gordon et al., 1973).

	$g\ l^{-1}$
Yeast extract (Difco)	3
DL-phenylalanine	2
Na_2HPO_4 (anhydrous)	1
NaCl	5
Agar (Oxoid, No. 1)	12
Distilled water	1,000 ml

Hippurate broth (Gordon et al., 1973).

	$g\ l^{-1}$
Tryptone (Difco)	10 g
Beef extract (Difco)	3
Yeast extract (Difco)	1
Glucose	1
Na_2HPO_4 (anhydrous)	5
Sodium hippurate	10
Distilled water	1,000 ml

Broth was dispensed as 10 ml aliquots in 1 oz bottles.

Litmus milk (Oxoid CN45). Litmus milk powder (100 g) was dissolved in 1 l of distilled water, strained through muslin, and distributed as 10 ml aliquots in 1 oz bottles. The medium was sterilised at 121 °C for 5 min, when hot the medium was colourless, but the purple colour returned on cooling.

Nutrient broth + 3% (w/v) or 5% (w/v) saline. Nutrient broth supplemented with 3% (w/v), or 5% (w/v) NaCl was dispensed as 10 ml aliquots in 1 oz bottles.

Nutrient broth + sodium azide. Nutrient broth was supplemented with 0.02% (w/v) sodium azide and dispensed as 10 ml aliquots in 1 oz bottles.

Nutrient broth + lysozyme. Nutrient broth was supplemented with 0.001% (w/v) lysozyme and dispensed as 10 ml aliquots in 1 oz bottles.

Nutrient broth at pH 7.0, 6.5, 6.0 and 5.5. Nutrient broth was adjusted to the appropriate pH and dispensed as 10 ml aliquots in 1 oz bottles.

Voges-Proskauer test media.

	$g\ l^{-1}$
Protease-peptone (Difco)	7
Glucose	5
NaCl	5
Distilled water	1,000 ml

The pH was adjusted to 7.2.

Broth was dispensed as 10 ml aliquots in 1 oz bottles.

Peptone water (Oxoid CM 9).

	$g\ l^{-1}$
Peptone (Oxoid)	10
NaCl	5
Distilled water	1,000 ml

The pH was adjusted to 7.4.

Broth was dispensed as 10 ml aliquots in 1 oz bottles.

Nitrate broth.

	$g\ l^{-1}$
Peptone (Evans)	5
Beef extract (Difco)	3
KNO_3	1
Distilled water	1,000 ml

The pH was adjusted to 7.2.

Broth was dispensed as 10 ml aliquots in 1 oz bottles containing Durham tubes.

Nitrite broth.

	$g\ l^{-1}$
Peptone (Evans)	5
Beef extract (Difco)	3
$NaNO_2$	0.01
Distilled water	1,000 ml

The pH was adjusted to 7.2.

Broth was dispensed as 10 ml aliquots in 1 oz bottles containing Durham tubes.

Citrate agar slopes (modification of Koser's citrate; Gordon et al., 1973).

	$g\ l^{-1}$
Sodium citrate	2
NaCl	1
$MgSO_4.7H_2O$	0.2
$(NH_4)_2HPO_4$	0.5
Agar	15
Phenol red (0.04%, w/v) sol'n	20 ml
Distilled water	1,000 ml

The pH was adjusted to 6.8, sterilised at 115 °C for 20 min and slopes prepared in 1 oz bottles.

Citrate agar slopes (Simmons, 1926; Cowan & Steel, 1974).

	$g\ l^{-1}$
NaCl	5
$MgSO_4.7H_2O$	0.2
$NH_4H_2PO_4$	1
K_2HPO_4	1
Citric acid	2
Agar	20
Bromothymol blue (0.2%, w/v)	40 ml

The salts were dissolved in water before the addition of the citric acid. The pH was adjusted to 6.8 and the medium sterilised at 115 °C

for 20 min. This medium was not used in the taxonomic studies since results were often inconclusive, the alternative medium of Gordon et al. (1973) was used instead.

Propionate agar slopes.

	$g\ l^{-1}$
Sodium propionate	2
NaCl	1
$MgSO_4.7H_2O$	0.2
$(NH_4)_2HPO_4$	0.5
Agar	15
Phenol red (0.04%, w/v) sol'n	20 ml
Distilled water	1,000 ml

The pH was adjusted to 6.8, sterilised at 115 °C for 20 min and slopes prepared in 1 oz bottles.

Peptone water sugars (Cowan & Steel, 1974).

Peptone water sugars were prepared by the method of Cowan and Steel using Andrades' indicator. Sucrose, maltose, glucose, lactose, mannitol, dulcitol and salicin peptone water sugars were obtained from Oxoid Ltd. as ready prepared media dispensed in bijou bottles.

2.1.4 Test reagents and buffers for taxonomic and genetic studies

The pH of buffers was adjusted with 2 N NaOH, or 2 N HCl unless otherwise indicated.

Lugols iodine.

Iodine	5 g
Potassium iodide	10 g
Distilled water	100 ml

The potassium iodide and iodine were dissolved in 10 ml of distilled water and adjusted to 100 ml. This stock concentrate was diluted 1/5 with distilled water before use.

Methyl red solution.

Methyl red	0.04 g
Ethanol	40 ml
Distilled water to	100 ml

The methyl red was dissolved in ethanol and diluted to 100 ml with distilled water.

α-naphthol solution.

5% (w/v) α-naphthol in ethanol

The solution was stored in brown glass bottles. If the colour became darker than straw colour the reagent was discarded.

Nitrite test reagents.

a) 0.8% (w/v) sulphanilic acid in 5 N-acetic acid.
Dissolved by gentle heating.

b) Dimethyl α-naphthylamine 5 N-acetic acid.
Dissolved by gentle heating.

PBMA buffer.

KH_2PO_4	4.5 g
NH_4Cl	0.5 g
$(NH_4)_2SO_4$	0.5 g
Manucol (sodium alginate (2.5%, w/v)	100 ml
GE silicones 60 (10%, w/v)	1 ml
Distilled water	1,000 ml

The pH was adjusted to 7.6 with 40% (w/v) NaOH.

Standard saline citrate buffer (SSC).

0.15 M NaCl
0.015 M tri-sodium citrate

The pH was adjusted to 7.0.

0.1 x SSC was a 1/10 dilution of SSC.

TS buffer.

50 mM Tris
25% (w/v) sucrose

The pH was adjusted to 8.0 with 4 N HCl.

TES buffer.

30 mM Tris
50 mM NaCl
5 mM Na_2 EDTA

The pH was adjusted to pH 8.0 with 4 N HCl.

TES.DS buffer.

50 mM Tris
1 mM Na_2EDTA
2% (w/v) sodium dodecyl sulphate

The pH was adjusted to 8.0 with 4 N HCl.

Formamide buffer.

200 mM Tris-HCl (pH 7.5)
10 mM Na_2 EDTA
50% Formamide

The pH was adjusted to 8.5 with 4 N HCl.

PMN buffer.

0.01 M KH_2PO_4
0.05 M NaCl
0.001 M $MgCl_2$

The pH was adjusted to 7.0.

Saline EDTA buffer.

0.15 M NaCl
0.1 M EDTA

The pH was adjusted to 8.0.

Restriction enzyme buffers.

(1) Low salt.

10 mM Tris
10 mM $MgSO_4$
1 mM Dithiothreitol

The pH was adjusted to 7.4.

(2) Medium salt.

10 mM Tris
50 mM NaCl
10 mM $MgSO_4$
1 mM Dithiothreitol

The pH was adjusted to 7.4.

(3) High salt.

50 mM Tris
100 mM NaCl
10 mM $MgSO_4$

The pH was adjusted to 7.4.

Bromophenol blue gel marker.

0.02% (w/v) Bromophenol blue
20% (w/v) sucrose
0.5% (w/v) ficoll
10 mM EDTA
5 mM Tris HCl (pH 7.5)

Tris borate buffer.

90 mM Tris
90 mM boric acid
3 mM Na_2 EDTA

pH 8.0 (not adjusted).

Tris maleic buffer (Adelberg et al., 1965).

	$g\ l^{-1}$
$(NH_4)_2SO_4$	1
$MgSO_4.7H_2O$	0.1
$Ca(NO_3)_2$	0.005
$FeSO_4.7H_2O$	0.025
0.05 M 2-amino-2(hydroxymethyl)-1,3-propanediol	
0.05 M maleic acid	

The pH was adjusted to 7.0.

2.1.5 Chemicals used in genetic and taxonomic studies

Chemicals not listed individually were of Analar grade and obtained from BDH.

Agarose (Standard Low -mr)	Bio Rad Laboratories Ltd., Caxton Way, Holywell Industrial Estate, Watford.
API-ZYM test strips and reagents	API, Grafton Way, Basingstoke.
Antibiotic sensitivity discs	Oxoid Ltd., Wade Rd., Basingstoke.
Amoxycillin trihydrate	Glaxo.
Ampicillin	Sigma London Chemical Company Ltd., Fancy Rd., Poole, Dorset.
Bacitracin	Sigma.
Benzyl penicillin (crystopen)	Glaxo.
Caesium chloride	BDH Ltd., Poole, Dorset.
D-cycloserine	Sigma.
Density marker beads	Pharmacia (Great Britain) Ltd., Prince Regent Rd., Hounslow, Middx.
DNA:	
E. coli	Sigma.
Micrococcus lysodeikticus	Sigma.
Clostridium perfringens	Sigma.
Calf thymus	Sigma.
Salmon sperm	Sigma.
Deoxyribonuclease I	BDH.
2-deoxyadenosine	BDH.
Ethylmethane sulphonate	Sigma.
Formamide	Fisons.
Hydrogen peroxide	BDH.
Hydroxylapatite (spheroidal)	BDH.
Kovacs' reagent	Hoffman-La Roche & Co., Basle, Switzerland.
Lysozyme (Grade I)	Sigma.
Methicillin	Sigma.
Mitomycin C	Sigma.
N-Methyl-N'-nitro-N-nitroso-guanidine	Sigma.

Percoll	Pharmacia.
Phenol	Bethesda Research Laboratories (BRL) (UK) Ltd., PO Box 145, Science Park, Cambridge.
Pronase	BDH.
Proteinase K	Sigma.
Restriction endonucleases	BRL, or New England Biolabs, 32 Tozer Rd., MA, USA.
Ribonuclease I	BDH.
Sodium lauryl sulphate (pure)	BDH.
Streptomycin	Sigma.
Sephadex G75	Pharmacia.
NNN'N'-Tetramethyl-p-phenylene-diamine, dihydrochloride	BDH.
Thymine	Sigma.
Thymidine	Sigma.
^{3}H Thymidine (45 Ci mmol^{-1})	Radiochemical Centre, Amersham, Bucks.
Trimethoprim	Sigma.
Tris (hydroxymethyl) methylamine (Analar)	BDH.

2.2 ISOLATION AND MAINTENANCE OF MICROORGANISMS

2.2.1 Isolation of Bacillus thermophiles

Samples of soil, water, mud, sand, sewage, compost and manure were collected over a four year period from a wide variety of localities in the United Kingdom, Europe, Africa and Asia. Where possible, samples were taken in sterile 1 oz bottles, or sterile 2 ml cryotubes (Sterilin).

Samples were generally treated by two or three different methods to maximise the isolation of any thermophilic strains present. Samples of water (10 µl and 500 µl) were spread across the surface of TSBA plates and Cal II agar plates (supplemented with 0.05% (w/v) casein hydrolysate, thiamine, nicotinic acid and biotin). Solid samples e.g. rock, sand and soils, were resuspended by shaking a few grams in 10 ml of 0.09% (w/v) saline for 5 min to free cells adhering to particulate matter. After allowing a few minutes for the larger material to sediment, the resulting suspension was plated as above. Solid materials were also plated directly onto agar plates, samples were transferred using a disposable 10 µl inoculating loop (Gibco) and spread across the agar surface.

Samples were also inoculated into 200 ml of BS broth and Cal II broth (supplemented as above) in 8 oz bottles. Approximately 2 g of solid material, or 2 - 5 ml of liquid was added to each bottle which were then incubated for 16 - 24 hours (h). Samples (10 µl and 50 µl) were then plated to the corresponding agar plate and incubated for 16 - 24 h, all incubations were carried out at 60 ^{o}C. Colonies isolated on Cal II medium were transferred to TSBA agar for comparison of colony morphologies. (In almost all cases transfer to a rich media did not result in any diminishing of growth and colony types isolated originally from Cal II media were almost always present in the initial isolations on TSBA.)

Strain selection was based on the variation in colony morphology on TSBA plates, strains were given a minimum of three successive single colony transfers on TSBA before being assigned a collection number and being transferred to TSBA slopes in McCartney bottles. Isolated strains are listed in Table 2.1; strains obtained from culture collections and other workers are listed in Table 2.2.

2.2.2 Maintenance of thermophilic isolates

Four stock slopes were prepared and maintained for each strain,

Table 2.1

Thermophilic strains of Bacillus isolated from a variety of sources and used in taxonomic and genetic studies

Strain numbers	Source of isolate
RS 1 - RS 3	Soil surrounding CAMR.
RS 4 - RS 8	Soil, Rhinog mountains, Wales.
RS 9 - RS 14	Forge cooling water, Boston, Lincolnshire.
RS 15 - RS 17	Contaminants from Thermus aquaticus cultures.
RS 18	Contaminant from a 400 l NCA 1503 fermentation.
RS 19 - RS 43	Soil from ICI, Bracknel, Berks.
RS 44 - RS 48	Sand from St Jean de Monts, France.
RS 49 - RS 62	Soil from St Jean de Monts, France.
RS 63 - RS 69	Soil from Ile de Re (La Rochelle), France.
RS 70	Sand from Ile de Re (La Rochelle), France.
RS 71	Water from Bath, Somerset.
RS 72	Water bath, CAMR.
RS 73 - RS 74	Contaminant from a 400 l NCA 1503 fermentation.
RS 75 - RS 84	Soil from La Mont St Michel, France.
RS 85 - RS 92	Soil from Les Eaux (Granville) France.
RS 93	Contaminant of plant hot water supply, CAMR.
RS 94 - RS 97	River Cam, Cambridge.
RS 98 - RS 113	Spring near St Albans.
RS 114 - RS 117	Soil from Kingston, Surrey. (1)
RS 118 - RS 129	Compost from Kingston, Surrey. (2)
RS 130 - RS 145	Compost heap, Porton.
RS 146 - RS 152	Soil from Greece. (1)
RS 153 - RS 158	Soil from Greece. (2)
RS 159 - RS 181	Soil from Calcutta, India.
RS 185 - RS 190	Soil 7 miles from Calcutta, India.
RS 191 - RS 196	Water from Tattapani (Hot springs), India.
RS 197 - RS 202	Compost, Salisbury.
RS 203 - RS 205	Soil from Pammakale, Turkey.
RS 206 - RS 216	Soil from Paris, France.

Table 2.1 (Cont'd)

Strain numbers	Source of isolate
RS 217 - RS 218	Poole Harbour.
RS 219	Polysaccharide producing strain from A. Bingham (LO_2).
RS 220 - RS 221	Cadmium resistant strains isolated by T. Marks.
RS 222 - RS 230	Pond mud from Zaire.
RS 231 - RS 232	Soil from Himalayas 14,000 ft.
RS 233	Soil from Himalayas 16,000 ft.
RS 234 - RS 235	Wet mud sample from area of Vesuvious.
RS 236 - RS 238	Soil from Himalayas 16,000 ft.
RS 239 - RS 242	Compost from Salisbury area, selected for streptomycin resistance.
RS 243 - RS 244	Soil from Ipsos, Corfu.
RS 245 - RS 247	Water from North Sea.
RS 248	Soil isolate from T. Marks.
RS 249	Soil isolate from T. Marks (produces bacteriocin).

Table 2.2

Strains of thermophilic and mesophilic bacteria used in taxonomic and genetic studies

Strain	Collection Numbers	Source or originator	Comments	References
B. stearothermophilus	NCA 1503, ATCC 7954, EP 170	NCA (canned peas)		
"	ATCC 12016, NCA 2184, EP 294	ATCC	Host for phage ATCC 12016B	
"	Strain NW 10, EP 307)	N. Welker, North Western	Host for phage TP 84	Epstein and Campbell (1975)
"	Strain NW 4S, EP 308)	University, Illinois	Host for phage TP 1C	Welker and Campbell (1965)
"	EP 136	Epstein & Grossowicz, Israel		Epstein and Grossowicz (1969)
"	EP 240	Chalky soil from Salisbury area		
"	EP 262	Icelandic hot water spring		
"	RS 93, NCIB 11400, EP 305	Plant hot water supply CAMR	Bacteriocin producer	Sharp et al. (1979)
"	LUDA T22)			Walker and Wolf (1971)
"	LUDA T42)			" " "
"	LUDA T60)			" " "
"	LUDA T141)	Dr. J. Wolf, The University of		" " "
"	LUDA T210, NCA 26)	Leeds	Type strain of Donk	" " "
"	LUDA T214, NCA 1518)			" " "
"	DSM 456	DSM (Sugar beet extractions)		Klaushofer & Hollaus (1970)
"	ATCC 8005, NCIB 8547	ATCC (Pasteurised milk)	B. kaustophilus	Prickett (1928)
"	ATCC 10149	ATCC	B. caldolactis	
"	ATCC 12976	ATCC		
"	NCIB 8919, NCA 1356	NCIB		
"	NCTC 10003, DSM 494	NCTC		

Table 2.2 (Cont'd)

Strain	Collection Numbers	Source or originator	Comments	References
B. stearothermophilus	NU 10)		Bacteriocin Producer	Yule and Barridge (1976)
"	NU 3)		Bacteriocin Producer	Shafia (1966)
"	NU 4)		Bacteriocin Producer	" "
"	NU 12)		Bacteriocin Producer	" "
"	NU 70)	B.D. Barridge, North Western	Bacteriocin Producer	" "
"	NU 234)	State University, USA	Bacteriocin Producer	" "
"	NU 1490)		Bacteriocin Producer	" "
"	NU 1503)		Bacteriocin Producer	" "
"	NU 1620)		Bacteriocin Producer	" "
"	NU 2184)		Bacteriocin Producer	" "
B. caldotenax	EP 263, DSM 406	W. Heinen, Yellowstone Park, USA		Heinen and Heinen (1972)
B. caldovelox	EP 295, DSM 411	W. Heinen, Yellowstone Park, USA		Heinen and Heinen (1972)
B. caldolyticus	EP 275, DSM 405	W. Heinen, Yellowstone Park, USA		Heinen and Heinen (1972)
B. thermocatenulatus	DSM 730	DSM, soil from geothermal region in the USSR		Golovacheva et al. (1975)
B. schlegelii	DSM 2000	DSM sediment of eutrophic lake, Switzerland	Chemolithotrophic organism	Schenk and Aragno (1979)
B. thermodenitrificans	DSM 465	DSM (Sugar Beet Extractions)		Klaushofer & Hollaus (1970)
" "	DSM 466	DSM (Sugar Beet Extractions)		Klaushofer & Hollaus (1970)
Bacillus thermophile	TB 118	Dr.A. Bingham		
" "	TB 124 (LO_2) EP 448	Dr.A. Bingham		
" "	TB 128	Dr.A. Bingham	All strains contain	Bingham et al. (1980)
" "	TB 144	Dr.A. Bingham	plasmid DNA	Bingham Ph.D. thesis (1980)
" "	TB 150	Dr.A. Bingham		
" "	TB 152	Dr.A. Bingham		

Table 2.2 (Cont'd)

Strain	Collection Numbers	Source or originator	Comments	References
B. alveii	NCIB 8198	NCIB	Decomposes tyrosine	Gordon et al. (1973)
B. alveii	ATCC 6344	ATCC	Production of indole	Gordon et al. (1973)
B. amyloliquefaciens H	EP 328	J. Spizizen, California, USA	Host phage ϕ1	
B. amyloliquefaciens H1	EP 277		(Hybridisation studies)	
B. coagulans	ATCC 8038, EP 338	ATCC)	Originally named	
B. coagulans	ATCC 12245, EP 341	ATCC)	B. thermoacidurans	
B. licheniformis	ATCC 14593	ATCC	Utilisation of propionate	Gordon et al. (1973)
B. megaterium	ATCC 89	ATCC	Decomposes tyrosine	Gordon et al. (1973)
B. megaterium	ATCC 4531	ATCC	Decomposes phenylalanine	Gordon et al. (1973)
B. megaterium KM	EP 207		(Hybridisation studies)	
B. pumilus	NCIB 9369	NCIB		
B. pumilus	ATCC 7061	ATCC	Utilisation of hippurate	Gordon et al. (1973)
B. sphaericus	DSM 463	DSM (Sugar Beet extraction)	Thermophiic strain	Klaushofer & Hollaus (1970)
B. subtilis	NCTC 3610	NCTC	Wild type strain	
B. subtilis	IG 20			
B. subtilis	ATCC 9372	ATCC	Pigment from tyrosine	Gordon et al. (1973)
B. subtilis	CU 1050)	Prof. S. Zahler, Cornell Univ.,	Host for phage SPβ	
B. subtilis	CU 1985)	Ithaca, New York	Lysogen for SPβ	Warner et al. (1977)
B. subtilis 12H	EP 321)	Dr. Reilly, Univ. of Minnesota,		
B. subtilis 168M	EP 322)	Minneapolis, USA	Host for phage ϕ29	
B. subtilis SB 11	EP 323	E. Hemphill, Syracuse, NY, USA	Host for phage SP82 and ϕ22	
B. subtilis 168 (trp⁻)	NCIB 10106, EP 330	NCIB	(Hybridisation studies)	

Table 2.2 (Cont'd)

Strain	Collection Numbers	Source or originator	Comments	References
Thermus aquaticus Strain YT-1	EP 244	D. Hocking, Cambridge	Gram negative thermophile	
Thermus aquaticus Strain B	EP 276)	R.A.D. Williams, London Hosp.	" " "	
Thermus aquaticus Strain X1	EP 353)	Medical School	" " "	
E. coli K_{12} HfrH	EP 249			(Hybridisation studies)
Bacteriophages				
TP 1C	)	N. Welker, North West Univ.,		Welker and Campbell (1965)
TP 84	)	Illinois, USA		Epstein and Campbell (1975)
ATCC 12016B		ATCC		
ϕ 1		J. Spizizen		
ϕ 29		Reilly, Univ. of Minnesota		
SPβ		Prof. S. Zahler, Cornell Univ.		Warner et al. (1977)

one was capped and sealed with tape and a second covered with 10 - 12 ml of sterile liquid paraffin to provide an anaerobic atmosphere. These two slopes were only used if the remaining two slopes, which were used as working stock, lost viability or were contaminated. Slopes were all stored at 4 °C and sub-cultured at approximately two yearly intervals. The majority (95%) of the strains tested have shown good viability after three years storage.

2.2.3 Preservation of mutant strains

Many of the strains used in this study (those with the EP collection numbers) were maintained by lyophilization as described by Breese and Sharp (1980). Mutant strains isolated (Section 2.19) were generally stored by adding 10% (w/v) glycerol to a broth culture, or resuspending plate cultures in 10 ml of suitable media (TSB or Cal II with supplements) plus 10% (w/v) glycerol. Samples (1 ml) were then loaded to 2 ml cryotubes (Sterilin) and stored at -180 °C over liquid nitrogen. Ampoules were thawed at room temperature when required. The genetic complement of strains of E. coli has been shown to be stable following storage for at least two years by this method (Ashwood-Smith, 1965; Breese & Sharp, 1980).

2.3 BIOCHEMICAL, PHYSIOLOGICAL AND MORPHOLOGICAL CHARACTERISATION

2.3.1 Biochemical and physiological characterisation tests

Inoculations. Tests utilising solid media in the form of plates, stabs or slopes were inoculated from overnight cultures grown on TSBA.

Liquid media were inoculated (three drops from a pasteur pipette) with a culture grown in peptone broth for 5 h following inoculation from an overnight culture grown on TSBA.

All incubations, unless otherwise indicated, were at 55 °C.

Starch hydrolysis (Cowan & Steel, 1974). Starch agar plates were streaked with colonies from an overnight culture and examined for evidence of hydrolysis after 1 day and 3 days incubation. To detect areas of starch hydrolysis some of the colonies from the streak were removed and the plates flooded with 1 - 2 ml of Lugol's iodine. Hydrolysis was indicated by clear colourless zones while unhydrolysed areas remained blue/black. Hydrolysis was either diffuse, resulting in hydrolysis of starch in the area surrounding the colonies, or restricted to the immediate area beneath the colonies.

Positive hydrolysis. (Restricted)	B. stearothermophilus NCA 1503 (Gordon et al., 1973; Walker & Wolf, 1971).
Positive hydrolysis. (Diffuse)	B. stearothermophilus NCIB 8923 (Walker & Wolf, 1971).
Negative hydrolysis.	B. stearothermophilus EP 136 (Epstein & Grossowicz, 1969).

Casein hydrolysis (modified from Gordon et al., 1973). Casein agar plates were streaked with colonies from overnight cultures grown on TSBA and examined for areas of casein hydrolysis after 1 day and 3 days incubation. Hydrolysis was indicated by clear zones in the white agar overlayer.

Positive hydrolysis.	B. stearothermophilus NCA 1503 (Gordon et al., 1973).
Negative hydrolysis.	B. stearothermophilus EP 136 (Epstein & Grossowicz, 1969).

Gelatin liquefaction (Gordon et al., 1973). Gelatin stabs were examined for liquefaction after 1 day and 3 days. Before recording results, the tubes were held at 20 ^{o}C for 1 h to allow unhydrolysed gelatin to harden. An uninoculated control was included with each batch of tests.

Positive control.	B. stearothermophilus NCA 1503 (Walker & Wolf, 1971).
Negative control.	B. stearothermophilus EP 136 (Epstein & Grossowicz, 1969).

Tyrosine decomposition (Gordon et al., 1973). Tyrosine stabs were examined after 1, 3, 5 and 10 days for the decomposition of the tyrosine crystals. Decomposition began at the top of the stab and progressed down through the agar. Observations were also made for the appearance of a brown pigment which appeared initially on the surface and progressed through the agar.

Positive control.	B. alveii NCIB 8198, B. megaterium ATCC 89 (Gordon et al., 1973).
	B. stearothermophilus strain EP 262 (Sharp, this thesis).

Production of pigment.	B. subtilis ATCC 9372 (Gordon et al., 1973).
	B. stearothermophilus DSM 465 (pigment only) (Sharp, this thesis).
	B. stearothermophilus EP 262 (Sharp, this thesis).
Negative control.	B. stearothermophilus NCA 1503 (Gordon et al., 1973).

Phenylalanine decomposition (Gordon et al., 1973). Phenylalanine slopes were streaked from an overnight culture grown on TSBA. After 1 day and 3 days incubation a few drops of 10% (w/v) $FeCl_3$ was run over the surface of the agar. The production of a green pigment in the agar immediately below the area of growth indicated the production of phenylpyruvic acid.

Positive control.	B. megaterium ATCC 4531 (Gordon et al., 1973).
Negative control.	B. stearothermophilus NCA 1503 (Gordon et al., 1973).

Hydrolysis of hippurate (Gordon et al., 1973). Hippurate broth (10 ml) was inoculated with three drops from a 5 h peptone broth culture. Samples were examined after 5 days and 10 days incubation. To 1.5 ml of 50% (v/v) H_2SO_4 was added 1 ml of culture. The appearance of crystals in the acid mixture was evidence of the formation of benzoic acid from the hippurate (Baird-Parker, 1963). Where dense growth in the broth obscured the formation of crystals, a 1 ml sample of culture was filtered through a 0.45 µm millex filter (Millipore).

Positive control.	B. pumilus ATCC 7061 (Gordon et al., 1973).
Negative control.	B. stearothermophilus NCA 1503 (Sharp, this thesis).

Litmus milk. The growth of cultures on litmus milk was observed after 1 day, 3 days, and 5 days. Reduction of the indicator was evident from the production of a white colour. Acid production was indicated by the production of a pink colour, and alkali by the production of a blue/brown colour. Acid clots became evident after 3 - 5 days, clots

were cream in colour, covered by a clear pink/grey liquid. The pH of the broths was recorded after 5 days incubation.

Unchanged control.	B. stearothermophilus EP 136 (Epstein & Grossowicz, 1969; Gordon et al., 1973).
Reduction control.	B. coagulans ATCC 12245 (Gordon et al., 1973).
Acid production control.	B. coagulans ATCC 12245 (Gordon et al., 1973).
	B. stearothermophilus ATCC 8005 (Sharp, this thesis).
Alkali production control.	B. stearothermophilus EP 262 (Sharp, this thesis).
Acid clot production.	B. coagulans ATCC 12245 (Gordon et al., 1973).

Sensitivity to saline (Gordon et al., 1973). Nutrient broth supplemented with 3% (w/v) and 5% (w/v) NaCl was inoculated as above and examined for growth after 1 day and 3 days incubation.

Controls:

Growth in 3% (w/v) NaCl, no growth in 5% (w/v) NaCl,

B. thermodenitrificans DSM 465 (Sharp, this thesis).

Growth in 3% (w/v) NaCl and growth in 5% (w/v) NaCl,

B. stearothermophilus EP 136 (Epstein & Grossowicz, 1969).

No growth in 3% (w/v) NaCl,

B. stearothermophilus ATCC 8005 (Gordon et al., 1973).

Sensitivity to sodium azide (Gordon et al., 1973). Nutrient broth, supplemented with sodium azide (0.02%, w/v), was inoculated as above and examined for growth after 1 day and 3 days incubation.

Positive control.	B. coagulans ATCC 8038 (Gordon et al., 1973).
Negative control.	B. stearothermophilus ATCC 8005 (Gordon et al., 1973).

Sensitivity to lysozyme (Gordon et al., 1973). Nutrient broth supplemented with lysozyme was inoculated from a 5 h peptone broth culture. Control broths without lysozyme were included for each test. Broths were examined after 1 day and 3 days incubation for evidence of growth.

Positive control.	B. pumilus NCIB 9369 (Gordon et al., 1973).
	B. stearothermophilus EP 136 (Sharp, this thesis).
Negative control.	B. stearothermophilus NCA 1503 (Gordon et al., 1973).

Growth at acidic pH. Nutrient broths adjusted to pH 7.0, 6.5, 6.0 and 5.5 were inoculated as above and examined for growth after 1 day and 3 days. If growth was not present at pH 7.0 the tests were repeated.

Controls (Sharp, this thesis):

Growth at pH 6.5, but not 6.0.	B. stearothermophilus ATCC 12016.
Growth at pH 6.0, but not 5.5.	B. stearothermophilus ATCC 8005.
Growth at pH 5.5.	B. coagulans ATCC 8038.
Growth at pH 7.0, but no growth at pH 6.5.	RS 13.

Growth on minimal media. Plates of SMS and Cal II minimal agar and minimal agar plus casein hydrolysate supplement, were streaked from an overnight culture grown on TSBA. Plates were examined for growth after 1 day and 3 days. Comparisons of growth on the minimal and supplemented plates were made.

Controls (Sharp, this thesis):

Positive growth on Cal II minimal agar B. caldotenax.

Negative growth on Cal II minimal agar B. coagulans ATCC 8038.

Positive growth on Cal II minimal agar plus supplement B. coagulans ATCC 8038 (weak growth).

Negative growth on Cal II minimal agar plus supplement thermophile RS 236.

Positive growth on SMS minimal agar B. stearothermophilus RS 98.

Negative growth on SMS minimal agar B. thermodenitrificans DSM 465.

Positive growth on SMS minimal agar plus supplement B. thermodenitrificans DSM 465.

Negative growth on SMS minimal agar plus supplement B. stearothermophilus LUDA T210.

Anaerobic growth on TSBA. TSBA plates were streaked from an overnight TSBA plate and incubated at 55 °C in an anaerobic jar in a CO_2 plus H_2 atmosphere in a BBL (Baltimore Biological Laboratories) Gas Pak anaerobic system. Plates were examined after 1 day and 3 days, positive growth was recorded when growth was similar to that on a complementary plate incubated under aerobic conditions.

Controls (Sharp, this thesis):

Positive.	B. stearothermophilus NCA 1503.
Positive.	B. thermodenitrificans DSM 465.
Negative.	B. stearothermophilus ATCC 12016.
Negative.	B. stearothermophilus EP 136.

Anaerobic growth in glucose peptone broth. Glucose peptone broths were inoculated and incubated at 55 °C in a BBL anaerobic jar as above. Broths were examined after 1 day and 3 days for evidence of growth and acid production.

Controls (Sharp, this thesis):

Growth positive.	B. stearothermophilus ATCC 8005.
Growth with acid production.	B. stearothermophilus NCA 1503.
Growth negative.	Thermophile RS 1.

Catalase production (Skerman, 1967). The production of catalase was detected by adding a few drops of hydrogen peroxide to a fresh overnight culture grown on TSBA. A positive reaction was indicated by the rapid evolution of bubbles.

Positive Control.	B. stearothermophilus ATCC 12016 (Gordon et al., 1973).
Negative Control.	B. stearothermophilus NCA 1503 (Gordon et al., 1973).

Oxidase (cytochrome C oxidase) activity (Kovacs, 1956). A few drops of 1% (w/v) tetramethyl-p-phenylenediamine dihydrochloride were placed on Whatman No. 1 filter paper in a petri dish. Colonies removed

from a fresh overnight culture grown on TSBA were streaked across the surface of the filter paper. A positive reaction was indicated by the production of a dark purple colour within 10 s.

Controls (Sharp, this thesis):

Positive.	B. stearothermophilus ATCC 12016.
Negative.	B. stearothermophilus NCA 1503.

MRVP test (acid and acetylmethylcarbinol production) (Cowan & Steel, 1974). Glucose peptone broth (Gordon et al., 1973) was inoculated from a 5 h peptone broth and examined after 1 day and 3 days for the production of acid and acetylmethylcarbinol. Acid production was detected by the production of a red colour in the broth following the addition of two drops of methyl red solution.

After completion of the methyl red test, the broth was examined for the production of acetylmethylcarbinol (Barritt, 1936). 0.6 ml of 5% (w/v) α-naphthol and 0.2 ml 40% (w/v) KOH were added to the broth and the tube observed after 1 h. A positive reaction was indicated by the production of a strong red colour.

The method of Barritt (1936) was used for characterisation tests following comparison with the alternative method used by Gordon et al. (1973), in which 3 ml of 40% (w/v) NaOH was added to the broth, followed by 0.5 - 1 mg of creatine. A positive reaction was detected by the production of a red colour in the broth.

Positive methyl red.	B. stearothermophilus ATCC 10149 (Gordon et al., 1973).
Negative methyl red.	B. stearothermophilus ATCC 8005 (Gordon et al., 1973).
Positive VP test.	B. coagulans ATCC 8038 (Gordon et al., 1973).
Negative VP test.	B. stearothermophilus ATCC 12016 (Gordon et al., 1973).

Indole production (Cowan & Steel, 1974). Duplicate peptone broths were inoculated and tested for indole production. After 1 day and 3 days 0.5 ml of Kovacs' reagent was added to one bottle which was shaken, after 1 min it was examined for the production of a red colour in the reagent layer which indicated indole production.

Positive Control.	B. alveii ATCC 6344 (Gordon et al., 1973).
Negative Control.	B. stearothermophilus NCA 1503 (Gordon et al., 1973).

Utilisation of citrate and propionate (Gordon et al., 1973). Citrate and propionate agar slopes were inoculated from an overnight culture grown on TSBA. The slopes were examined for the utilisation of organic acids at 1 day, 3 days and 5 days. This was demonstrated by the production of a red colour in the agar due to the alkaline reaction of the phenol red indicator.

Controls (Gordon et al., 1973):

Citrate positive.	B. stearothermophilus ATCC 8005.
Citrate negative.	B. stearothermophilus NCA 1503.
Propionate positive.	B. licheniformis ATCC 14593.
Propionate negative.	B. pumilus ATCC 7061.

Utilisation of Simmons' citrate (Cowan & Steel, 1974). Slopes were inoculated and examined as above. A positive reaction was indicated by the production of a blue colour in the agar. A negative reaction was indicated when the agar remained its original green colour.

Controls (Gordon et al., 1973):

Citrate positive.	B. stearothermophilus ATCC 8005.
Citrate negative.	B. stearothermophilus NCA 1503.

Reduction of nitrate and nitrite (Gordon et al., 1973; Cowan & Steel, 1974). Duplicate bottles of nitrate broth and nitrite broth were inoculated from a 5 h peptone broth culture. One bottle from each pair was examined after 1 day for the presence of nitrite, the second was examined after 3 days incubation. To test for the presence of nitrite, 1 ml of sulfanilic acid reagent and 1 ml of dimethyl α-naphthylamine reagent was added to each bottle, nitrite was indicated by the presence of a deep red colour. If nitrite was not indicated in the nitrate broths, a small quantity of powdered zinc was added, production of a red colour indicated the presence of residual nitrate.

Controls:

Reduction of NO_3 to NO_2.	B. stearothermophilus NCA 1503 (Gordon et al., 1973).
Reduction of NO_3, NO_2 and gas not detected.	B. stearothermophilus EP 240 (Sharp, this thesis).
Reduction of NO_3 to gas.	B. stearothermophilus ATCC 12016 (Sharp, this thesis).
Reduction of NO_2 but gas not detected.	B. stearothermophilus LUDA T210 (Sharp, this thesis).
Reduction of NO_2 to gas.	B. stearothermophilus ATCC 12016 (Sharp, this thesis).
No reduction of NO_3.	B. coagulans ATCC 8038 (Gordon et al., 1973). B. stearothermophilus EP 136 (Sharp, this thesis).
No reduction of NO_2.	B. coagulans ATCC 8038, B. stearothermophilus EP 136 (Sharp, this thesis).

Anaerobic reduction of nitrate. Freshly steamed nitrate broth of Gordon et al. (1973) was inoculated from a 5 h peptone broth and then covered with 4 ml of sterile liquid paraffin to maintain anaerobiosis. The broths were examined after 1 day and 3 days for the production of gas in the Durham tube. Reduction of NO_3 was confirmed using the tests outlined above.

Positive controls.	B. thermodenitrificans DSM 465 (Klaushofer & Hollaus, 1970). B. stearothermophilus ATCC 12016 (Sharp, this thesis). B. stearothermophilus LUDA T22 (Walker & Wolf, 1971).
Negative controls.	B. stearothermophilus NCA 1503 (Sharp, this thesis). B. stearothermophilus EP 136 (Sharp, this thesis). B. stearothermophilus LUDA T141 (Walker & Wolf, 1971).

Acid production from carbohydrates and sugar alcohols. Peptone water sugars were inoculated in duplicate, incubated at 55 °C and examined for acid production. (Andrade's indicator turns red at acid pH.)

	Positive Control		Negative Control	
Adonitol	B. stearo.	RS 93	B. stearo.	NCA 1503
Arabinose	B. stearo.	ATCC 12016	B. stearo.	NCA 1503
Dextrin	B. stearo.	NCA 1503	B. stearo.	ATCC 12016
Dulcitol			B. stearo.	NCA 1503
Erythritol	B. stearo.	EP 136	B. stearo.	NCA 1503
Fructose	B. stearo.	NCA 1503	B. stearo.	EP 262
Galactose	B. stearo.	ATCC 12016	B. stearo.	EP 136
Glucose	B. stearo.	NCA 1503		
Glycerol	B. stearo.	NCA 1503	B. stearo.	EP 262
Glycogen	B. stearo.	NCA 1503	B. stearo.	EP 136
Inositol	B. stearo.	EP 136	B. stearo.	NCA 1503
Inulin			B. stearo.	NCA 1503
Lactose	B. coagulans	ATCC 8038	B. stearo.	NCA 1503
Maltose	B. stearo.	NCA 1503	B. stearo.	RS 93
Mannose	B. stearo.	NCA 1503	B. stearo.	EP 136
Mannitol	B. stearo.	EP 136	B. stearo.	NCA 1503
Raffinose	B. stearo.	NCA 1503	B. stearo.	ATCC 12016
Rhamnose	B. coagulans	ATCC 8038	B. stearo.	NCA 1503
Salicin	B. coagulans	ATCC 8038	B. stearo.	NCA 1503
Sorbitol	B. stearo.	EP 136	B. stearo.	NCA 1503
Starch	B. stearo.	NCA 1503	B. stearo.	ATCC 12016
Sucrose	B. stearo.	NCA 1503	B. stearo.	ATCC 12016
Trehalose	B. stearo.	NCA 1503	B. stearo.	ATCC 12016
Xylose	B. stearo.	ATCC 12016	B. stearo.	NCA 1503

Temperature growth tests. Growth temperatures above 37 °C were recorded following 3 days incubation on TSBA slopes in a thermostatically controlled water bath. At 37 °C and below, growth temperatures were recorded following growth on TSBA plates in a thermostatically controlled air circulating incubator.

2.3.2 Morphological characterisation

Colony morphology. Colony morphology was examined using cultures grown on TSBA plates for 16 h. (Plates were inoculated from cultures also incubated for 16 h at 60 °C on TSBA plates.) The plates,

containing 25 ml of agar, were dried at 40 °C for 48 h before use. Morphological descriptions were restricted to the elevation, form, margin, surface, and degree and form of spreading. The various categories of elevation, form and edge are indicated in Fig. 2.1. The colony surface was described as either smooth and shiny, smooth and matt, or rough. Spreading of colonies was dependent upon the time of incubation and the amount of water bound by the agar. The degree of spreading of colonies was observed on TSBA plates incubated for 16 h at 60 °C. Incubation for longer periods increased the degree of spreading, while extra drying of the plates reduced spreading. The degrees of spreading were categorised as:

V - No evidence of spreading after 16 h incubation at 60 °C.
W - Dry colonies showing evidence of moist spreaders.
X - A small number of colonies showing evidence of spreading from original colony.
Y - Most colonies producing spreading areas.
Z - All of agar surface covered in spreading growth with no evidence of the original streak.

Controls

Elevation	
Flat	B. stearothermophilus EP 136,
	B. thermodenitrificans DSM 465
Raised	Bacillus thermophile RS 8
Convex	B. stearothermophilus NCA 1503
Pulvinate	-
Form	
Circular	B. stearothermophilus NCA 1503
Irregular	B. thermodenitrificans DSM 465
Margin	
Entire	B. stearothermophilus NCA 1503
Undulate	B. stearothermophilus LUDA T22
Erose	B. thermodenitrificans DSM 465

Surface

Smooth/shiny	B. stearothermophilus NCA 1503
Smooth/matt	B. coagulans ATCC 12245
	B. thermocatenulatus DSM 730
Rough	B. thermodenitrificans DSM 465,
	B. sphaericus DSM 463

Spreading

V	-	B. stearothermophilus NCA 1503
W	-	Bacillus thermophile RS 14
X	-	B. stearothermophilus LUDA T141
Y	-	Bacillus thermophile RS 3
Z	-	Bacillus thermophile RS 5

2.3.3 Examination of cell motility

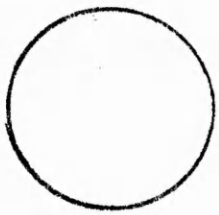
Cultures grown for 5 h at 55 °C in peptone broth were examined for evidence of motility by the "hanging drop" method.

2.3.4 Examination for the presence of spores

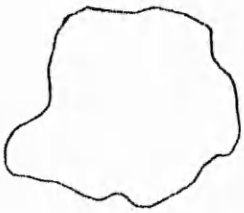
Colonies from 2 day old cultures grown on TSBA plates at 55 °C were examined for the presence of spores using the method of Schaeffer and Fulton (1933). After fixing, the slide was flooded with 5% (w/v) aqueous malachite green and steamed for 1 min. After washing for 1 min it was counterstained with 0.5% (w/v) safranin for 15 s and rinsed with water. The vegetative cells or cell bodies stained red and the spores green.

Figure 2.1

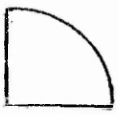
Colony Morphology



Circular

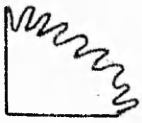


Irregular



Entire

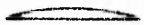
Undulate



Erose

Flat

Raised



Convex

Pulvinate

2.4 ANTIBIOTIC SENSITIVITY TESTING OF BACILLUS THERMOPHILES

Antibiotic sensitivity was determined using Oxoid sensitivity discs dispensed onto seeded agar plates by an Oxoid hand held eight cartridge dispenser unit. Oxoid Sensitest agar (CM409) was initially used, but found unsuitable for the growth of many strains, and was superceded by TSBA (Section 3.1.6).

Thermophilic strains were incubated for 16 h on TSBA plates and used to prepare suspensions in 0.09% (w/v) saline. To standardise the cell density, suspensions were adjusted to the opacity of a solution of 0.01 g of finely powdered $CaCO_3$ in 10 ml of saline. This gave a viable cell count between 10^7 and 2×10^8 viable cells ml^{-1} (based on counts of eight strains). TSBA plates were freshly poured and dried at 70 °C for 15 min. Aliquots (1 ml) of the suspension were applied to duplicate plates, spread across the agar surface and allowed to dry. Four discs were applied to each of the two duplicate plates (eight different antibiotics in total) which were then incubated for 16 h at 60 °C before recording the zones of inhibition.

2.5 NUMERICAL TAXONOMY OF THERMOPHILIC SPECIES OF BACILLUS

2.5.1 Strains and tests

Phenetic data on 102 thermophilic Bacillus were examined and analysed using numerical taxonomic methods. Seventeen marker strains (Table 2.3) representing the various taxonomic groups previously observed by Klaushofer and Hollaus (1970), Walker and Wolf (1971) and Gordon et al. (1973) and several more recently reported species (Heinen & Heinen, 1972; Golovacheva et al., 1975; Sharp et al., 1979, 1980; Bingham et al., 1980), were included. The source of the remaining cultures which were isolated from a range of natural environments are listed in Table 2.1.

The tests used were all described in Sections 2.3 and 2.4. Each strain was examined for 96 characters based on the results presented in Table 3.6, 3.7 and 3.8. Some of these results, including aesculin utilisation (all strains negative), Gram stain (results variable and inconsistent), litmus milk (test complicated by interacting reactions), pigment production (only two strains produced pink pigment, RS 93 and LO_2, and one strain RS 222 produced yellow pigment) and motility and spore morphology (only a few strains examined), were excluded from the numerical study. The majority of results were recorded following 3 days incubation, the exceptions were propionate and citrate utilisation (5 days), tyrosine and hippurate (10 days), and catalase, oxidase and colony morphology which were examined on overnight cultures grown on TSBA. All tests were carried out in duplicate and where the results were inconclusive tests were repeated. Eight strains selected at random were given a code letter to hide their original identity and were then re-examined to provide a check on the reproducibility of the tests.

2.5.2 Coding of data

The final n x t matrix contained 96 characters and 102 strains. Most of the data assembled were in two states, i.e. the results were either positive or negative. Starch hydrolysis was an exception with results in one of three states, negative, hydrolysis restricted and hydrolysis diffuse. The data was therefore coded using multistate numerical coding, this permitted the separate coding of weak positive reactions and strong positive reactions.

Table 2.3

Marker strains included in the numerical taxonomic study

Strains	Previous taxonomic studies		
	K & H	W & W	G, H & P
B. stearothermophilus			
NCA 1503	+	+	+
LUDA T214 (NCA 1518)	-	+	-
LUDA T210 (NCA 26)	+	+	+
ATCC 8005	+	+	+
ATCC 12016	-	-	+
NCTC 10003	-	-	-
ATCC 10149	-	+	+
NCIB 8919 (NCA 1356)	-	+	-
DSM 456	+	-	-
EP 136	-	-	+
LUDA T22	-	+	-
LUDA T42	-	+	-
LUDA T60	-	+	-
LUDA T141	-	+	-
NW 10	-	-	-
RS 93 (NCIB 11400)	-	-	-
B. thermodenitrificans			
DSM 465	+	-	-
DSM 466	+	-	-
B. caldotenax	-	-	-
B. caldovelox	-	-	-
B. thermocatenulatus DSM 730	-	-	-
B. sphaericus DSM 463	+	-	-
B. coagulans ATCC 8038	-	-	+
B. coagulans ATCC 12245	-	-	+

Legend

K & H, Klaushofer and Hollaus (1970); W & W, Walker and Wolf (1971); G, H & P, Gordon, Haynes and Pang (1973).

e.g. Test	Result	Code
Mannitol fermentation	negative	0
	weak positive	1
	strong positive	2
Starch hydrolysis	negative	0
	restricted	1
	diffuse	2
Streptomycin sensitivity	Zone size 0 mm	0
	Zone size <10 mm	1
	Zone size 10 mm or >10 mm	2

Erythromycin, chloramphenicol, tetracycline, ampicillin, gentamicin and trimethoprim were all coded using the same limits as streptomycin (see example above). Naladixic acid, to which the majority of the strains were more susceptible, was coded as 0 - no sensitivity, 1 - zone diameter up to 4 mm, 2 - zone diameter 4 mm or greater.

Cell morphology was generally in one of two exclusive states, i.e. a colony was either circular or irregular and was coded as 0 for absence of this character and 2 for its presence.

e.g.		
	Colonies circular	2
	Colonies not circular	0
	Colonies irregular	2
	Colonies not irregular	0

Cell diameter was coded 0 for cells 0.75 µm or less and 2 for cells of greater than 0.75 µm diameter. Cell length was coded 0 for cells 5 µm or less and 2 for cells greater than 5 µm in length.

The cell form, i.e. growth as single cells, pairs, chains or filaments, was recorded 0 for absence of character and 2 for its presence. In some cases, cultures produced a mixture of single cells, pairs, chains and filaments, when this occurred each character was scored as 2 (four strains from cluster (3) grew as single cells, pairs, and chains). Where data was unavailable the entry was coded as X.

2.5.3 Computation of resemblance

Computations were carried out using an Acorn system 4 microcomputer with a 48K memory and interfaced with a RICOH RP1600 printer (model 8744 RICOH Company Ltd., Japan) and a Watanabe MIPLOT XY plotter (Model WX4671, Watanabe Instrument Corporation, Tokyo, Japan). All programmes were written in BASIC (by Dr. J. Woodrow, CAMR, Porton) specifically for this study.

The calculation of similarity coefficients was based on the coefficient of Gower (1971), in which negative matches were scored. Matching pairs of positive characters, matching pairs of weak positive characters and matching pairs of negative characters between two OTU's were scored 1. Mismatches of positive and negative tests were scored 0. Half mismatches, i.e. positive with weak positive, and weak positive with negative scored 0.5.

Characters	OTU (A) coded results	OTU (B) coded results	Similarity score
I	0	0	1
II	0	1	0.5
III	0	2	0
IV	1	0	0.5
V	1	1	1
VI	1	2	0.5
VII	2	0	0
VIII	2	1	0.5
IX	2	2	1

2.5.4 Clustering analysis

The similarity values were sorted using single linkage analysis and a shaded, sorted similarity matrix prepared using a Watanabe XY plotter. Levels of shading intensity were set at 85 - 100% similarity, 80 - 84% similarity, 75 - 79% similarity, 70 - 74% similarity and 65 - 69% similarity, below 65% remained unshaded.

2.5.5 Programmes

PNT-1 - was designed to check the validity of the coded data typed into the computer (i.e. all the data should be coded as 0, 1, 2 or X).

PNT-2 - computed the percentage similarity of each OTU with all other OTU's in the study.

PNT-3 - tested the symmetry of the matrix by comparing the corresponding values at each side of the 100% line of similarity.

PNT-4 - was designed to enable the printer to print out the unordered similarity matrix.

PNT-5 - sorted the strains by single linkage analysis and re-sorted the matrix using the new established order.

PNT-6 - instructed the plotter to produce a shaded similarity matrix based on a sorted matrix prepared in PNT-5.

PNT-7 - enabled sorting of the OTU's into groups based on the clusters established in the sorted similarity matrix.

Programme PNT-1

```
  1 REM RESTEST-Check results all valid
  9 REM Open file and allocate storage
 10 R=FIN"RESULTS"
 20 A=TOP
 29 REM Get result
 30 DO SGETR,A
 40 FORZ=5 TO LENA-1
 49 REM Check all characters X,0,1,2
 50 IF A?Z=88 OR A?Z=48 OR A?Z=49 OR A?Z=50 GOTO 70
 59 REM If not print strain code number position and character
 60 FORX=0TO4;P.$A?X;NEXTX;P.Z,"  '",$A?Z,"'"'
 70 NEXTZ
 79 REM End of results-Stop
 80 UNTIL PTRR=EXTR-1
 90 P."O.K."'
100 END
>
```

Programme PNT-2

```
  1 REM CALC S-calculates S values
  9 REM Allow to ignore replicates
 10 INPUT"How many strains to be used"A
 19 REM Open file to store S values
 20 P."Give name of matrix storage file"'
 30 P."These are the files already on the disc"''
 40 *CAT
 50 INPUT$TOP
 60 B=FOUT$TOP
 70 *LOAD"RESULTS"4000
 79 REM C=strain1 results;D=strain2
 80 C=#4000;D=#4000
 90 FOR E=1 TO A
100 FOR F=1 TO A
110 IF LENC<>LEND P.$C'$D';END
120 X=0;Y=0
130 FOR Z=5 TO LENC-1
140 IF C?Z=88 OR D?Z=88 GOTOa
149 REM Count total valid tests
150 X=X+2
159 REM Count total similar tests
160 Y=Y+2-ABS(C?Z-D?Z)
170 aNEXTZ
179 REM Calculate S-round to nearest %
180 S=(1000*Y/X+5)/10
189 REM Save value
190 BPUTB,S
199 REM Show progress
200 P.(C-#4000)/(LENC+1),(D-#4000)/(LENC+1),S'
210 D=D+LEND+1
219 REM Get next strain2
220 NEXTF
230 C=C+LENC+1;D=#4000
239 REM Get next strain1
240 NEXTE
249 REM All combinations tested-Stop
250 SHUTB
260 END
>
```

Programme PNT-3

```
  1 REM MTXTEST-tests matrix symmetry
 10 *CAT
 20 INPUT"Name of matrix for test"$TOP
 30 INPUT"Number of strains"S
 39 REM *LOAD"Matrix name"4000
 40 A=#0100
 50 $A="LOAD "
 60 $A+LENA=$TOP
 70 $A+LENA=" 4000"
 80 LINK#FFF7
 90 Z=#4000
 99 REM Wait until loaded
100 *DIR
110 FORB=0 TO S-1
120 FORC=B TO S-1
129 REM Get number in top half
130 X=S*B+C
139 REM Get number in bottom half
140 Y=S*C+B
149 REM If not equal print coordinates
150 IFZ?X<>Z?Y P.B,C'
160 NEXTC
169 REM Show progress
170 P.B'
180 NEXTB
189 REM All numbers tested-Stop
190 P."O.K."'
200 END
>
```

Programme PNT-4

```
  1 REM PMATRIX-Prints out matrix in 40 column blocks
 10 *CAT
 20 INPUT"Matrix to be printed"$TOP
 30 INPUT"Number of strains"S
 39 REM *LOAD"Matrix name"4000
 40 A=#0100
 50 $A="LOAD "
 60 $A+LENA=$TOP
 70 $A+LENA=" 4000"
 80 LINK#FFF7
 89 REM Switch on printer
 90 *DW
100 @=3
110 P.$2''
119 REM A=page;B=row;C=column
120 FOR A=0 TO S-1 STEP 40
130 FOR B=0 TO (S-1)*S STEP S
140 FOR C=0 TO 39
150 D=A+B+C
160 IF A+C>=S GOTO190
170 P.D?#4000
180 NEXTC
190 P.'
200 NEXTB
210 FORZ=1 TO 66-(S%66);P.';NEXTZ
220 NEXTA
229 REM All results printed-Stop
230 P.$3
240 END
>
```

Programme PNT-5

```
  1 REM SNGLINK-sorts strains by a single linkage procedure
 10 *DW
 20 *CAT
 30 INPUT"Name of matrix for sorting"$TOP
 40 INPUT"Number of strains"S
 49 REM *LOAD"Matrix name"4000
 50 A=#0100
 60 $A="LOAD "
 70 $A+LENA=$TOP
 80 $A+LENA=" 4000"
 90 LINK#FFF7
100 INPUT"Name of sorted matrix"$TOP
110 T=FOUT$TOP
120 INPUT"Name of sorted order of strains"$TOP
130 U=FOUT$TOP
199 REM P=current %age: X=group storage location
200 P=100;X=#9800;@=4
209 REM Fill group storage with end of group marker(255)
210 FORZ=0 TO 255;X?Z=255;NEXTZ
219 REM Printer on
220 P.$2'
230 DO
239 REM Get each matrix value in turn
240 FOR B=0 TO S-2
250 FOR C=B+1 TO S-1
260 D=B*S+C
270 E=D?#4000
279 REM If=current %age store strains
280 IF E=P;GOSUBa
290 NEXTC
300 NEXTB
309 REM Print current %age and groups
310 P.P''
320 Z=1;W=0
330 DOY=X?Z
340 IFY=255 P.';W=0;GOTOd
350 P.Y
360 W=W+1
369 REM If >30 strains in group print-> and start new line
370 IF W=30 P."->"';W=0
380 dZ=Z+1
390 UNTIL Y=X?Z
400 P.'
409 REM Set Z to end of first group
410 Z=0
420 DOZ=Z+1
430 UNTIL X?Z=255
439 REM Go to next %age level
440 P=P-1
449 REM Start again unless all strains in one group
450 UNTIL Z=S+1
459 REM Printer off
460 P.$3
599 REM Resort matrix in correct strain order and store
600 FOR B=1 TO S
610 D=X?B
620 FOR C=1 TO S
630 E=X?C
640 F=D*S+E
650 G=F?#4000
660 BPUTT,G
>
```

```
 670 NEXTC
 680 P.B'
 690 NEXTB
 700 SHUTT
 799 REM Store strain order
 800 FOR B=1 TO S
 810 C=X?B
 820 BPUTU,C
 830 NEXTB
 840 SHUTU
 850 END
 999 REM Look for strains in current groups
1000 aZ=-1;G=255;H=255
1010 DO Z=Z+1;F=X?Z
1019 REMIf end of all groups without both found GOTOb
1020 IF F=255 UNTILX?(Z+1)=255;GOTOb
1029 REM If strains found store positions in G and H
1030 IF F=B G=Z
1040 IF F=C H=Z
1049 REM Until1 both found
1050 UNTIL G<255 AND H<255
1059 REM Put first strain in G
1060 IF G>H Z=G;G=H;H=Z
1069 REM Find end of G's group unless H already in same group
1070 DO G=G+1
1080 UNTIL X?G=255 OR G=H
1090 IF G=H RETURN
1099 REM Find start of H's group
1100 DO H=H-1
1110 UNTIL X?H=255
1119 REM Take out groups in between
1120 Z=G-1
1130 DO Z=Z+1
1140 Z?#9C00=X?Z
1150 UNTIL Z=H
1159 REM Join H's group to G's
1160 Z=G-1
1170 DO H=H+1
1180 Z=Z+1
1190 X?Z=X?H
1200 UNTIL X?H=255
1209 REM Bring back other groups
1210 DO G=G+1
1220 Z=Z+1
1230 X?Z=G?#9C00
1240 UNTIL Z=H
1249 REM Move others up one to allow for missing 255
1250 DO X?H=X?(H+1)
1260 H=H+1
1270 UNTIL X?H=X?(H+1)
1280 RETURN
1999 REM If neither found make new group at end
2000 bIF G=H X?(Z+1)=B;X?(Z+2)=C;RETURN
2009 REM Z=location of strain found;N=number of other strain
2010 IFH=255 Z=G;N=C;GOTOc
2020 Z=H;N=B
2029 REM Find end of group of strain found
2030 cDO Z=Z+1;UNTIL X?Z=255
2039 REM Insert new strain and move rest along one
2040 DOM=X?Z;X?Z=N;N=M
2050 Z=Z+1
2060 UNTIL N=255 AND X?Z=255
2070 RETURN
```


>

Programme PNT-6

```
  1 REM MTXPLOT-Gives labelled shaded similarity matrix
 10 *CAT
 20 INPUT"Matrix to be plotted"$TOP
 30 INPUT"Number of strains"S
 39 REM *LOAD"Matrix name"4000
 40 A=#0100
 50 $A="LOAD "
 60 $A+LENA=$TOP
 70 $A+LENA=" 4000"
 80 LINK#FFF7
 89 REM Open strain name and order files
 90 INPUT"Name of strains file"$TOP
100 N=FIN$TOP
110 INPUT"Name of strain order file"$TOP
120 O=FIN$TOP
130 P.$2
140 @=3;Y=TOP+5
149 REM Set character size and pen position
150 P."S1"'"M0,2575"'
160 FOR A=0 TO S-1
169 REM Get next strain number
170 W=BGETO
180 FOR X=0 TO W
189 REM Get strain name
190 SGETN,TOP
200 NEXTX
209 REM Print strain name and start new line
210 P."P",A,"  "$Y'
220 P."M0,",2551-24*A'
230 PTRN=0
240 NEXTA
249 REM Number bottom axis
250 P."R250,-45"'
260 P."Q1"'
270 FOR A=0 TO S-1
280 P."P",A'
290 P."R24,-42"'
300 NEXTA
309 REM Go to position of first square and wait
310 P."M230,2570"'
320 P.$3,$12
330 P."Change pen"'"Press space bar to continue"'
340 DO;UNTIL ?#E21=32
350 P.$2
360 FOR A=0 TO S-1
370 FOR B=0 TO A
380 C=A*S+B
389 REM Get similarity coefficient
390 D=C?#4000
399 REM Draw square
400 P."I0,24,24,0,0,-24,-24,0"'
409 REM Select correct shading
410 D=D/5-11
420 IF D>6 D=6
430 IF D<1 D=1
440 GOSUB(1000*D)
450 NEXTB
459 REM Start new line
460 P."R",-24*(A+1)",-24"'
470 NEXTA
480 P."H"'
490 P.$3
500 END
```


>

Programme PNT-6 (Cont'd)

```
 999 REM Shading subroutines
1000 P."I24,0"';RETURN
2000 P."I24,24,0,-24"';RETURN
3000 P."I16,0,-16,16,0,8,8,0,16,-16,0,-8"';RETURN
4000 P."I0,12,12,12,12,0,-24,-24,12,0,12,12,0,-12"';RETURN
5000 P."I6,0,0,24,6,0,0,-24,6,0,0,24,6,0,0,-24"';RETURN
6000 FOR Z=1 TO 4
6010 P."I3,0,0,24,3,0,0,-24"'
6020 NEXTZ
6030 RETURN
>
```

Programme PNT-7

```
  1 REM PGROUPS-Prints results of selected groups
 10 *LOAD"RESULTS"4000
 20 *DW
 30 aP."Input strain numbers in group"'
 40 P."To end input enter 255"'
 50 A=TOP;@=3
 59 REM Store strain numbers
 60 DO INPUT B
 70 IF B=255 GOTOb
 80 ?A=B
 90 A=A+1
100 UNTIL0
110 bP.$2'
120 FOR C=TOP TO A-1
129 REM Print strain numbers
130 P.?C
140 NEXTC
150 P.''
159 REM Find number of results per strain
160 D=LEN#4000
169 REM Get each result from each strain in turn
170 FOR E=0 TO D-1
180 FOR F=TOP TO A-1
190 G=?F
200 H=G*(D+1)+E
210 P."  ",$H?#4000
220 NEXTF
230 P.'
240 NEXTE
250 P.$3'
260 GOTOa
>
```

2.6 API ZYM ANALYSIS

The API ZYM system (API, Grafton Way, Basingstoke) is a semi-quantitative micromethod designed for the detection of enzymatic activities from a wide range of biological specimens. The system was used as part of a larger study by Logan and Berkeley (1981) to investigate the taxonomy of the genus Bacillus. The strip is composed of 20 microtubes which permit the study of 19 enzymatic reactions using very small quantities. The bottom of the microtube forms a support, designed to contain the enzymatic substrate and buffer. This support permits contact between the enzyme and the substrate.

Organisms were grown for 16 h on TSBA plates at 60 °C. Suspensions of cells were prepared in distilled water to give an absorbance of 1.5 at 450 nm and 60 µl added to each reaction cupule. The strips were incubated at 60 °C for 10 h before reading the results. Enzyme production was assessed quantitatively after the addition of API ZYM reagents A and B (one drop of each to each reaction cupule). The strips were placed in bright sunlight for 10 min before reading results to eliminate any yellow colour produced due to an excess of fast blue dye which had not reacted. Colour development was compared with a standard API ZYM colour comparability chart and results recorded on a scale 0 to 5 (0 indicating no enzyme production and 5 high enzyme production).

2.7 EXAMINATION OF ESTERASE ENZYMES BY STARCH GEL ELECTROPHORESIS

All strains were grown overnight at 60 °C in the medium of Sargeant et al. (1971). Bacterial cells were harvested at 10,000 x g for 30 min at 4 °C, washed twice by resuspension in 0.15 M NaCl and then suspended in 0.15 M NaCl at a concentration of 3 g wet cells in 15 ml saline. The suspensions were disrupted by sonication (20 kHz, 5 x 1 min) on ice and cell debris removed by centrifugation at 48,000 x g for 1 h at 4 °C. The cell extracts were concentrated twenty-five fold in a Minicon IB15 (Amicon, UK) and stored at -20 °C.

Gels were prepared by dissolving 23 g soluble starch in 200 ml of electrophoresis buffer (90 mM Tris, 90 mM boric acid, 3 mM EDTA, pH 8.0) and heating with continuous stirring until maximum viscosity was attained. The viscous solution was then poured onto plates

(190 x 140 mm) to give a 4 mm deep flat bed and graphite electrodes inserted 150 mm apart. Wells were formed with a well-former designed to give 9 x 1 mm wells, 6 mm apart. The plates were stored overnight at 4 °C covered with clingfilm.

Bromophenol blue as a marker was added to each extract prior to electrophoresis. 100 µl of each sample were loaded and electrophoresis carried out at 120 volts for 2.5 h at ambient temperature. Electrophoresis buffer was dropped on to the gel surface intermittently to prevent excess heating or drying out.

The gels were stained as described by Baillie and Norris (1963) and the Rf of esterase bands determined with respect to the bromophenol blue front.

2.8 PYROLYSIS MASS SPECTROMETRY

Twelve strains (B. caldotenax, B. caldovelox, B. caldolyticus, NCA 1503, NW 10, EP 240, EP 136, EP 262, ATCC 12016, RS 93, B. coagulans ATCC 8038 and B. coagulans ATCC 12245) cultured on TSBA were examined by Dr. C. Gutteridge using pyrolysis mass spectrometry (Gutteridge & Norris, 1979).

2.9 BACTERIOCIN TYPING

Various methods were examined for the bacteriocin typing of thermophilic strains with the aim of establishing a rapid method with a high degree of reproducibility. Two methods have been studied, one involving the culturing of bacteriocin producing strains on solid media with the diffusion of bacteriocin into the surrounding agar, and secondly the culturing of producing strains in broth cultures yielding active bacteriocin in the culture supernatant.

2.9.1 Growth of bacteriocin producers on solid media

i. The initial and probably simplest technique, involved the streaking of four to six test strains approximately 5 mm apart across a TSBA plate and then streaking a bacteriocin producing strain at right angles across the centre of the plate. After overnight incubation at 60 °C, the plates were examined for evidence of growth inhibition where the two streaks were in contact. This procedure was not suitable for any quantitative comparison and was not suitable for organisms which had a tendency to form

spreading colonies.

ii. An alternative method involved the inoculation of bacteriocin producing strains onto an agar plate using a grid to enable 30 different strains to grow on one plate. Following 16 h incubation these colonies (areas of growth) were transferred by replica plating using velvet pads to a number of fresh TSBA plates (up to 30 plates from one master plate). The replica plates were incubated for 16 h at 60 °C and cells then killed by inverting the plates for 90 min over a pad of filter papers soaked with 1 - 2 ml of chloroform. After allowing the chloroform vapour to diffuse the colonies were overlaid with 3 ml of trypticase soft agar containing approx 10^8 viable cells of the test organism. When the seeded overlayer had set, the plates were inverted and incubated for 16 h at 60 °C. Growth inhibition of the test strain seeded on the plate was observed in the vicinity of the patches of growth of the bacteriocin producing strains.

Exposure to chloroform did not successfully kill all of the cells, probably due to the resistance of bacterial spores, thus resulting in the spread of bacteriocin producing colonies following addition of the overlayer. Some bacteriocins did not appear to diffuse readily through the agar and zones were not easily detected, this became apparent since zones produced were very small, although culture supernatant had previously indicated the production of bacteriocin. Alternative methods were examined to solve those problems.

iii. Removal of bacteriocin producing colonies. Replica plates were produced as outlined above but before exposure to chloroform, colonies were removed with the aid of a microscope slide. Plates were then treated as in (ii.).

iv. Growth on cellophane. Discs of cellophane were cut to exactly the agar surface and gently pressed onto the agar avoiding wrinkling of the film. Bacteriocin producing strains were streaked onto the cellophane (eight per plate) and incubated for 16 h at 60 °C. The cellophane was then carefully removed taking with it the bacterial growth leaving any products of diffusion in the agar. The plate was then overlaid as in (ii.) and zones of inhibition observed.

2.9.2 Production of bacteriocin in broth culture

Bacteriocin producing strains were inoculated to 200 ml of broth culture in 1,000 ml Erlenmeyer flasks and incubated at 60 °C for 16 h with shaking at 150 r.p.m. in a Gallenkamp orbital shaking incubator. The culture supernatant was collected by centrifugation at 9,000 x g for 20 min in a Sorval RC2B centrifuge; 20 - 40 ml samples were filtered using 0.45 µm Millex (Millipore (UK) Ltd.) filter units. This crude bacteriocin preparation was then stored at 4 °C until required. Bioassay plates (Nunc, Stone, Staffordshire) were prepared containing 200 ml of TSBA and dried at 70 °C for 1 h. The plates were then seeded with 2 ml of a suspension of the test organism (10^7 - 10^8 viable cells ml^{-1}) which was spread across the surface of the plate. After allowing 1 h for the plate to dry, wells (up to 36 per plate) were cut into the plates with a sterile No. 4 cork borer. The wells were inoculated with 0.1 ml of crude bacteriocin. The plates were then incubated at 60 °C for 16 h and zones of inhibition recorded.

2.10 BACTERIOPHAGE TYPING OF THERMOPHILIC BACTERIA

Phages were harvested after growth on B. caldotenax, NCA 1503, or RS 239 and titres were adjusted to 10^6 plaque forming units (p.f.u.) ml^{-1}. Dilutions of the bacteriophage were mixed with 0.1 ml of a 6 h culture of the bacteria in 2 ml of trypticase (BBL) soft agar (0.6%, w/v) containing 0.015 M $CaCl_2$ at 50 °C. After mixing, the soft agar was poured over the surface of TSBA plates and incubated at 55 °C overnight. The presence and number of phage plaques was recorded.

2.11 DETERMINATION OF DNA MELTING TEMPERATURE (Tm) AND CALCULATION OF THE PERCENTAGE GUANINE AND CYTOSINE (% G+C)

DNA melting temperatures were measured using a Pye Unicam SP8 100 spectrophotometer fitted with a jacketted four place cell holder. The temperature of the silica cells was raised by circulation of liquid paraffin with a Haake PF3 circulating pump. The rate of temperature increase was controlled by a Haake temperature programmer model PG10. The method used was essentially that described by Marmur and Doty (1962). DNA was isolated by the method described in Section 2.13.2 and generally dissolved in SSC buffer. The DNA concentration was adjusted to approximately 20 $\mu g\ ml^{-1}$ to give an absorbance of approximately 0.4 at 260 nm. Silica cells with a 1 cm light path were used; SSC buffer was used as a blank in the spectrophotometer. The temperature of the DNA solution within the cuvette was monitored by both the internal spectrophotometer probe and a second reference probe from a YSI telethermometer placed within one of the cells. The temperature measured by the spectrophotometer probe was recorded by the spectrophotometer chart recorder. The readings from the second probe were recorded manually on the chart. Using the multirange accessory coupled to the automatic cell changer enabled the change in absorbance of the four cells to be followed simultaneously.

Standard DNA reference samples obtained from Sigma, were used in one of the four cells during each Tm determination as an additional control. DNA from E. coli strain B (Sigma D2001) with a Tm of 91.4 ^{o}C in SSC was generally used as a control. Where Tm values were expected to be below 90 ^{o}C, salmon sperm DNA (Sigma D1501) with a Tm of 87.5 ^{o}C was used; at higher temperatures DNA from Micrococcus lysodeikticus (Sigma D8259) with a Tm in SSC of 99.5 ^{o}C was used as a control. Tm values above 95 ^{o}C were re-examined with the DNA dissolved in 0.1 SSC.

The circulating oil temperature, was stabilised at 85 - 90 ^{o}C before beginning Tm determinations. Since there was a heat loss of 8 - 10 ^{o}C between the circulatory oil bath and the cell holder, DNA samples within the cells were raised to 75 - 80 ^{o}C before the controlled heating cycle was started.

After stabilisation of the cell temperature, the programmer was switched on to raise the circulating oil temperature by 1 ^{o}C min^{-1}, this resulted in an increase within the cells of 0.85 ^{o}C min^{-1}. As the temperature was increased the samples were monitored for a rapid increase in absorbance at 260 nm as the DNA began to melt. The melting temperature was that temperature corresponding to a 50% change in hyperchromicity.

The % G+C in SSC was determined by the formulae of Marmur and Doty (1962):

$$\text{mole \% G+C} = \frac{Tm - 69.3}{0.41}$$

Samples were repeated to obtain three Tm values within 1 ^{o}C and the average value used to calculate the mole % G+C content of the DNA.

2.12 DNA HYBRIDISATION BETWEEN MESOPHILIC AND THERMOPHILIC STRAINS OF BACILLUS

Preparation and analysis of DNA. ^{32}p DNA was prepared from B. stearothermophilus NCA 1503 and B. caldotenax grown at 60 ^{o}C and from B. subtilis 168 I^{-} and B. coagulans ATCC 8038 grown at 35 ^{o}C in low phosphate medium plus $^{32}PO_4^{3-}$ (1.2 mCi l^{-1}; 44 MBq l^{-1}). For unlabelled DNA, all strains were grown at 60 ^{o}C in the BS medium of Sargeant et al. (1971), but with 1% (w/v) glucose replacing sucrose as the major carbon source. B. subtilis 168 (trp^{-}) B. amyloliquefaciens H1, B. megaterium KM, B. coagulans ATCC 8038 and 12445 and E. coli K 12 HfrH were grown at 35 ^{o}C in this medium.

Cells, washed with 0.15 M NaCl, 0.01 M Na_2EDTA, 0.01 M Tris-HCl (pH 8.0) were treated with lysozyme (0.2 mg ml^{-1}; 22,000 units mg^{-1}; Boehringer) in an identical buffer, but containing 0.05 M Na_2EDTA for 20 min at 25 ^{o}C and lysed with sodium dodecyl sulphate (20 g l^{-1}) at 70 ^{o}C for 15 min. The lysed material was incubated with Proteinase K (20 units mg^{-1}, 50 µg ml^{-1}; Boehringer) for 16 h at ambient temperature. DNA was isolated as described by Marmur (1961) and further purified as described by Shah et al. (1976).

Thermal denaturation of purified DNA was carried out by heating to 121 ^{o}C for 10 min in standard saline citrate buffer.

DNA-DNA hybridisation was carried out essentially as described by

Denhardt (1966) on Millipore HADP 25 mm diam. filters. Filters, pre-loaded with 25 µg ultra-sonicated, heat-denatured ^{32}p DNA at 65 °C for 16 h under paraffin oil. Filters were washed with 2 x SSC, dried and counted. DNA from E. coli K 12 HfrH was used as a blank in hybridisation experiments.

Reassociation values (α) were defined as:

$$100 \times \frac{(\text{Counts on heterol. filters}) - (\text{Counts on E. coli filters})}{(\text{Counts on homol. filters}) - (\text{Counts on E. coli filters})}$$

2.13 DNA ISOLATION AND PURIFICATION

2.13.1 Chromosomal DNA isolation

Cultures were grown in L broth or BS broth (200 ml) in 1 l Erlenmeyer flasks for 6 h at 60 °C and 150 r.p.m. Cells were harvested by centrifugation yielding 2 - 3 g of cell paste, and washed in 50 - 100 ml, 10 mM $CaCl_2$. Cells were resuspended in 20 ml of saline EDTA pH 8.0 (10 - 12 ml g^{-1} of cells).

Cells were lysed following the addition of 1 mg ml^{-1} of lysozyme (Sigma) and incubation at 37 °C for 30 min. If cells failed to lyse 0.5 ml of sodium lauryl sulphate (SDS; 10%, w/v) was added and shaken gently.

An equal volume of 90% (v/v) phenol (freshly distilled), equilibrated against TES buffer, was added to the lysed cells. The tubes were gently shaken for 15 - 20 min to produce a stable emulsion and then centrifuged at 3,000 x g for 5 min. The top aqueous layer was collected in a separating funnel and 4 ml of diethyl-ether added. After shaking, the lower aqeuous layer was collected and the ether layer discarded. The ether wash was repeated twice and the aqeuous layer centrifuged at 12,000 x g for 20 min to precipitate residual protein.

Two volumes of 95% (v/v) ethanol (-20 °C) was added to precipitate DNA. The DNA was collected by spooling on glass rods and washed in 95% (v/v) ethanol. The DNA was dried by passing nitrogen gas over the spools and then redissolved in SSC or 0.1 SSC buffer.

Further purification was carried out when required. 50 µg ml^{-1} of ribonuclease (BDH) (heat inactivated at 60 °C for 30 min to remove DNase activity) was added and incubated for 1 h at 37 °C. Pronase (self digested; 50µg ml^{-1}; BDH) was added and incubated for 2 h at 37 °C. Phenol deproteinisation was then repeated or alternatively 10 ml of chloroform:isoamyl alcohol (24:1) was added. After shaking

gently for 5 min and centrifugation at 5,000 r.p.m. for 5 min, the aqueous layer was removed. Chloroform extraction was repeated three times and the DNA finally reprecipitated from the aqueous layer with 95% (v/v) ethanol.

The resuspended DNA was dialysed for 3 days against 3 x 1,000 volumes of SSC or 0.1 SSC buffer.

The DNA was assayed by absorbance at 260 nm and reference to the standard curve, the purity was examined by measuring absorbance at 230 nm and 280 nm.

The DNA used for the examination of DNA hybridisation was isolated using the method of Marmur (1961).

2.13.2 The examination of thermophilic bacteria for plasmid DNA

Bacillus strains were grown overnight at 60 °C in 20 ml of BS broth in 100 ml shaking (150 r.p.m.) Erlenmeyer flasks.

The media were supplemented with 0.1 ml (^{3}H) thymidine, 1 mCi ml^{-1} (45 Ci mmol^{-1}, Radiochemical Centre, Amersham) and 0.25 ml of deoxyadenosine (10 mg ml^{-1}).

Strains of Thermus aquaticus were grown in the medium of Ramaley and Hixon (1970). Cultures were grown for 3 - 4 days at 70 °C without shaking and supplemented with 0.2 ml of (^{3}H) thymidine (1 mCi ml^{-1}) and 0.5 ml of deoxyadenosine. The cells were harvested by centrifugation and resuspended in 1.5 ml of TS buffer and 0.1 ml of lysozyme (10 mg ml^{-1} in TS) added. Following 10 min incubation on ice, 0.1 ml of Na_2 EDTA (0.25 M, pH 8.0) was added and incubation continued for a further 5 min at 20 °C. TES.DS buffer (1.5 ml) was added and the lysate produced vortexed for 2 min to produce an homogenous suspension which was then left on ice overnight. A cleared lysate was obtained following centrifugation at 45,000 x g for 60 min at 4 °C. The cleared lysates were then examined by isopycnic centrifugation in caesium chloride/ethidium bromide density gradients.

Caesium chloride (3.8 g) was dissolved in 0.5 ml of ethidium bromide (1 mg ml^{-1} in TES buffer) and 2.5 ml of TES buffer. The cleared lysate (1 ml) was then added and the mixture transferred to a 10 ml polycarbonate centrifuge tube and centrifuged at 150,000 x g (10 °C) for 24 h in an MSE 65 ultracentrifuge (MSE Scientific Ltd., Crawley, UK).

The presence of satelite bands of DNA in the density gradients were observed by their fluorescence under ultra-violet (u.v.) light using a 320 nm u.v. source (Ultra Violet Products Ltd., Winchester, UK).

Each ultracentrifuge tube was transferred to a tube piercing device, (MSE Scientific Ltd.) and five drop fractions collected from the bottom of the tube under gravity until a complete gradient was collected (about 40 fractions). A sample (25 µl) of fractions was placed on 2.5 cm glass fibre discs (Whatman Ltd., Maidstone, UK) and dried in an oven at 80 °C. Each glass fibre disc was washed with 1 x 10 ml of cold (4 °C) 5% (w/v) tri-chloroacetic acid, 1 x 10 ml 95% (v/v) ethanol and 1 x 10 ml diethyl ether. After drying for 5 min at 80 °C, each glass fibre disc was transferred to a glass scintillation vial (Searle Ltd., High Wycombe, UK) with 6 ml of scintillation fluid, NE260 (Nuclear Enterprises Ltd., London). The samples were then counted for (^{3}H) activity in an LKB 1215 Rackbeta liquid scintillation counter.

2.13.3 Bacteriophage DNA isolation

Following overnight dialysis against 1,000 volumes of PMN buffer, phage preparations were adjusted to a minimum of 10^{11} p.f.u. ml^{-1}. Proteinase K (BDH) dissolved in PMN buffer was added to the phage dialysate (20 µg ml^{-1} of phage dialysate) and incubated at 37 °C for 30 min.

The phages were then dialysed for 6 h at 4 °C against 500 volumes of PMN buffer. The dialysis buffer was then changed to formamide buffer for 16 h, followed by 48 h dialysis against 2 x 1,000 volumes of 20 mM Tris 1 mM EDTA buffer pH 7.5.

Phage DNA was assayed by absorbance at 260 nm and purity assessed by the absorbance ratio at 260:280:230 nm.

An alternative phenol extraction derived from the method of Saunders and Campbell (1965) was also used. An equal volume of 90% (v/v) phenol equilibrated twice against PMN buffer, was added to the phage suspension in saline EDTA (0.15 M NaCl - 0.1 M EDTA, pH 8.0) and shaken for 30 min at 4 °C. The aqueous phase was extracted three times with diethyl ether to remove traces of phenol and the DNA was precipitated with two volumes of 95% (v/v) ethanol.

The DNA fibres were dissolved in 0.1 SSC buffer and dialysed for 48 h at 4 °C against 2 x 1,000 volumes 20 mM Tris, 1 mM EDTA buffer pH 7.5.

Further purification of the DNA was occasionally carried out using CsCl/ethidium bromide density centrifugation. A total of 5 ml of DNA suspension in 20 mM Tris, 1 mM EDTA (pH 7.5) was added to 5 g of CsCl with 50 µl of ethidium bromide solution (10 mg ml^{-1}). The mixture was then transferred to 10 ml polycarbonate centrifuge tubes

and centrifuged at 150,000 x g at 6 °C for 20 h in an MSE 65 ultracentrifuge. The DNA bands were visualised under u.v. light and were collected using a 2 ml syringe with a hypodermic needle. The ethidium bromide was removed from the DNA by washing twice with an equal volume of iso-amyl alcohol and discarding the upper purple layer. The DNA was then dialysed for 24 h against 2,000 volumes of PMN buffer.

2.14 ISOLATION OF THERMOPHILIC BACTERIOPHAGES

2.14.1 Selection of bacteriophage from soil and compost samples using a range of indicator strains

Samples (5 g) of soil, mud or compost, etc., were added to 100 ml of TSB and incubated at 55 °C for 7 h. The cells and debris were removed by centrifugation and 10 ml samples inoculated with a range of potential phage sensitive indicator strains. Phages were then detected by plating dilutions of filtered culture supernatant with the respective potential host strains, using the soft agar double layer plating technique. Single plaques were picked off with tooth picks or a straight wire and transferred to 5 ml of PMN buffer for replating. Three successive reselection steps were made for each plaque type isolated, to ensure the purity of the phage stock.

2.14.2 Isolation of bacteriophage for specific strains using a modification of the method of Romig and Brodetsky (1961)

Samples (5 g) of soil, mud or compost were added to 15 ml of tap water and incubated at 50 °C for 24 h. After incubation, the samples were shaken and then stood for 30 min to allow debris to settle. An aliquot (5 ml) from the top layer was then added to 10 ml of TSB (supplemented with 0.015 M $CaCl_2$) and incubated at 55 °C. After 4 h, 10 ml of fresh TSB, 1.5 ml of a 6 - 8 h culture of a Str^r strain of B. stearothermophilus and 50 µg ml^{-1} of streptomycin were added. Incubation was then continued for a further 6 h. The culture supernatant was then collected after centrifugation at 4,000 x g for 20 min, and dilutions plated on the appropriate Str^r strain using the soft agar technique (Section 2.15). Single plaques were selected and purified as described earlier.

2.14.3 Isolation of spontaneously induced bacteriophage from thermophilic strains of Bacillus

The isolation of small numbers of bacteriophages from large volumes of liquid, such as culture supernatant or samples of river and pond water, required their concentration before successful detection using a conventional plate assay. Two methods were investigated based on adsorption of phage particles to hydroxylapatite.

i. Column adsorption. 10 ml hydroxylapatite columns were equilibrated in 5 mM KH_2PO_4, pH 6.8. A 200 ml volume of phage suspension (culture supernatant or water sample) was passed through the column by gravity at a rate of 15 - 60 ml h^{-1}. Phage

bound to the column were eluted with 20 ml of 1 M K_2HPO_4, pH 9.

ii. Batch adsorption. Screening culture supernatants from a large number of potentially lysogenic strains using column adsorption was rather slow and was superceded by a batch adsorption method. Organisms under investigation were cultured in 200 ml of TSB in 1 l Erlenmeyer flasks shaken at 150 r.p.m. at 55 °C for 8.5 h. Cells were removed by centrifugation at 12,000 x g and supernatant collected. An aliquot (10 ml) was taken for examination of bacteriocin production, (Section 2.17) the remaining 190 ml was added to 5 g of hydroxylapatite in a 1 l Erlenmeyer flask and gently shaken in an orbital shaker. After 1 h the flasks were removed and held at an angle for 20 min to allow settling of the hydroxylapatite. The supernatant was then discarded by pumping away and the hydroxylapatite washed with 100 ml of 5 mM KH_2PO_4, pH 6.8. After swirling for 5 min the pellet was again allowed to settle (1 h). After discarding the buffer, 7 ml of 2 M KH_2PO_4, pH 6.8 was added followed by gentle mixing for 5 min, the suspension was then centrifuged for 1 min at 2,000 x g to pellet the hydroxylapatite. The supernatant was then plated with a range of potential host strains using the soft agar technique to detect the presence of any viable phage particles.

2.14.4 Lysogenic induction of thermophilic strains of Bacillus using mitomycin C and u.v. radiation

Organisms to be examined for phage induction were grown overnight in static cultures of BS broth at 55 °C. The cultures were diluted with BS broth to give an OD of 0.1 at 460 nm and then 10 ml were added to the growth tubes. The growth tubes (Fig. 2.2) were fitted with suba-seal caps (size 25) pierced with a hypodermic needle plugged with non-absorbent cotton wool. The tubes were incubated at 55 °C in a Gallenkamp reciprocating shaking water bath. OD readings on each tube were read directly using the end of the tube which comprised the OD tube at 30 min intervals. Bacteriophage induction was carried out when the culture reached early to mid log phase, generally at an OD of 0.3 to 0.5 (460 nm).

u.v. induction. The contents of the reaction tubes were poured into prewarmed glass petri dishes and exposed to u.v. irradiation (3.1 J m^{-2} s^{-1}) for periods of 60 s, 80 s, 100 s and 120 s. The cultures were then transferred back to the respective tubes and incubation continued for a

further 4 - 5 h.

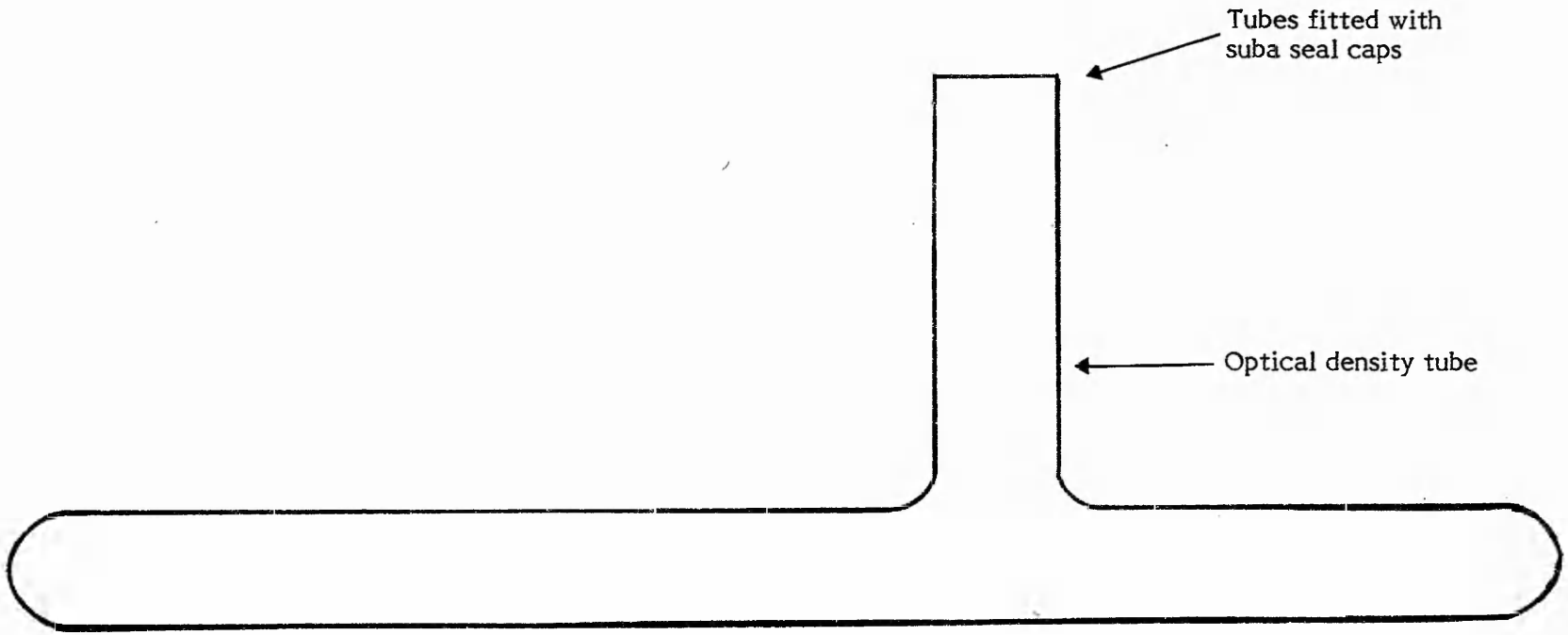
Mitomycin C induction. Mitomycin C (Sigma) was dissolved in sterile distilled water and added to the cultures to give final concentrations of 0.5, 0.1, 0.05 and 0.01 μg ml^{-1}. Incubation was then continued for a further 4 - 5 h.

The contents of the tubes were centrifuged at 6,000 x g and the supernatant retained to examine for the presence of bacteriophage.

Detection of bacteriophages. A range of indicator strains B. caldotenax, B. caldovelox, B. caldolyticus, B. stearothermophilus strains NCA 1503, NW 4S, EP 262, EP 240, ATCC 12016, RS 93, RS 15, RS 38, RS 108 and RS 125, grown overnight on TSBA, were sub-cultured in 10 ml of BS broth and incubated at 60 °C for 6 h. Using the soft agar double layer plating technique and a range of potential phage hosts, 500 μl, 100 μl, and 50 μl of the culture supernatants were examined for the presence of induced bacteriophages. Plates were incubated at 55 °C for 14 - 16 h.

Figure 2.2

T tube used in phage induction and mutation studies



Legend

The T tubes were closed with suba seal caps fitted with hypodermic needles plugged with non-absorbent cotton wool. The tubes were inoculated with 10 ml of medium and incubated in a Gallenkamp reciprocating shaking water bath. Additions were made to the tubes via a hypodermic syringe. Optical density measurements were made directly by inserting the optical density tube end of the T tube into a Corning colorimeter.

2.15 BACTERIOPHAGE MAINTENANCE AND PRODUCTION

2.15.1 Maintenance and assay

Bacteriophages were maintained as suspensions in culture media or PMN buffer. Sterility was maintained by the addition of four to five drops of chloroform to the suspension (this was not effective against spores from certain strains, e.g. RS 93 conversely some phage isolates were found to be chloroform sensitive), or alternatively by filtration through an 0.45 µm millex disposable filter unit (Millipore). TSBA plates inoculated with host and phage in 2 ml of soft agar and incubated at 55 °C for 16 h, were sealed and stored at 4 °C; they retained viability for up to three years.

Bacteriophages were titred by the soft agar double layer technique of Adams (1959) using 0.6% (w/v) trypticase soft agar supplemented with 0.015 M $CaCl_2$. Phage dilutions were made in PBMA, 0.09% (w/v) saline, or PMN buffer and incubation was carried out at 55 °C unless otherwise stated. Phage dilutions (500 µl) were added to a series of 5 ml capped pyrex test tubes containing 2 ml of soft molten agar in a 55 °C water bath. The host strain was incubated in 10 ml of BS media or peptone water for 5 h and 100 µl added to each tube. After mixing, the contents of the tubes were poured over the surface of fresh undried TSBA plates on a previously levelled bench and then incubated overnight.

2.15.2 Production of bacteriophage lysate

Production on solid medium. Phage suspensions were prepared by collecting phages from the soft agar layer of double layered plates. Production of phage on 85 mm agar plates was a relatively quick and easy means of obtaining a phage suspension up to 10^9 p.f.u. ml^{-1}. This method was used for producing all phage preparations used in transduction studies.

The host strain was incubated in 10 ml of BS broth at 55 °C for 5 - 6 h giving a cell density of $5 \times 10^7 - 10^8$ viable cells ml^{-1}. An aliquot (0.5 ml) of cell suspension and 0.5 ml of phage suspension ($10^5 - 10^6$ p.f.u. ml^{-1}) was added to 3 ml of trypticase soft agar in 5 ml pyrex tubes in a 55 °C water bath. The contents of the tubes were mixed and spread over the surface of freshly poured TSBA plates and incubated overnight at 55 °C. The soft agar layer was removed from the plates using a 10 µl disposable loop (Nunc) and added to a 50 ml centrifuge tube (Sorval). The soft agar layer was mixed and broken up with 10 ml

of PMN buffer (approximately 5 ml additional buffer was added for each extra plate used) and centrifuged at 6,000 r.p.m. for 15 min. For transduction studies, or stock suspensions, the supernatant was generally sterilised by filtration and not purified any further.

Production in Erlenmeyer flasks. The host strain (B. caldotenax, RS 93 or RS 239) was grown in TYF broth in 500 ml or 1,000 ml Erlenmeyer flasks at 55 °C and 150 r.p.m. At mid log phase (OD 0.55 - 0.6 at 420 nm) the phage suspension was added (to give an m.o.i. of 0.1) together with $CaCl_2$ to give 0.015 M and 50% (w/v) glucose to give 0.4% (w/v). Incubation was continued for a further 3 - 4 h before collecting the culture supernatant, phage titres were in the region of 10^{10} - 10^{11} p.f.u. ml^{-1}.

Where phage preparations were used for EM studies, DNA isolation, or buoyant density studies, the preparations were concentrated and further purified. The lysate supernatant was treated with 10 µg ml^{-1} of DNase, RNase and lysozyme, 203 µg ml^{-1} of $MgCl_2.6H_2O$, and incubated for 2 h at 37 °C. The phage were then pelleted at 30,000 x g for 3 h in a Sorval RC2B centrifuge. The pellet was resuspended in 2 - 5 ml PMN buffer and dialysed against 2 x 2 l of PMN buffer for 48 h. The phage particles were then banded for 24 h on a 5 ml CsCl gradient (7.5 M CsCl in 0.02 M Tris buffer) at 160,000 x g in an MSE 65 high speed centrifuge at 6 °C. The phage bands were collected and dialysed for 24 h against 2 x 2 l of PMN buffer.

Production in a 700 ml fermenter. Phage TP 1C was produced in a 700 ml Biotec fermenter (LKB) equipped with aeration, stirring, temperature control and OD monitoring through a Corning 253 colorimeter with flow cell. TSB medium (600 ml) was added asceptically to the sterilised vessel and was inoculated from a fresh plate culture of B. caldotenax. The increase in OD was monitored to 0.58 (420 nm) at which point the culture was inoculated with 5×10^6 p.f.u. ml^{-1} of phage TP 1C, 50% (w/v) glucose, to give a final concentration of 0.4% (w/v) and $CaCl_2$ to give a final concentration of 0.015 M. The viable count and bacteriophage level in the culture was monitored for 3.5 h and the culture supernatant then collected by centrifugation. Phage concentration was then carried out by either centrifugation or precipitation with $(NH_4)_2SO_4$.

Production in 20 l aerated bottles. Pyrex glass bottles containing 17 l of TSB medium were pre-warmed to 60 °C in a water bath. The seed bottle was fitted with ports for inlet and exit air, inoculation and

sampling. Seed cultures of B. caldotenax were prepared from overnight growth on four TSBA Roux bottles. Cells were washed from the surface of the agar with 50 ml of sterile water and glass beads. The cell suspensions were bulked and inoculated to the 20 l bottle. The aeration was set to 12 l min^{-1} and the OD monitored at intervals until it reached 0.68. The bottle was then inoculated with 500 ml of TP 1C suspension at 10^8 p.f.u. ml^{-1}, 170 ml of 1.5 M $CaCl_2$ and 136 ml of 50% (w/v) glucose. The OD, viable cell count and phage count were monitored at 15 min intervals for a further 4.5 h.

After removal from the water bath the bottle was cooled to room temperature and 100 mg of DNase RNase and lysozyme added together with 203 mg l^{-1} of $MgCl_2.6H_2O$. Digestion was continued for 2 h before adding 600 ml of 4 M NaCl, after a further 30 min digestion the culture (now 12 - 13 l) was centrifuged in an Alpha Laval continuous centrifuge. $(NH_4)_2SO_4$ (1,888 x g) was added to 4 l of supernatant and was then held overnight at 4 °C to precipitate the bacteriophage. The upper supernatant layer was decanted and the lower layer was centrifuged at 13,000 x g for 30 min to collect the $(NH_4)_2SO_4$ and bound phage. The $(NH_4)_2SO_4$ precipitate was taken up in 160 ml of PMN buffer and left at 4 °C overnight. After centrifugation at 4,000 x g for 20 min the precipitate was taken up in 10 ml of PMN buffer and dialysed for 48 h against 2 x 4 l of PMN buffer. The dialysate was centrifuged at 30,000 x g for 4 h and the pellet resuspended in 10 ml of PMN buffer.

2.16 BACTERIOPHAGE CHARACTERISATION

2.16.1 Determination of phage host range

The host range of phages was established as they were isolated. Phage suspensions (10^6 p.f.u. ml^{-1}) were prepared following infection of the most suitable host available (generally B. caldotenax, B. stearothermophilus NCA 1503, or strains RS 239 - RS 242). The preparation was serially diluted and dilutions plated against a range of indicator strains using soft agar. The host strain used to prepare the phage suspension was considered to have an efficiency of plating (e.o.p.) of 1. The other strains were considered in relation to this using five categories, an e.o.p. of 1, 0.1 - 0.01, 0.01 - 0.001, less than 0.001 (a few plaques observed on neat or low dilutions) and no evidence for phage infection.

2.16.2 Examination of host restriction and modification of bacteriophages

The host range of bacteriophages is dependent on phage adsorption and the presence of restriction and modification systems. The presence of a restriction and modification system becomes evident from studies of the e.o.p. data. Bacteriophages were harvested following lysis on two independent host strains. The two phage populations were then titred on both host strains. The phage titres on the original host strains were considered to have an e.o.p. of 1, comparison with the e.o.p. on the second host strain gave an indication of the presence of a restriction and/or modification system in the two host strains.

2.16.3 Study of phage plaque morphology

Plaque morphology was examined after plating phage dilutions with the appropriate indicator strain. Conditions were those described in Section 2.15.1, plates were examined following overnight incubation (16 - 18 h).

2.16.4 The effect of metal ion concentration, incubation temperature and exposure to u.v. radiation on the p.f.u. of bacteriophage JS 017

Calcium chloride or magnesium chloride solutions were added to trypticase soft agar to give a range of concentrations between 0.001 M and 0.5 M. Phage titres were then determined as described earlier.

The effect of incubation temperature on p.f.u. was examined by incubating a series of replica phage count plates overnight at a range of temperatures between 50 ^{o}C and 70 ^{o}C.

The effect of u.v. radiation was examined by exposing 10 ml of

phage suspension to 6.8 J m^{-2} s^{-1} for 2 min taking 0.1 ml samples for assay at 30 s intervals. The phage suspension was swirled gently in a glass petri dish during exposure.

2.16.5 Thermal inactivation of thermophilic bacteriophages

Suspensions of bacteriophage in PMN buffer were adjusted to 10^7 - 10^8 p.f.u. ml^{-1} and titred on the appropriate host. Aliquots (3 ml) were placed in 5 ml pyrex tubes in 50 °C, 60 °C and 70 °C water baths. Samples (0.5 ml) were titred at intervals up to 4.5 h.

2.16.6 Studies on the kinetics of phage infection

Phage infection studies were carried out on cultures grown in a 700 ml Biotec fermenter (LKB). Culture conditions are detailed in Section 2.15.2. The kinetics of infection of phages TP 1C, JS 001, JS 002 and JS 003 were examined on B. caldotenax. Cultures of B. caldotenax were infected with bacteriophage at an m.o.i. of 0.1. The viable cell count, OD and free unbound phage in the supernatant were assayed following removal of cells by centrifugation. Viable phage particles within the cells were assayed after washing the cells in fresh media and lysis by sonication. The cell suspensions were maintained on ice and sonicated for 4 x 30 s at 8 A, 20 Wc s^{-1} (viable count of B. caldotenax was reduced by more than 99.9%). The remaining intact cells were removed by centrifugation and the lysate supernatant assayed for phage.

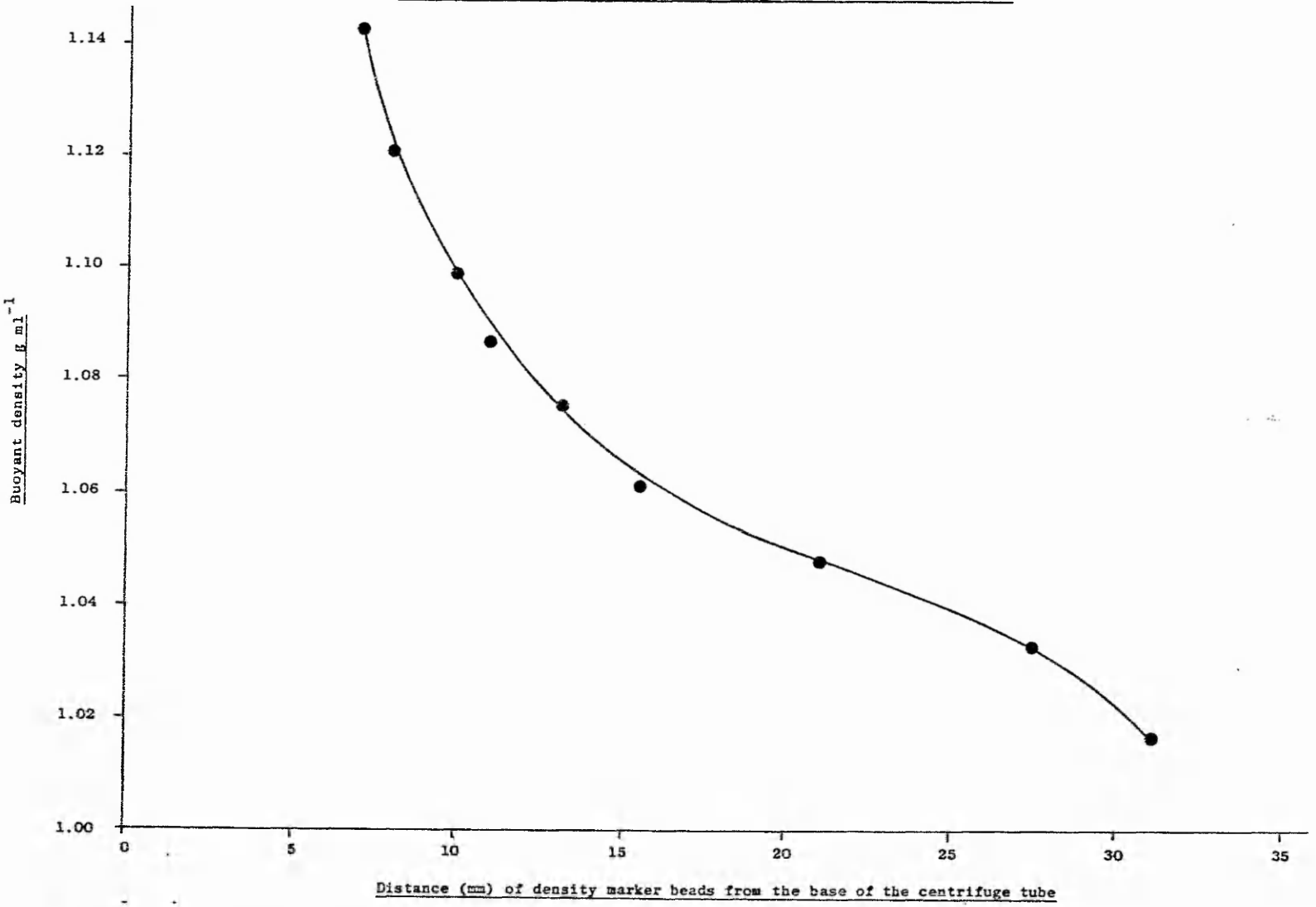
2.16.7 Determination of buoyant density of bacteriophage particles in percoll gradients using density marker beads

Percoll (Pharmacia) consists of colloidal silica particles 15 -30 nm diameter and coated with polyvinylpyrrolidone. It is able to form self generated gradients in the range 1.0 - 1.3 g ml^{-1} and is non-toxic to cells. (Further data and information is available in "Percoll methodology and applications", Pharmacia.)

An aliquot of percoll (49.5 ml) was mixed with 5.5 ml of 1.5 M NaCl to prepare an iso-osmotic stock solution. This stock solution was mixed with an equal volume of 0.1 M NaCl and 7 ml added to MSE 7 ml ultracentrifuge tubes. Concentrated phage preparations (100 µl) were loaded gently on the top of percoll gradients. To one or two control tubes was added 10 µl of each type of density marker bead. Marker beads (Pharmacia) were colour coded to indicate their buoyant density.

Figure 2.3

Percoll gradient calibration using density marker beads



Legend

Density marker beads were suspended in 7 ml of percoll and centrifuged at 60,000 x g for 15 min. The distance of each band of marker beads from the base of the tube was recorded.

Blue	1.143 g ml^{-1}
Yellow	1.121 g ml^{-1}
Green	1.097 g ml^{-1}
Red	1.087 g ml^{-1}
Blue	1.075 g ml^{-1}
Yellow	1.062 g ml^{-1}
Green	1.048 g ml^{-1}
Red	1.033 g ml^{-1}
Violet	1.017 g ml^{-1}

The tubes were centrifuged at 60,000 x g in an MSE "High Speed 65" ultracentrifuge for 15 min.

The control tube was examined and the distance of the various marker bead bands from the base of the tube was recorded. A standard curve (Fig. 2.3) of marker bead buoyant density against the distance of the marker bead bands from the base of the tube was plotted for each centrifuge run. The distance of the phage bands in the percoll gradients from the base of the tube was measured and buoyant densities determined with reference to the standard calibration curve.

2.16.8 Examination of bacteriophage by Electron Microscopy

Phage suspensions were purified by banding on caesium chloride followed by 48 h dialysis against 2 x 1 l of PMN buffer.

A drop of phage suspension (10^{10} p.f.u. ml^{-1}) was placed on a formvar/carbon filmed, 200 mesh, copper specimen grid. After approximately 30 s the drop was gently removed by touching the edge of the grid with a piece of filter paper. A drop of negative strain (1% phosphotungstic acid, pH 6.8) was then placed on the grid and similarly removed after approximately 10 s.

The grids were examined in either a Philips EM300 or a Philips EM400T electron microscope operated at accelerating voltage of 80 kV. Micrographs were recorded on Ilford EM technical film at magnifications of x 28,000 - x 46,000. Negatives were developed in Kodak D163 developer and printed on Ilford print photographic paper enlarging x 2 or x 3.

2.16.9 Examination of bacteriophage DNA with restriction endonucleases

The buffers used during the digestion of DNA with restriction endonucleases are described in Section 2.1.4 and a particular buffer used for each enzyme is outlined in Table 2.4. Before examination each DNA sample was dialysed overnight against a low salt buffer

Table 2.4

Restriction endonucleases and their activity on phage λDNA

Enzyme	Number of bands with λDNA	Buffer used
BalI	15	low
BamHI	5	medium
BclI	7	medium
BglI	22	medium
EcoRI	5	high
HhaI	>50	medium
HindIII	6	medium
KpnI	2	low
SalI	2	high
SmaI	3	*
SstI	2	low
SstII	3	low
PstI	18	medium
PvuI	15	high
XbaI	1	high

*Buffer used 20 mM KCl, 10 mM Tris, 10 mM $MgSO_4$, 1 mM Dithiothreitol.

(Section 2.1.4). DNA (1 µg) was incubated in 20 µl of buffer with 1 - 2 units of enzyme for 3 h at 37 °C, or 60 °C when using endonuclease BclI. Following incubation, the digested samples were heated at 70 °C for 5 min to inactivate the enzyme. Electrophoresis was carried out using a Protean double slab electrophoresis cell (Bio Rad Laboratories). Vertical slab gels with 15 wells (3 mm wide) were prepared using 0.8% (w/v) agarose (Bio Rad standard low m_r) dissolved in Tris borate buffer. An equal volume of bromophenol blue/sucrose/ficol was added to each digested sample which were then loaded to wells in agarose gel. Tris borate buffer was used in the electrophoresis tank. Gels were run for 3 h at 30 mA and then stained in 2 µg ml^{-1} ethidium bromide for 0.5 h. Destaining was carried out for 16 h before examining gels with a u.v. transilluminator (254 nm) (Ultraviolet Products Ltd., Winchester, UK). Gels were photographed using a polaroid MP-4 land camera with an orange Wratten gelatin filter (Kodak). The activity of all restriction enzymes was examined periodically using λ DNA.

2.17 THE DETECTION OF BACTERIOCIN (THERMOCIN) PRODUCTION FROM THERMOPHILIC BACILLUS

Bacteriocins are generally named after the species from which they are produced. Bacteriocins from B. subtilis, B. megaterium and B. stearothermophilus follow this nomenclature and are termed subtilicins, megacins, and thermocins respectively.

Organisms examined for the production of thermocin were grown in 200 ml of TSB in 1 l Erlenmeyer flasks (Section 2.14.3) shaken at 150 r.p.m. at 55 °C for 8.5 h. The culture supernatant was examined for evidence of thermocin activity. Bio-assay plates (Nunc (UK), Stone, Stafford.) containing 200 ml of TSBA were seeded with 2 ml of a 6 h culture of a bacteriocin indicator strain. Fifteen indicator strains were used to detect evidence of bacteriocin production. After allowing 1 h for the plates to dry, 20 wells were cut into each plate (using a No. 4 cork borer). Twenty supernatant samples (100 µl) were inoculated into the wells, following overnight incubation at 55 °C the plates were examined for evidence of growth inhibition of the indicator strains.

2.18 CHARACTERISATION OF THERMOCINS

2.18.1 Activity range

The activity range of thermocins was determined using the procedure detailed in Section 2.9.2. Crude bacteriocin (culture supernatant) was added (100 µl) to wells cut into a series of TSBA plates (using a No. 4, cork borer) seeded with the appropriate indicator strains.

2.18.2 Determination of size and nature of thermocins

To categorise the thermocins into the two groups described by Bradley (1967), a sample of crude bacteriocin was centrifuged at 40,000 x g for 3 h to sediment any "phage like" particles. The pellet was resuspended in PMN buffer to the original volume. Samples (5 ml) of crude bacteriocin were dialysed for 48 h against 2 x 500 volumes of PMN buffer. The original control sample, the resuspended pellet, supernatant and dialysate were assayed for activity using the well diffusion assay with the appropriate indicator strain.

2.18.3 Sensitivity to proteolytic and nuclease activity

Crude thermocin 93 was examined for sensitivity to DNase, RNase and Pronase. DNase and RNase ((BDH Ltd.) 200 µg ml^{-1} in

0.05 M Tris buffer, pH 8.2) was added to an equal volume (0.2 ml) of crude thermocin preparation. Controls of DNase + medium, RNase + medium, buffer + medium and thermocin + buffer were also prepared. Pronase (20 µg ml^{-1}; 100 µg ml^{-1}; 200 µg ml^{-1}) was added to an equal volume of crude thermocin (0.2 ml). Controls of pronase + medium and thermocin + buffer were also prepared.

All samples were incubated at 37 °C for 4 h followed by the addition of PMFS and further incubation for 30 min. Controls of PMFS + buffer and thermocin + buffer at 4 °C were also examined. Each sample was assayed for thermocin activity using the well diffusion method with B. caldolyticus as the indicator strain.

The degree of degradation of the thermocin was assessed using a standard curve of thermocin activity (assessed by zone size with dilutions of crude thermocin (Fig 6.1)).

2.18.4 Molecular weight determination by gel filtration

A crude preparation (20 ml) of thermocin 93 was concentrated ten fold (2 ml) by rotary film evaporation at 70 °C for 2 h. After centrifugation at 10,000 x g for 15 min an equal volume of 0.05 M potassium phosphate (pH 6.8) was added to 0.75 ml of the supernatant and loaded onto a Sephadex G-75 column (85 x 1.5 cm). The column was pre-equilibrated with 25 mM potassium phosphate, pH 6.8, containing 50 mM KCl. Precalibration of the column was carried out with chymotrypsinogen (24,500), myoglobin (17,000), lysozyme (14,400) and cytochrome C (12,500). Fractions (100 x 1 ml) were collected at a flow rate of 8 ml h^{-1} and assayed for activity by the well diffusion assay using Nunc bio-assay plates.

2.19 ISOLATION OF MUTANTS FROM B. CALDOTENAX AND B. STEAROTHERMOPHILUS

2.19.1 The use of penicillin enrichment for the isolation of auxotrophs from B. caldotenax

Penicillin enrichment is a standard technique used in the isolation of auxotrophic mutants from a wild type population following exposure to mutagenic agents (Davis, 1948). B. caldotenax, while being sensitive to penicillin on complex media, was less sensitive when grown on minimal Cal II medium. To increase the susceptibility to β-lactam antibiotics, the effect of various adjustments to the medium composition was examined. Other antibiotics inhibitory to cell wall synthesis were also examined.

Studies were made in T tubes (Fig 2.2) to allow in situ monitoring of the OD (400 nm for minimal medium, 420 nm for complex medium). The media used was either BS (complex) or Cal II (defined). A range of penicillin antibiotics were examined including benzyl penicillin, ampicillin, cloxacillin, methicillin, amoxycillin trihydrate, together with cycloserine, bacitracin and vancomycin. Antibiotic sensitivity in modified minimal media was examined using reduced phosphate levels and the addition of the cell wall amino acids, lysine, alanine and glutamate (10 μg ml^{-1}) to the media.

2.19.2 Mutagenesis of B. caldotenax with N-methyl-N'-nitro-N-nitroso-guanidine (NTG)

The use of NTG to induce mutations in E. coli was first described by Mandell and Greenberg (1960) and Adelberg et al. (1965).

Two general procedures are available, one involving exposure of washed cells to NTG and the second in which NTG is added to the growing culture. Both methods were examined for the induction of auxotrophic mutants from B. caldotenax.

i. NTG mutagenesis of washed cells of B. caldotenax. A 100 ml culture of B. caldotenax in BS broth was incubated at 60 °C and 150 r.p.m. for 4 h; 20 ml of culture was centrifuged and the cells washed in Tris-maleate buffer before resuspending in 20 ml of buffer. NTG (100 μg ml^{-1}) dissolved in distilled water was added to 2 ml aliquots of the washed cells. The tubes were allowed to stand for 15 min (99.99% kill, Fig. 7.5) before washing the cells to remove NTG. The cells were added to 100 ml of pre-warmed BS medium and incubation continued for a further 4 - 5 h. Cells were

then centrifuged, washed in Cal II medium and resuspended in 100 ml of Cal II medium in a 1,000 ml Erlenmeyer flask. Incubation was continued for 90 min before the addition of 100 μg ml^{-1} of bacitracin. Incubation was continued for 60 min before harvesting the cells, washing in Cal II medium and resuspending in 100 ml of Cal II medium supplemented with 0.01% (w/v) leucine, histidine, tryptophan, tyrosine, methionine, lysine, isoleucine, proline, arginine, glutamine and alanine. Incubation was then continued for 4 h to allow growth of mutants. The culture was then serially diluted to 10^{-7} and 0.5 ml of dilutions 10^{-5}, 10^{-6} and 10^{-7} plated onto ten Cal II agar plates supplemented with the above amino acids. Plates were incubated for 24 h at 60 °C before replicating suitable plates (40 - 60 colonies) onto Cal II minimal and Cal II + the above amino and supplement.

Following 24 h incubation, colonies showing growth on supplemented media, but no growth on minimal medium were selected and plated onto a grid, on supplemented Cal II agar (25 - 35 colonies per plate). Following overnight incubation, the grids were replica plated to the following amino acid combinations in Cal II agar, tyrosine and tryptophan, lysine, methionine and isoleucine, alanine and leucine, and proline, arginine and histidine.

By plating on suitable amino acid combinations the various auxotrophic requirements of the mutant isolates were determined.

ii. NTG mutagenesis of actively growing cells. Cultures of B. caldotenax grown for 4 h in BS medium at 60 °C in Erlenmeyer flasks were inoculated with 100 μg ml^{-1} of NTG. Incubation was continued for a further 50 min and then cells washed to remove NTG. Cells were returned to 100 ml of pre-warmed BS media and the procedure continued as above.

An alternative procedure, which avoided the growing up and enrichment of the mutant population, involved the direct plating of the freshly exposed "mutant" population. Dilutions were plated on TSBA to give 40 - 60 colonies per plate, these were replica plated to minimal medium and medium supplemented with a range of amino acids and vitamins. Colonies indicating growth on supplements, but no growth on minimal medium, were further examined.

2.19.3 Mutagenesis of B. stearothermophilus with ethyl methane sulphonate

EMS was used at a level of 17.5 µl ml^{-1} in place of NTG using the above procedures. An alternative method was described by Sundaram (1973) using B. stearothermophilus EP 136.

2.19.4 Mutagenesis of B. caldotenax with u.v. irradiation

A 100 ml culture of B. caldotenax in BS media was incubated at 60 °C and 150 r.p.m. in an orbital shaking incubator to an OD of 1.3 (420 nm). 10 ml were removed to a pre-warmed glass petri dish and the contents of the dish exposed to 930 J m^{-2} of u.v. irradiation which was sufficient to kill 99% of B. caldotenax cells (Fig. 7.7). The contents of the petri dish were returned to 100 ml of fresh pre-warmed BS medium and incubation continued for 4 - 5 h, before direct plate selection of mutants, or penicillin/bacitracin enrichment.

2.19.5 Isolation of thymine requiring strains

Mutants deficient in thymidylate metabolism were isolated using trimethoprim as a selective agent. Cal II or SMS defined agar plates were prepared containing necessary auxotrophic requirements and supplemented with trimethoprim (25 µg ml^{-1}) and thymine (50 µg ml^{-1}). A suspension of cells in 0.09% (w/v) saline was prepared from an overnight plate culture to give a density of 10^7 - 10^8 viable cell ml^{-1}. An aliquot (0.5 ml) was spread onto the surface of the thymine/trimethoprim plates which were then incubated for 3 days at 55 °C. Large surviving colonies appearing after 2 - 3 days incubation amongst a faint background of growth covering the plate were transferred using a straight wire and replated onto a fresh plate. The isolates were arranged on a grid (approx 40 colonies/plate), and incubated for 2 days at 55 °C. The isolates were characterised for the amount of thymine required for their normal growth and their resistance to trimethoprim. The colonies were emulsified in 2 ml of sterile distilled water and a loopful transferred to various test plates (as detailed below):

Cal II agar
Cal II agar + thymine 50 µg ml^{-1}
Cal II agar + thymine 20 µg ml^{-1}
Cal II agar + thymine 5 µg ml^{-1}
Cal II agar + thymine 2 µg ml^{-1}
Cal II agar + thymine 0.5 µg ml^{-1}
Cal II agar + thymine 50 µg ml^{-1} + trimethoprim 100 µg ml^{-1}
Cal II agar + thymine 50 µg ml^{-1} + trimethoprim 200 µg ml^{-1}
Cal II agar + thymidine 50 µg ml^{-1}

Plates were incubated for 48 h at 55 °C before recording their minimum requirement for exogenous thymine and resistance to trimethoprim.

2.20 EXAMINATION OF THYMINELESS DEATH IN THERMOPHILIC BACTERIA

Thymineless death, which occurs following starvation for thymine, is usually evident by a rapid decrease in the ability to form colonies and the formation of long filaments (Stacey, 1976).

Thy⁻ strains of B. stearothermophilus RS 93 and B. caldotenax were cultured in L broth supplemented with thymine (100 μg ml^{-1}), in 1,000 ml Erlenmeyer flasks, at 150 r.p.m. on a Gallenkamp orbital shaking incubator at 60 °C. On reaching an OD of 1.0 (420 nm), 40 ml of culture was centrifuged to collect the cells which were washed and transferred to two flasks of L broth, one supplemented with 100 μg ml^{-1} thymine. The cells were examined by Gram staining at intervals for evidence of elongation and chain formation.

2.21 TRANSDUCTION IN THERMOPHILIC STRAINS OF BACILLUS

2.21.1 Screening of thermophilic phage for transducing ability between strains of B. stearothermophilus and B. caldotenax

The majority of the phages listed in Table 5.1 able to infect B. stearothermophilus NCA 1503, RS 93, or B. caldotenax together with phages TP 1C TP 84 and ATCC 12016B were all examined for their ability to act as transducing phages. The initial screen was restricted due to the limited range of markers available. These included, thy$^-$, Str^r, protease$^-$, amylase$^-$ and thermocin production.

For the initial screen, putative transducing phages were harvested from wild type strains using the soft agar overlayer technique. Phage preparations were filtered through a Swinenex 0.45 µm disposable filter (Millipore) and titres adjusted to 10^8 p.f.u. ml^{-1}. The mutant strains were cultured in 200 ml of BS broth (supplemented as necessary e.g. thymine 50 µg ml^{-1} or thy$^-$ strains) in 1 l Erlenmeyer flasks at 60 °C in an orbital shaking incubator (Gallenkamp) at 150 r.p.m. The growth was monitored by OD at 420 nm to an OD of 1.0 (corresponding to a v.c. of 1 x 10^8 cell ml^{-1}), aliquots of cell suspension were removed from the shake flask and added to 5 ml capped pyrex tubes held in a 55 °C water bath. Phage suspensions (0.5 ml) were added to the tubes to give an m.o.i. of 0.5.

After 30 min the suspensions were centrifuged and washed in BS broth to remove free phage particles and then resuspended on 0.2 ml of the appropriate plating broth; Cal II broth for B. caldotenax and SMS broth for B. stearothermophilus NCA 1503 and RS 93. The cells were then spread onto the surface of either Cal II minimal or SMS minimal plates which, when dry, were incubated at 60 °C for 2 days. Controls were included for mutant reversion using 0.5 ml of PMN buffer in place of phage. Phage contamination controls replaced 1 ml of cells with 1 ml of L broth.

Transduction of thermocin 93 production was examined using phage harvested from strain RS 93 and used to infect B. caldotenax. Plating was carried out on TSBA plates and potential transductants examined for bacteriocin production by overlayer of colonies (killed by exposure to chloroform) with B. stearothermophilus NCA 1503 in 2 ml of trypticase soft agar. Bacteriocin producing colonies would be surrounded by a clear halo in the NCA 1503 overlayer. NCA 1503 was resistant to the phages used but sensitive to thermocin 93, interference

from secondary phage infections would therefore not arise.

Transduction of streptomycin resistance was examined using phage plated from B. caldotenax Str^r (50 μg ml^{-1}) and B. stearothermophilus NCA 1503 Str^r (50 μg ml^{-1}). Transduction was examined using wild type strains of B. caldotenax and B. stearothermophilus NCA 1503. After washing the cells to remove residual phage, they were incubated for 3 h in 10 ml of BS broth in 1 oz bottles at 60 °C. Samples (0.5 ml) were then plated onto TSBA plates supplemented with 50 μg ml^{-1} of streptomycin.

2.21.2 Optimisation of thy^+ transductants using phage JS 017

The effect of media. The effect of growth media on the level of transduction was examined following growth in double strength L broth, TSB, BS broth, TYF broth, and Cal II broth. All media were supplemented with thymine (50 μg ml^{-1}) and trimethoprim (15 μg ml^{-1}). The latter inhibited the growth of thy^- revertants. On reaching an OD of 1.2 at 420 nm 1 ml samples were added to 1 ml of phage suspension (1×10^8 p.f.u. ml^{-1}) in 5 ml pyrex tubes in a 55 °C water bath. After 30 min, cells were washed using the medium in which they were cultured before resuspending in 0.2 ml of Cal II broth and spreading over Cal II agar plates. Plates were incubated 48 h at 60 °C. Comparisons were made between plating transductants on Nutrient agar, TSBA, Cal II minimal and Cal II supplemented with 0.1% (w/v) casein hydrolysate.

The effect of $CaCl_2$. $CaCl_2$ was added to the reaction tubes to give final concentrations of 0.1 M, 0.05 M, 0.01 M, 0.005 M, 0.001 M in the transducing mixture of cells and phage.

The effect of temperature. The effect of temperature was examined using water baths set at 70 °C, 65 °C, 60 °C, 55 °C and 50 °C. Plates were incubated at 60 °C.

The effect of cell density. The effect of the age of the culture was examined by transducing samples of cells taken at various stages of the growth cycle. The effect of cell concentration was determined by diluting the cell suspension with broth to give a range of dilutions from neat to 10^{-1}.

The effect of transducing phage concentration. The effect of phage concentration was examined using phage preparations diluted in sterile PMN buffer. A range of phage concentrations from 10^6 - 10^9 p.f.u. ml^{-1} were examined for transforming ability.

The effect of u.v. irradiation. The effect of exposure of transducing phage to u.v. irradiation was observed following exposure to 6.8 J m^{-2} s^{-1} for 30 - 120 s.

2.21.3 Analysis of the B. caldotenax thy$^-$ transducing system

The effect of sub-culture on the survival of thy$^+$ transductant and revertant colonies. Transductant and thy$^+$ revertant colonies were successively replica plated to Cal II minimal agar, Cal II supplemented with thymine (50 µg ml^{-1}) and TSBA. (The level of thymine in TSBA is not sufficient for the growth of low thymine requiring strains, i.e. it contains less than 2 µg ml^{-1}.)

Examination of the cell morphology of transduced cells. Cells from transduced colonies were examined by Gram stain for evidence of filament formation or cell elongation as evidence of thymine starvation.

The effect of DNase on transducing ability. Transductions were carried out following pre-incubation of the phage for 30 min at 37 °C with DNase (BDH) at levels of 2, 10 and 25 mg ml^{-1}. A control phage preparation was inoculated at 37 °C without added DNase.

Examination of transductants for the presence of lysogenic phage. Thy$^+$ transductant colonies were sub-cultured from Cal II agar to TSBA agar plates and examined for the presence of lysogenic phage by two alternative methods.

i. The transductant colonies were overlaid with 2 ml of trypticase soft agar seeded with wild type B. caldotenax and observed for clearing around the transductant colonies.

ii. The transductants were sub-cultured three times on TSBA to eliminate the presence of residual phage from the transduction and then inoculated into 10 ml of L broth.

The broths were incubated overnight and the filtered culture supernatants examined for phage by spreading 0.1 ml samples on TSBA plates. The plates were overlaid with 2 ml of trypticase soft agar supplemented with 0.015 M $CaCl_2$ and wild type B. caldotenax cells. The plates were examined following overnight incubation at 60 °C for areas of clearing in the vicinity of the supernatant spread onto the plate.

Thy$^+$ transduction using phage isolated from a thy$^-$ strain of B. caldotenax. Phage JS 017 was harvested following lysis of a thy$^-$ strain of B. caldotenax using the soft agar technique. The phage was harvested following two successive cycles of infection in the thy$^-$

strain. Transduction was then carried out in the usual way.

Thy$^+$ transduction using phage JS 017 selected from a thy$^+$ transductant colony. In an attempt to select a high transducing phage variant, phage were isolated from transduced colonies. High titre phage preparations were prepared from a lysate of a wild type B. caldotenax and transductions carried out as normal.

Transduction of high and low thymine requiring strains of B. caldotenax. Transduction was carried out with several thy$^-$ mutants which were differentiated by the level of thymine required for normal growth on Cal II agar.

Thy$^+$ transduction using alternative thy$^-$ strains. Transduction was examined using phage JS 017 harvested from B. caldovelox, B. stearothermophilus ATCC 8005 and B. thermocatenulatus DSM 730. Attempts to transduce thy$^-$ strains of these thermophiles were also examined using the method previously described.

2.21.4 Transduction of auxotrophic markers

Transduction was examined using met, his, ilv and ade auxotrophs of B. caldotenax. The transducing phage population was prepared on wild type B. caldotenax and the mutant strains cultured in double strength L broth.

Cotransduction with thy$^-$ markers was examined following growth in L broth supplemented with thymine (100 µg ml^{-1}) and trimethoprim (20 µg ml^{-1}). Putative transductant colonies were examined by replica plating to suitable selective plates. Controls for reversion and contamination of transducing phage population were included with each test.

CHAPTER 3

BIOCHEMICAL, PHYSIOLOGICAL, MORPHOLOGICAL AND GENETIC TYPING OF THERMOPHILIC STRAINS OF BACILLUS

3. BIOCHEMICAL, PHYSIOLOGICAL, MORPHOLOGICAL AND GENETIC TYPING OF THERMOPHILIC STRAINS OF BACILLUS

3.1 SELECTION OF SUITABLE MEDIA AND TESTING METHODS FOR BIOCHEMICAL CHARACTERISATION

3.1.1 Litmus milk

In an attempt to define the optimum time for recording the test results, the growth of eight strains of thermophilic Bacillus was examined in litmus milk medium over 10 days (Table 3.1). B. coagulans and EP 262 both showed changes in the pH of the media within 24 - 48 h however, ATCC 8005 and B. caldolyticus did not show any significant change for 4 - 6 days, when both indicated a more acid reaction. Clotting of the milk was evident after 2 - 4 days; B. coagulans ATCC 12245 produced a typical acid clot which was soluble in alkali. Other strains produced rennet clots and were generally white to grey in colour and covered by a clear grey/red liquid. Three strains (ATCC 8005, B. caldolyticus, LUDA T210) appeared to digest the clot which either became smaller or disappeared.

Reduction of the litmus indicator appeared to be unstable, strain NCA 1503 reduced litmus after 4 days incubation but after 8 days the reaction appeared to be reversed, strain LUDA T210 showed similar results. On the basis of these results the activity of strains on litmus milk was recorded after 1 day, 3 days and 5 days.

Due to the number of different biochemical reactions with interfering and simultaneously appearing reaction products (Glucose produced from lactose during sterilisation, may ferment, giving a strong acid reaction; degradation of protein may lead to an alkaline reaction due to production of ammonia and amines; coagulation of casein may occur and proteins may become hydrolysed and litmus may be reduced) the litmus milk test was omitted from the subsequent numerical study. Some of these reactions, such as fermentation of glucose, galactose and lactose, and the degradation of casein were repeated as single tests within the study.

3.1.2 Citrate utilisation

Simmons' and Koser's media were examined for their reproducibility in the citrate utilisation test (Table 3.2). Koser's media indicated a high degree of reproducibility in most cases with the differences in the triplicate inoculations only varying between weak and

Table 3.1

Activity of Bacillus thermophiles on litmus milk over 10 day incubation

	B. coagulans ATCC 12245			B. stearo. NCA 1503			B. stearo. ATCC 8005			B. caldo-lyticus			B. stearo. LUDA T210			B. stearo. EP 136			B. thermo. DSM 465			B. stearo. EP 262			Uninoculated control		
	pH	clot	red	pH	clot	red	pH	clot	red	pH	clot	red	pH	clot	red	pH	clot	red	pH	clot	red	pH	clot	red	pH	clot	red
1 day	5.8	-	+	6.3	-	-	6.4	-	W	6.3	-	W	6.3	-	W	6.4	-	-	6.4	-	W	NT	-	-	6.3	-	-
2 day	4.7	+	+	6.4	-	-	6.5	+	W	6.6	-	W	6.5	-	-	6.6	-	-	6.5	-	+	7.2	-	W	6.5	-	-
3 day	4.5	+	+	6.3	-	-	6.3	+	W	6.3	+	W	6.3	-	-	6.7	-	-	6.4	-	-	7.5	-	W	6.4	-	-
4 day	4.5	+	+	6.3	+	+	6.1	+	-	6.4	+	-	6.2	+	+	6.7	-	-	6.4	-	-	7.8	-	+	6.3	-	-
5 day	4.6	+	+	6.1	+	+	6.0	+	-	6.4	+	W	6.3	+	+	6.7	-	-	6.3	-	-	8.2	-	+	6.3	-	-
6 day	4.4	+	+	6.1	+	+	5.6	+	-	6.0	+	-	6.1	+	+	7.0	-	-	6.4	-	W	8.4	-	+	6.1	-	-
8 day	4.6	+	W	6.1	+	-	5.5	W	-	6.0	W	-	6.1	+	+	7.0	-	-	6.4	-	W	7.9	-	-	6.1	-	-
10 day	4.5	+	W	5.9	+	-	5.3	W	-	5.9	W	-	6.1	-	-	6.1	-	-	6.3	-	W	7.9	-	-	6.1	-	-

Legend

Litmus milk was inoculated with 100 µl from a 5 h culture grown in peptone broth and incubated at 55 °C. Ten broths were inoculated for each strain and were discarded following recording of pH and reaction at 24 h intervals.

negative or weak and positive reactions. Simmons' media was less consistent with some triplicates indicating negative, weak and strong positive reactions. In most cases citrate utilisation was evident after 2 days on Koser's medium, although B. coagulans ATCC 12245 required 5 days for full development of the reaction. Simmons' medium required up to 6 days (DSM 466) for full development of the colour reaction. The reactions on Koser's medium were usually negative or strong positive, contrasting with the less discernable reactions on Simmons' medium which were generally regarded as weakly positive. Simmons' medium also indicated several false positive (NCA 1503) and false negative reactions (B. coagulans, DSM 465).

In the taxonomic study Koser's modified medium was used and the reactions were recorded after 5 days.

3.1.3 MRVP test

Two methods of testing for acetoin production were compared (Table 3.3) following growth in the media of Cowan and Steel (1974) and Gordon et al. (1973). The method of Barritt (1936) appeared to give a stronger reaction than the creatine reaction method of Gordon et al. (1973). B. coagulans ATCC 12245 appeared negative for acetoin production using the latter method although Gordon et al. (1973) reported a positive reaction. ATCC 8038 gave a strong positive reaction after 2 days using the medium of Cowan and Steel (1974), although ATCC 12245 appeared negative after 2 days incubation in this medium. In the taxonomic study the medium of Cowan and Steel and the method of Barritt were used.

3.1.4 Indole production

The indole test appeared to give a number of false positive results and was disregarded in the numerical study. Examination of a random sample of uninoculated peptone broths for the presence of indole, using Kovacs' reagent, indicated several false positive reactions. Closer examination of these broths indicated that the tops were sealed during autoclaving. Preparation of peptone broths using Evans, Oxoid, Difco and BBL peptone indicated that where the media was autoclaved with the tops tightly sealed, the majority gave false positive indole reactions, when the caps were loosened false positives were not detected. The false positive reaction gave the typical red colour in the reagent layer but diffused through the broth within 15 min. It appeared that sterilisation at 121 ^{o}C resulted in conversion of the tryptophan present to indole; where the tubes or bottles were vented the indole

Table 3.2

Comparison of Koser's (modified) and Simmons' media for the examination of citrate utilisation in thermophilic strains of Bacillus

Time of incubation	B. coagulans ATCC 12245		B. coagulans ATCC 8038		B. stearo. NCA 1503		B. stearo. ATCC 8005		B. caldolyticus		B. stearo. LUDA T210		B. stearo. EP 136		B. thermo. DSM 465	
	Kos	Sim	Kos	Sim	Kos	Sim	Kos	Sim	Kos	Sim	Kos	Sim	Kos	Sim	Kos	Sim
	W	-	-	-	-	-	+	-	-	-	-	-	W	-	-	VW
	W	W	-	VW	-	-	+	VW	W	W	-	-	W	-	-	VW
1 day	W	W	-	VW	-	-	+	W	+	W	-	-	W	VW	-	VW
	W	W	-	W	-	W	+	-	+	-	-	-	+	-	-	-
	W	W	-	W	-	W	+	W	+	W	-	-	+	VW	W	-
2 day	+	W	+	W	-	W	+	W	+	+	-	-	+	+	W	W
	W	W	W	W	-	W	+	-	+	-	-	-	+	-	-	-
	W	W	W	-	W	+	W	+	+	-	-	+	W	W	-	
3 day	+	W	+	+	-	W	+	W	+	+	-	-	+	+	W	W
	W	W	+	W	-	-	+	W	+	W	-	-	+	W	W	-
	W	W	+	W	-	W	+	+	+	+	-	-	+	+	W	-
4 day	+	W	+	W	-	W	+	+	+	+	-	-	+	+	+	W
	+	W	+	W	-	-	+	W	+	W	-	-	+	W	W	-
	+	W	+	W	-	W	+	W	+	+	-	-	+	+	W	-
5 day	+	W	+	+	-	W	+	+	+	+	-	-	+	+	+	W

Table 3.2 (Cont'd)

Time of incubation	B. coagulans ATCC 12245		B. coagulans ATCC 8038		B. stearo. NCA 1503		B. stearo. ATCC 8005		B. caldolyticus		B. stearo. LUDA T210		B. stearo. EP 136		B. thermo. DSM 465	
	Kos	Sim	Kos	Sim	Kos	Sim	Kos	Sim	Kos	Sim	Kos	Sim	Kos	Sim	Kos	Sim
	W	W	+	W	-	-	+	W	+	W	-	-	+	+	W	W
	W	W	+	W	-	W	+	W	+	+	-	-	+	+	W	W
6 day	+	W	+	+	-	W	+	+	+	+	-	-	+	+	+	+
	+	-	+	-	-	-	+	+	+	+	-	-	+	+	W	-
	+	W	+	+	-	W	+	+	+	+	-	-	+	+	W	W
7 day	+	W	+	+	-	W	+	-	+	+	-	-	+	+	+	W

Legend

All slopes were inoculated from an overnight culture grown on TSBA and incubated at 55 °C. Results are from triplicate tubes.

Table 3.3

Production of acetoin from B. coagulans ATCC 8038 and ATCC 12245

		B. coagulans ATCC 8038		B. coagulans 12245	
Time of incubation days	Testing method	GHP media (1973)	CS media (1974)	GHP media (1973)	CS media (1974)
2	GHP	-	W	-	-
	Barritt	+	++	W	-
3	GHP	W	W	-	-
	Barritt	+	+	W	W
5	GHP	W	W	-	-
	Barritt	+	+	W	W

Legend

A comparison of the media of Gordon et al. (GHP, 1973) and Cowan and Steel (CS, 1974) was made. Media were inoculated in triplicate from a 5 h culture grown in peptone broth and incubated at 55 °C. Three tubes of each medium were examined for acetoin production using the methods of Barritt (1936) and Gordon et al. (1973) after 2 days, 3 days and 5 days incubation.

diffused, when the caps were sealed indole remained in solution. In the taxonomic study any positive indole reactions were repeated using medium prepared in bottles on which the caps were kept loose during sterilisation.

3.1.5 Nitrate reduction (anaerobic)

Anaerobic reduction of nitrate was examined in the medium of Gordon et al. (1973) and Cowan and Steel (1974). Strains ATCC 12016, DSM 465, DSM 466, and LUDA T22, all indicated the production of gas in both media (medium was steamed before inoculation). Strains LUDA T42, LUDA T60, DSM 730 and NCA 1503 did not produce gas after 3 days incubation at 55 ^{o}C. In the taxonomic study the media of Gordon et al. (1973) was used to determine anaerobic gas production from nitrate.

3.1.6 Antibiotic sensitivity testing

For initial studies, Oxoid sensitest agar (CM 409) was used, but it became evident that a number of strains, including B. caldotenax, B. caldovelox, EP 240, ATCC 12016, B. caldolyticus, ATCC 10149 and LUDA T42, grew poorly on this medium. TSBA medium was examined as a possible alternative (Table 3.4). Zone diameters were generally smaller, however reproducibility using TSBA plates (Table 3.5) appeared satisfactory. In the taxonomic study TSBA was used for antibiotic sensitivity testing.

Table 3.4

Comparison of antibiotic sensitivity zones using Oxoid sensitivity discs on Oxoid sensitest agar and TSBA

Strains Plating	Media	Antibiotic sensitivity discs							
		Te30	E10	PN10	C30	Na30	S25	W5	CN10
B. stearothermophilus NCA 1503	TSBA	15	11	19	10	1	8	12	10
	Sensitest	15	13	18	9	3	9	17	12
B. stearothermophilus RS 262	TSBA	14	12	21	8.5	2	8	15	9
	Sensitest	12	12	21	9	0	6	16	13
B. stearothermophilus ATCC 12016	TSBA	12	11	23	8	0	8	15	11
	Sensitest	15	17	31	14	0	13	22	-
B. stearothermophilus RS 93	TSBA	11	7	17	9	1	10	1	11
	Sensitest	13	9	20	10	1.5	13	11	20
B. coagulans ATCC 12246	TSBA	12	11	13	9	0	10	3	11
	Sensitest	13	14	18	11	0	15	6	18
B. stearothermophilus EP 136	TSBA	11	4	17	9	6	10	4	9
	Sensitest	13	5	20	10	3	11	6.5	20

Legend

TSBA and sensitest agar (Oxoid) plates were seeded with the appropriate strain and allowed to dry before inoculation with the sensitivity discs (4 discs to each plate. The plates were incubated at 55°C and the diameter (mm) of the inhibition zones recorded.

Te30, tetracycline 30 µg; E10, erythromycin 10 µg; PN10, ampicillin 10 µg; C30, chloramphenicol 30 µg; Na30, naladixic acid 30 µg; S25, streptomycin 25 µg; W5, trimethoprim 5 µg; CN10, gentamicin 10 µg.

Table 3.5

Reproducibility study of antibiotic sensitivity on TSBA plates using Oxoid antibiotic sensitivity discs

Strains	Antibiotic sensitivity discs							
	Te30	E10	PN10	C30	Na30	S25	W5	CN10
B. caldotenax	17	19	27	14	0	18	18	15
	17	13	21	13	0.5	13	15	14
	17	17	23	-	0	16	16	15
RS 239	2	12	14	0	0	1	8	10
	2	10	16	1	0	1	8	8
	3	12	18	1	0	0	9	9
RS 238	9	21	0	6	10	12	15	15
	11	21	0.5	7	10	12	16	14
	11	22	1h	8	9	13	15	13

Legend

Six plates were seeded with the appropriate strain (Section 2.4) and allowed to dry before inoculation with the sensitivity discs (four discs to each plate). The plates were incubated overnight at 55 °C and the diameter (mm) of the inhibition zones recorded.

Te30, tetracycline 30 µg; E10, erythromycin 10 µg; PN10, ampicillin 10 µg; C30, chloramphenicol 30 µg; Na30, naladixic acid 30 µg; S25, streptomycin 25 µg; W5, trimethoprim 5 µg; CN10, gentamicin 10 µg.

3.2 BIOCHEMICAL, PHYSIOLOGICAL AND MORPHOLOGICAL CHARACTERISATION OF THERMOPHILIC STRAINS OF BACILLUS; ANALYSIS OF RESULTS BY NUMERICAL TAXONOMY

The results of biochemical, physiological and morphological characterisation of 102 strains of Bacillus thermophiles are presented in Table 3.6, 3.7 and 3.8.

3.2.1 Reproducibility of tests

The eight strains selected at random and examined a second time using coded identities (BB1 - T60, BB2 - RS 2, BB3 - RS 16, BB4 -RS 47, BB5 - RS 42, BB6 - RS 51, BB7 - RS 210, BB8 - RS 173) had similarity coefficients between 93% and 97% (average 95.3%) with the corresponding duplicate strains. The majority of these variations were due to differences in sugar fermentation results, represented by changes between weak and strong reactions, and weak and negative reactions. The control data which comprised identical duplicate sets of results from ten strains showed 100% similarity for each of the ten pairs.

Most of the strains examined were allocated to one of seven major clusters defined at the level of 80 - 85% similarity. The quantitative taxonomic relationships between and within the various clusters are indicated in the similarity matrix (Fig. 3.1). The dendrogram derived from this matrix is presented in Fig. 3.2. The percentage of strains giving positive reactions for each character within the various clusters was calculated and is presented in Table 3.9, 3.10 and 3.11. The strains used in the numerical taxonomy study are listed in their sorted order in a pull-out Table in Appendix I.

3.2.2 Cluster (1)

Cluster (1) contains 32 strains within two sub-groups or sub-clusters; (1a) contains 13 strains and (1b) contains 19 strains. The latter group contains the marker strains EP 136 isolated by Epstein and Grossowicz (1969) and reported to be an atypical strain of B. stearothermophilus due to its inability to hydrolyse starch. This strain was also described as a sub-species of B. coagulans (Cazzulo et al., 1969). Strain LUDA 141, one of a number of starch negative strains isolated by Walker and Wolf (1971) was also included within sub-group (1b) together with the starch negative strain RS 93 reported by Sharp et al. (1979).

Biochemical and physiological characterisation of Bacillus thermophiles

Legend (Table 3.6)

All results were recorded following 3 days incubation at 55 °C unless otherwise indicated. Weak positive reactions were recorded as W.

Starch hydrolysis was recorded: +, for a diffuse zone of hydrolysis and R, for hydrolysis in the immediate vicinity of the colonies.

Tyrosine hydrolysis was recorded following 10 days incubation.

Litmus milk was recorded: R, reduction of litmus; C, evidence of clotting.

Catalase and oxidase reactions were examined using cultures grown overnight on TSBA.

Citrate and propionate utilisation were recorded following 5 days incubation.

Table 3.6

	NCA 1503 (901)	ATCC 12016 (902)	EP 136 (903)	EP 240 (904)	EP 262 (905)	RS 93 (906)	NW 10 (907)	tenax (908)	velox (909)	lyticus (910)
Decomposition										
Starch	+	+	-	R	R	-	+	R	R	+
Casein	W	W	-	+	+	-	+	+	+	+
Gelatin	+	+	-	-	+	-	+	+	+	+
Tyrosine	-	-	-	-	+	-	-	-	-	-
Pigment	-	-	-	+	+	-	W	W	-	-
Phenyl alanine	-	-	-	-	+	-	-	-	-	-
Hippurate	-	+	-	+	+	-	+	+	+	+
Litmus milk	C	-	R	-	R	-	WC	-	-	WC
Litmus milk pH	6.1	6.0	6.4	6.1	8.2	6.8	6.1	6.35	6.45	6.0
Growth										
3% (w/v) NaCl	+	-	+	+	+	+	+	-	-	-
5% (w/v) NaCl	-	-	+	-	-	+	-	-	-	-
Azide	-	-	-	W	+	-	-	-	-	-
Lysozyme	-	-	+	+	-	-	-	-	-	-
pH 6.5	+	+	+	+	+	+	+	+	+	+
pH 6.0	+	-	W	+	+	W	+	+	+	W
pH 5.5	-	-	-	+	NT	-	+	-	+	-
Cal min	VW	+	+	NT	+	W	W	+	+	+
Cal + CA	W	+	+	NT	+	W	W	+	+	+
SMS min	-	+	W	NT	+	W	-	-	-	W
SMS + CA	VW	+	W	NT	+	+	-	W	+	+
TSBA anaerobic	+	-	W	+	W	W	+	W	W	W
Glu. anaer. growth	+	W	W	+	+	W	+	+	+	+
Glu. anaer. acid	+	-	W	+	+	-	+	W	W	+
Production										
Catalase	-	-	+	+	+	+	-	-	-	-
Oxidase	-	+	+	+	+	+	-	+	+	+
Acid (MR)	-	-	-	-	-	-	-	-	-	W
Acetoin (VP)	-	-	-	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-	-	-
Utilisation										
Citrate	-	-	+	-	+	+	-	+	+	+
Propionate	-	-	-	-	-	-	-	-	-	-

Table 3.6 (Cont'd)

	ATCC 12245 (911)	ATCC 8038 (912)	T22 (913)	T42 (914)	T60 (915)	T141 (916)	T210 (917)	T214 (918)	DSM 456 (919)	DSM 463 (920)
Decomposition										
Starch	-	-	R	R	R	-	+	+	+	-
Casein	W	-	-	-	-	-	+	+	-	-
Gelatin	-	-	+	+	+	-	+	-	+	+
Tyrosine	-	-	-	-	+	-	-	-	-	-
Pigment	-	-	-	-	+	-	-	-	-	-
Phenyl alanine	-	-	-	-	-	-	-	-	NT	-
Hippurate	-	-	-	+	+	-	-	-	-	-
Litmus milk	WR.C	NT	-	-	-	WR	WR.C	C	C	-
Litmus milk pH	6.8	NT	6.4	6.4	6.25	6.5	6.3	6.1	6.5	6.3
Growth										
3% (w/v) NaCl	-	-	-	+/-	+	+	-	-	-	-
5% (w/v) NaCl	-	-	-	-	-	+	-	-	-	-
Azide	+	+	-	+	+	-	-	-	-	-
Lysozyme	-	-	-	-	-	-	-	-	-	-
pH 6.5	+	+	+	+	+	+	+	+	+	-
pH 6.0	+	+	-	-	+	W	-	+	-	-
pH 5.5	+	+	-	-	-	-	-	-	-	-
Cal min	W	-	+	+	+	W	+	W	W	-
Cal + CA	+	W	+	+	+	W	+	+	W	-
SMS min	-	-	+	W	+	W	-	W	-	-
SMS + CA	W	W	+	W	+	W	-	W	-	-
TSBA anaerobic	+	+	+	+	+	W	+	+	+	+
Glu. anaer. growth	+	+	W	+	+	W	+	+	+	-
Glu. anaer. acid	+	+	W	+	+	-	+	+	+	-
Production										
Catalase	+	+	+	+	-	+	-	-	-	+
Oxidase	-	-	+	+	+	+	W	W	+	+
Acid (MR)	W	-	-	-	-	-	-	-	W	-
Acetoin (VP)	W	+	-	-	-	-	-	-	-	-
Indole	-	+	W	-	+	-	+	-	-	+
Utilisation										
Citrate	NT	-	+	-	-	+	-	-	-	-
Propionate	-	-	-	-	-	-	-	-	-	-

Table 3.6 (Cont'd)

	DSM 730 (921)	DSM 465 (922)	DSM 466 (923)	ATCC 8005 (924)	ATCC 10149 (925)	NCIB 8919 (926)	NCTC 10003 (927)	LO_2 516 (928)
Decomposition								
Starch	R	R	R	+	+	+	+	+
Casein	+	-	-	+	+	+	+	+
Gelatin	+	-	+	+	-	+	+	-
Tyrosine	-	-	-	-	-	-	-	-
Pigment	-	+	+	+	-	-	-	-
Phenyl alanine	-	-	-	-	-	-	-	-
Hippurate	-	+	-	+	-	-	-	-
Litmus milk	-	R	R	RC	-	-	RC	-
Litmus milk pH	6.8	6.2	7.0	5.8	6.3	6.3	6.0	6.3
Growth								
3% (w/v) NaCl	-	+	+	-	-	-	-	W
5% (w/v) NaCl	-	-	-	-	-	-	-	W
Azide	+	-	-	-	-	-	-	-
Lysozyme	-	-	-	NT	-	-	-	+
pH 6.5	+	+	+	+	+	+	+	W
pH 6.0	+	+	W	+	+	-	+	-
pH 5.5	+	-	-	-	-	-	+	-
Cal min	+	+	+	+	W	+	W	NT
Cal + CA	+	+	+	+	W	+	W	NT
SMS min	-	-	+	W	-	-	-	NT
SMS + CA	-	+	+	+	-	-	-	NT
TSBA anaerobic	-	+	+	W	+	+	W	NT
Glu. anaer. growth	W	W	+	+	+	+	+	W
Glu. anaer. acid	W	-	W	-	+	+	+	-
Production								
Catalase	-	+	+	+	-	-	-	+
Oxidase	+	+	+	+	-	W	+	+
Acid (MR)	-	-	-	-	+	-	+	-
Acetoin (VP)	-	-	-	-	-	-	-	+
Indole	-	-	-	+	-	-	-	NT
Utilisation								
Citrate	+	W	+	+	-	-	-	+
Propionate	-	-	-	-	-	-	-	-

Table 3.6 (Cont'd)

	RS 1	RS 2	RS 3	RS 4	RS 5	RS 6	RS 7	RS 8	RS 9	RS 10
Decomposition										
Starch	R	R	-	+	NT	-	+	+	+	-
Casein	-	-	-	+	NT	-	+	+	-	-
Gelatin	-	+	-	-	NT	-	+	+	+	-
Tyrosine	-	-	-	-	-	-	-	-	-	-
Pigment	W	-	-	-	W	-	-	-	-	-
Phenyl alanine	-	-	-	-	-	-	W	-	-	-
Hippurate	W	+	-	-	-	-	+	-	+	-
Litmus milk	-	-	-	-	-	-	C	-	-	-
Litmus milk pH	5.65	6.1	6.1	5.85	5.96	7.7	6.1	5.95	6.0	5.9
Growth										
3% (w/v) NaCl	+	+	+	+	-	-	W	+	+	+
5% (w/v) NaCl	+	-	+	+	-	-	-	+	+	W
Azide	+	+	-	-	-	-	+	+	-	-
Lysozyme	-	-	-	+	+	+	-	+	-	-
pH 6.5	+	+	+	+	+	+	+	+	+	NT
pH 6.0	-	+	-	W	-	+	+	+	W	-
pH 5.5	-	+	-	-	-	-	-	+	-	-
Cal min	+	+	-	W	W	W	+	W	-	-
Cal + CA	+	+	W	W	+	+	+	+	W	W
SMS min	+	+	-	-	NT	+	-	+	-	-
SMS + CA	+	+	W	-	NT	+	-	+	W	W
TSBA anaerobic	NT	W	-	W	-	-	-	+	W	VW
Glu. anaer. growth	-	+	+	+	-	W	W	-	+	+
Glu. anaer. acid	-	+	-	+	-	-	-	-	W	-
Production										
Catalase	+	+	+	NT	-	-	-	+	+	+
Oxidase	+	+	+	NT	+	+	+	+	+	+
Acid (MR)	-	-	-	-	-	-	-	-	-	-
Acetoin (VP)	-	-	-	W	-	-	-	+	-	-
Indole	-	NT	NT	NT	NT	NT	NT	NT	NT	NT
Utilisation										
Citrate	-	+	-	-	+/-	+/-	+	-	-	-
Propionate	-	-	-	-	-	-	-	-	-	-

Table 3.6 (Cont'd)

	RS 11	RS 12	RS 13	RS 14	RS 15	RS 16	RS 17	RS 18	RS 19	RS 20
Decomposition										
Starch	-	+	-	-	R	R	+	-	-	R
Casein	-	-	-	-	+	+	-	-	-	W
Gelatin	-	+	-	-	-	+	-	-	-	+
Tyrosine	-	-	-	-	-	-	-	-	-	+
Pigment	-	-	-	-	-	W	-	-	-	+
Phenyl alanine	-	W	-	-	-	W	W	-	-	VW
Hippurate	-	+	-	-	+	+	-	-	-	+
Litmus milk	-	-	-	-	R	R	-	-	C	R
Litmus milk pH	6.0	6.06	6.1	6.1	NT	6.1	6.1	6.7	NT	NT
Growth										
3% (w/v) NaCl	+	+	+	+	-	-	+	+	+	-
5% (w/v) NaCl	W	+	+	+	-	-	+	+	+	-
Azide	-	-	-	W	-	+	+	-	-	W
Lysozyme	-	-	-	-	-	-	-	-	-	-
pH 6.5	+	+	W	+	+	+	+	+	W	W
pH 6.0	-	W	-	-	W	+	+	-	-	-
pH 5.5	-	-	-	-	-	-	+	-	-	-
Cal min	W	W	-	+	+	+	+	+	W	+
Cal + CA	W	+	W	+	+	+	+	+	W	+
SMS min	-	-	-	+	+	W	W	+	-	-
SMS + CA	-	+	W	+	+	+	+	+	+	-
TSBA anaerobic	VW	VW	-	-	-	VW	+	W	-	NT
Glu. anaer. growth	+	W	W	W	W	W	W	W	W	NT
Glu. anaer. acid	-	-	-	-	-	-	W	-	-	NT
Production										
Catalase	+	-	+	+	-	-	+	+	+	+
Oxidase	+	NT	+	+	+	+	+	+	+	+
Acid (MR)	-	-	-	-	-	-	-	-	-	NT
Acetoin (VP)	-	-	W	-	-	W	+	-	-	NT
Indole	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Utilisation										
Citrate	-	-	-	-	+	+	-	+	-	+
Propionate	-	-	-	-	-	-	-	+	-	-

Table 3.6 (Cont'd)

	RS 21	RS 22	RS 23	RS 37	RS 42	RS 43	RS 44	RS 45	RS 47	RS 48
Decomposition										
Starch	-	-	-	-	-	-	+	+	WR	WR
Casein	-	-	-	-	-	-	W	W	-	-
Gelatin	-	-	-	-	-	-	+	+	-	-
Tyrosine	-	W	-	-	-	-	-	-	-	-
Pigment	-	-	-	-	-	-	-	-	-	-
Phenyl alanine	-	-	-	-	-	-	-	-	-	-
Hippurate	W	-	-	-	-	-	-	-	-	-
Litmus milk	-	-	-	-	-	-	-	-	-	-
Litmus milk pH	6.3	6.24	6.15	6.13	6.1	6.05	6.1	6.1	6.5	6.1
Growth										
3% (w/v) NaCl	+	+	+	+	+	+	+	+	+	+
5% (w/v) NaCl	+	+	+	+	+	+	+	+	+	-
Azide	-	-	-	-	-	-	+	+	-	+
Lysozyme	W	-	-	-	-	W	+	-	-	-
pH 6.5	+	+	+	+	+	+	+	-	+	+
pH 6.0	-	+	-	-	-	-	+	-	+	+
pH 5.5	-	-	-	-	-	-	+	-	-	-
Cal min	W	W	-	+	-	-	+	NT	W	+
Cal + CA	W	W	W	+	-	-	+	NT	W	+
SMS min	+	+	-	+	-	-	+	NT	+	W
SMS + CA	+	+	W	+	-	-	+	NT	+	+
TSBA anaerobic	W	W	W	W	VW	VW	W	VW	VW	W
Glu. anaer. growth	NT	NT	W	+	-	+	W	-	W	+
Glu. anaer. acid	NT	NT	-	W	-	-	W	-	W	+
Production										
Catalase	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+
Acid (MR)	-	-	-	NT	NT	NT	-	NT	-	-
Acetoin (VP)	-	-	-	NT	NT	NT	+	NT	-	-
Indole	NT	NT	NT	NT	NT	NT	NT	NT	NT	-
Utilisation										
Citrate	+	+	-	+	-	-	-	-	W	-
Propionate	W/-	-	-	-	-	-	-	-	-	-

Table 3.6 (Cont'd)

	RS 49	RS 51	RS 53	RS 54	RS 56	RS 57	RS 58	RS 70	RS 80	RS 85
Decomposition										
Starch	+	R	-	+	R	-	+	-	WR	+
Casein	+	-	-	+	W	-	+	-	+	+
Gelatin	+	-	-	+	+	-	+	-	+	+
Tyrosine	-	+	-	-	-	-	+	-	-	-
Pigment	-	+	-	-	+	-	+	-	-	W
Phenyl alanine	NT	-	-	-	-	-	-	-	-	-
Hippurate	-	+	-	-	+	-	+	NT	-	+
Litmus milk	-	-	-	-	-	-	RWC	-	-	NT
Litmus milk pH	6.1	6.25	6.2	6.1	6.3	6.6	7.3	7.9	6.15	7.1
Growth										
3% (w/v) NaCl	+	W	+	+	-	+	-	+	-	-
5% (w/v) NaCl	+	-	+	+	-	-	-	+	-	-
Azide	+	-	-	+	+	-	+	W	-	-
Lysozyme	-	-	-	+	-	-	-	-	-	-
pH 6.5	+	+	+	+	+	+	-	+	+	+
pH 6.0	+	-	-	+	+	-	-	+	-	-
pH 5.5	+	-	-	+	-	-	-	-	-	-
Cal min	+	W	W	+	-	VW	-	+	W	+
Cal + CA	+	W	W	+	-	VW	VW	+	W	+
SMS min	NT	W	+	+	-	W	W	+	-	+
SMS + CA	NT	+	+	+	-	W	W	+	W	+
TSBA anaerobic	W	+	VW	W	W	VW	W	W	W	-
Glu. anaer. growth	+	-/W	+	+	+	+	+	+	NT	+
Glu. anaer. acid	W	-/W	-	+	+	-	+	W	NT	+
Production										
Catalase	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	-	+
Acid (MR)	NT	-	-	-	-	-	W	-	-	W
Acetoin (VP)	NT	-	-	+	-	-	-	-	-	-
Indole	NT	NT	-	NT	-	-	NT	-	-	NT
Utilisation										
Citrate	-	+	+	-	-	-	-	+	-	+
Propionate	-	-	-	-	-	-	-	+	-	-

Table 3.6 (Cont'd)

	RS 86	RS 87	RS 98	RS 99	RS 155	RS 166	RS 168	RS 173	RS 174	RS 182
Decomposition										
Starch	+	R	WR	WR	WR	R	R	R	+	-
Casein	+	-	-	-	-	+	-	-	-	-
Gelatin	+	+	-	-	-	+	-	-	-	+
Tyrosine	-	+	-	-	-	+	-	+	+	-
Pigment	W	+	-	-	-	+	-	W	+	-
Phenyl alanine	-	-	-	-	-	-	-	-	-	-
Hippurate	+	+	-	-	-	+	+	W	-	-
Litmus milk	R	RC	-	-	RC	WR	-	WR	-	-
Litmus milk pH	6.15	5.4	6.35	6.6	5.8	5.7	6.3	NT	5.8	6.1
Growth										
3% (w/v) NaCl	-	-	+	+	-	-	+	-	-	+
5% (w/v) NaCl	-	-	+	+	-	-	-	-	-	+
Azide	-	-	-	-	+	+	-	+	+	-
Lysozyme	+	-	-	-	-	-	-	-	-	-
pH 6.5	+	-	+	+	+	-	+	-	-	-
pH 6.0	-	-	+	+	+	-	W/-	-	-	-
pH 5.5	-	-	W	+	+	-	-	-	-	-
Cal min	+	W	+	+	W	+	VW	+	W	-
Cal + CA	+	+	+	+	+	+	W	+	+	-
SMS min	-	-	+	+	-	W	-	-	-	-
SMS + CA	-	-	+	+	W	W	+	+	-	-
TSBA anaerobic	+	+	W	VW	+	W	+	W	W	VW
Glu. anaer. growth	+	+	+	+	+	+	+	+	+	W
Glu. anaer. acid	+	+	+	W	+	+	+/-	+	+	-
Production										
Catalase	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	-	+	+	+	-	+
Acid (MR)	W	-	-	-	W	-	-	W	W	-
Acetoin (VP)	-	-	-	-	-	-	-	-	-	-
Indole	-	-	-	-	-	NT	NT	-	NT	NT
Utilisation										
Citrate	+	+	+	-	-	+	+	-	+	-
Propionate	-	-	-	-	-	-	-	-	-	-

Table 3.6 (Cont'd)

	RS 187	RS 203	RS 204	RS 205	RS 209A	RS 209B	RS 210	RS 211	RS 215	RS 216
Decomposition										
Starch	R	R	R	-	-	R	-	R	R	-
Casein	-	-	-	-	-	-	-	-	+	-
Gelatin	+	+	+	+	-	-	-	-	+	-
Tyrosine	-	-	-	+	-	+/-	-	+	-	-
Pigment	-	W	+	+	-	W	-	+	+	-
Phenyl alanine	-	W	-	-	-	-	-	-	-	-
Hippurate	-	-	+	+	-	-	-	-	+	-
Litmus milk	-/R	-	NT	WR	-	-	-	R	-	-
Litmus milk pH	NT	6.3	NT	7.8	6.1	6.3	6.2	6.3	NT	6.1
Growth										
3% (w/v) NaCl	+	+	+	-	+	+	+	+	+	+
5% (w/v) NaCl	+	W	+/-	-	+	-	W	-	-	W
Azide	+	+	-	+	+	W	-	W	+	-
Lysozyme	-	-	-	-	-	-	-	-	+	-
pH 6.5	+	+	+	+/-	+	+	+	+	+	-
pH 6.0	-	-	-	+/-	W	-	-	W/-	+	-
pH 5.5	-	-	-	-	-	-	-	-	-	-
Cal min	+	+	+	W	W	W	W	+	+	+
Cal + CA	+	+	+	W	W	W	W	+	+	+
SMS min	+	-	-	-	+	VW	W	VW	-	-
SMS + CA	+	+	+	-	+	VW	W	+	VW	VW
TSBA anaerobic	+	+	+	W	W	+	W	-	VW	VW
Glu. anaer. growth	W	+	+	+	W	W/-	W	W	W	W
Glu. anaer. acid	-	+/-	+	+	+	-	W	-	+	-
Production										
Catalase	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	W	+	W	+	+	+	+
Acid (MR)	-	-	-	W	-	-	-	-	-	-
Acetoin (VP)	-	-	-	-	-	-	-	-	-	VW
Indole	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Utilisation										
Citrate	+	+	+	-	+	+	+	+	+	-
Propionate	-	-	-	-	W	-	-	-	-	-

Table 3.6 (Cont'd)

	RS 217	RS 222	RS 236	RS 237	RS 238	RS 239	RS 240	RS 241	RS 242	RS 243
Decomposition										
Starch	WR	+	WR	-	R	-	-	-	-	+
Casein	-	W	-	-	-	-	-	-	-	-
Gelatin	-	+	+	+	+	-	-	-	-	+
Tyrosine	-	-	-	-	-	-	-	-	-	-
Pigment	-	+/-	-	-	-	-	-	-	-	-
Phenyl alanine	-	-	-	-	-	-	-	-	-	W/-
Hippurate	-	+	-	-	+	-	-	-	-	-
Litmus milk	-	-	-	-	WR	-	-	-	R	-
Litmus milk pH	7.5	6.1	5.9	6.0	5.9/ 6.7	6.0	6.1	NT	6.6	6.3
Growth										
3% (w/v) NaCl	+	+/-	-	-	+	+	+	+	+	W
5% (w/v) NaCl	+	-	-	-	W	W	+	W	W	-
Azide	-	W	-	-	-	-	+	-	+	+
Lysozyme	-	+/-	-	-	-	-	-	-	-	-
pH 6.5	+	+	W	W	+	W/-	+	-	W	-
pH 6.0	+	-	-	-	+	-	W	-	-	-
pH 5.5	-	-	-	-	-	-	W	-	-	-
Cal min	+	W	-	-	+	-	-	-	VW	VW
Cal + CA	+	W	-	-	+	VW	VW	VW	VW	VW
SMS min	NT	-	-	-	W	-	-	-	-	-
SMS + CA	NT	-	-	-	+	VW	VW	VW	-	VW
TSBA anaerobic	-	W	-	+	W	-	-	W	W	+
Glu. anaer. growth	+	+	W	W	W	-	+	W	W	+
Glu. anaer. acid	+	+/-	-	-	-	-	-	-	W	+
Production										
Catalase	+	-	+	+	+	+	+	NT	+	+
Oxidase	+	+	+	-	+	+	+	NT	+	+
Acid (MR)	-	-	NT	-	-	NT	NT	NT	-	NT
Acetoin (VP)	-	-	NT	-	-	NT	NT	NT	-	NT
Indole	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Utilisation										
Citrate	+	-	-	-	+	-	-	-	-	-
Propionate	+	-	-	-	-	-	-	-	-	-

Table 3.6 (Cont'd)

	RS 244	RS 245	RS 248	RS 249
Decomposition				
Starch	-	+	+	WR
Casein	-	+	+	-
Gelatin	-	+	+	-
Tyrosine	-	-	+/-	-
Pigment	-	-	+	-
Phenyl alanine	-	-	W	-
Hippurate	-	-	NT	-
Litmus milk	R	-	R	R/-
Litmus milk pH	7.1	6.3	6.5	6.2
Growth				
3% (w/v) NaCl	+	+	-	+
5% (w/v) NaCl	+	+	-	+
Azide	W	W	W	-
Lysozyme	-	W	-	-
pH 6.5	+	+	+	+
pH 6.0	-	+	+	-
pH 5.5	-	W	+/-	-
Cal min	W	W	+	W
Cal + CA	W	+	+	W
SMS min	W	W	W	W
SMS + CA	+	+	+	W
TSBA anaerobic	VW	+	+	W
Glu. anaer. growth	W	+	W	+
Glu. anaer. acid	W	+	-	-
Production				
Catalase	+	NT	-	NT
Oxidase	+	NT	+	+
Acid (MR)	-	-	W	-
Acetoin (VP)	-	W	-	-
Indole	NT	NT	NT	NT
Utilisation				
Citrate	+	-	W	+
Propionate	-	-	+	-

Table 3.6 (Cont'd)

Duplicate tests on a random selection of strains to examine reproducibility

	T60	RS 2	RS 16	RS 47	RS 42	RS 51	RS 210	RS 173
Decomposition								
Starch	R	R	R	R	-	R	-	R
Casein	-	-	+	-	-	W	-	-
Gelatin	+	+	+	-	-	-	-	-
Tyrosine	+	-	NT	-	-	+	-	+
Pigment	+	-	W	-	-	+	-	W
Phenyl alanine	-	-	-	-	-	-	-	-
Hippurate	+	W	+	-	-	+	-	+
Litmus milk	NT	NT	NT	NT	NT	NT	NT	NT
Litmus milk pH	NT	NT	NT	NT	NT	NT	NT	NT
Growth								
3% (w/v) NaCl	+	+	-	+	+	+	+	-
5% (w/v) NaCl	-	-	-	W	+	-	+	-
Azide	+	+	+	-	-	-	-	+
Lysozyme	-	-	-	-	-	-	-	-
pH 6.5	+	+	-	+	NT	+	W	-
pH 6.0	+	+	+	+	-	-	-	-
pH 5.5	+	+	+	+	-	-	-	-
Cal min	NT	NT	NT	NT	NT	NT	NT	NT
Cal + CA	NT	NT	NT	NT	NT	NT	NT	NT
SMS min	NT	NT	NT	NT	NT	NT	NT	NT
SMS + CA	NT	NT	NT	NT	NT	NT	NT	NT
TSBA anaerobic	W	W	W	W	NT	+	W	W
Glu. anaer. growth	+	+	W	W	-	W	W	+
Glu. anaer. acid	+	+	-	+	-	W	W	+
Production								
Catalase	-	+	-	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+
Acid (MR)	-	-	-	-	-	-	-	NT
Acetoin (VP)	-	-	-	NT	NT	NT	NT	NT
Indole	NT	NT	NT	NT	NT	NT	NT	NT
Utilisation								
Citrate	-	+	+	NT	-	+	+	-
Propionate	-	-	-	-	-	-	-	-

Biochemical characterisation of Bacillus thermophiles

Legend (Table 3.7)

All results were recorded following 3 days incubation at 55 ^{o}C.

Table 3.7

Tests	NCA 1503 (901)	ATCC 12016 (902)	EP 136 (903)	EP 240 (904)	EP 262 (905)	RS 93 (906)	NW 10 (907)	tenax (908)	velox (909)	lyticus (910)
Reduction										
NO_3 - NO_2	+	-	-	-	+	-	+	+	+	+
NO_3 - NO	-	-	-	+	-	-	-	-	-	-
NO_3 - gas	-	+	-	-	-	-	-	-	-	-
NO_2 - NO	-	-	-	+	-	-	+	-	-	-
NO_2 - gas	-	+	-	-	-	-	-	-	-	-
Anaer. gas	-	+	-	-	-	-	-	-	-	-
Acid Prod.										
Adonitol	-	-	-	-	-	+	-	-	-	-
Aesculin	-	-	-	-	-	-	-	-	-	-
Arabinose	-	+	-	-	-	-	W	-	-	-
Dextrin	+	-	+	+	+	-	+	+	+	+
Dulcitol	-	-	-	-	-	-	-	-	-	-
Erythritol	-	-	+	-	-	-	-	-	-	-
Fructose	+	W	+	+	-	W	+	+	W	W
Galactose	W	+	-	-	-	-	W	-	-	-
Glucose	+	+	+	+	+	W	+	+	+	+
Glycerol	+	+	W	+	-	+	+	+	+	W
Glycogen	+	W	-	+	+	-	+	-	-	+
Inositol	-	-	W	-	-	W	-	-	-	-
Inulin	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	W	-	-	-
Maltose	+	+	+	+	+	-	+	+	+	W
Mannose	+	+	-	+	W	W	+	+	+	+
Mannitol	-	W	+	-	-	+	-	W	+	W
Raffinose	+	-	-	-	-	-	+	W	W	-
Rhamnose	-	-	W	-	-	-	-	-	-	-
Salicin	-	W	W	-	-	-	-	-	-	-
Sorbitol	-	-	+	-	-	W	+	-	-	-
Starch	+	-	-	+	W	-	+	-	-	+
Sucrose	+	-	+	+	+	+	+	+	+	+
Trehalose	+	-	W	+	-	W	+	-	+	+
Xylose	-	+	-	-	-	-	+	-	-	-

Table 3.7 (Cont'd)

Tests	ATCC 12245 (911)	ATCC 8038 (912)	T22 (913)	T42 (914)	T60 (915)	T141 (916)	T210 (917)	T214 (918)	DSM 456 (919)	DSM 463 (920)
Reduction										
NO_3 - NO_2	+	-	-	+	W	-	+	+	-	-
NO_3 - NO	-	-	-	-	W	-	-	-	-	-
NO_3 - gas	-	-	+	-	-	-	-	-	+	-
NO_2 - NO	-	-	-	-	+	-	+	+	-	-
NO_2 - gas	-	-	+	-	-	-	-	-	+	-
Anaer. gas	-	-	+	-	-	-	-	-	-	-
Acid Prod.										
Adonitol	-	-	-	-	-	-	-	-	-	-
Aesculin	-	-	-	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-	-	-	W/-
Dextrin	+	+	W	-	NT	-	+	W	+	-
Dulcitol	-	-	-	-	-	VW	-	-	-	-
Erythritol	-	-	-	-	-	NT	-	-	-	-
Fructose	+	+	W	W	W	W	W	W	+	-
Galactose	+	+	-	-	-	W	W	W	W	-
Glucose	+	+	W	W	W	VW	+	+	+	-
Glycerol	W	+	+	W	W	-	W	+	+	-/W
Glycogen	-	W	-	-	-	-	+	+	+	-
Inositol	-	-	-	-	-	-	-	-	-	-
Inulin	-	-	-	-	-	-	-	-	-	-/W
Lactose	+	+	-	-	-	-	-	-	-	-
Maltose	+	+	+	+	+	+	+	+	+	VW
Mannose	+	+	W	W	W	-	W	W	+	W/-
Mannitol	-	W	W	-	+	W	-	-	-	-
Raffinose	-	-	-	-	-	-	+/-	+	+	-
Rhamnose	-	+	-	-	NT	-	-	-	W	VW
Salicin	+	+	+	W	+	W	-	+	-	-
Sorbitol	-	+	W	-	-	-	-	-	-	-
Starch	W	W	+	-	+	-	+	+	+	-
Sucrose	+	-	W	+	+	W	+	+	+	-
Trehalose	+	+	+	+	+	VW	+	+	+	W
Xylose	W	W	W	-	W	-	-	-	-	W

Table 3.7 (Cont'd)

Tests	DSM 730 (921)	DSM 465 (922)	DSM 466 (923)	ATCC 8005 (924)	ATCC 10149 (925)	NCIB 8919 (926)	NCTC 10003 (927)	LO_2 516 (928)
Reduction								
NO_3 - NO_2	+	-	-	NT	+	+	+	NT
NO_3 - NO	-	-	-	NT	-	-	-	NT
NO_3 - gas	-	+	+	NT	-	-	-	NT
NO_2 - NO	W	-	-	-	+	-	+	NT
NO_2 - gas	-	+	+	-	-	-	-	NT
Anaer. gas	-	+	+	-	-	-	-	NT
Acid Prod.								
Adonitol	-	-	-	-	-	-	-	-
Aesculin	-	-	-	-	-	-	-	-
Arabinose	-	-	-	-	-	-	-	+
Dextrin	W	W	W	+	+	W	+	W
Dulcitol	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-
Fructose	+	W	W	+	W	W	W	+
Galactose	-	-	-	-	-	W*	W*	-
Glucose	+	W	+	W	+	+	+	+
Glycerol	-	W	W	-	+	W	+	W
Glycogen	-	-	-	-	+	+	+	W
Inositol	-	NT	NT	-	NT	NT	-	-
Inulin	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-
Maltose	+	+	+	W	+	+	+	+
Mannose	W	W	-	W	W	W	+	+
Mannitol	W	-	W	-	-	-	-	+
Raffinose	-	-	+	-	+	-	+	-
Rhamnose	-	NT	NT	-	-	NT	NT	-
Salicin	W	+	-	W	+	+	+	+
Sorbitol	-	-	-	-	-	-	-	W
Starch	-	NT	+	W	+	+	+	-
Sucrose	-	-	W	-	+	+	+	+
Trehalose	+	+	+	W	+	+	+	+
Xylose	-	+	W	+	-	-	-	+

Table 3.7 (Cont'd)

Tests	RS 1	RS 2	RS 3	RS 4	RS 5	RS 6	RS 7	RS 8	RS 9	RS 10
Reduction										
NO_3 - NO_2	-	-	-	+	+	+	+	+	+	-
NO_3 - NO	-	-	-	-	-	-	-	-	-	-
NO_3 - gas	+	+	-	-	-	-	-	-	-	-
NO_2 - NO	-	-	-	+	-	-	-	-	-	-
NO_2 - gas	+	+	-	-	-	-	-	-	-	-
Anaer. gas	+	+	-	-	-	-	-	-	-	-
Acid Prod.										
Adonitol	-	-	-	-	-	-	-	-	-	-
Aesculin	-	-	-	-	-	-	-	-	-	-
Arabinose	+	W	-	-	-	-	-	W	-	-
Dextrin	W	+	-	-	-	-	W	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-
Fructose	+	-	-	-	-	-	+	W	-	-
Galactose	-	-	-	-	-	-	-	+	-	-
Glucose	+	+	-	W	-	-	+	+	-	-
Glycerol	W	-	-	-	-	-	+	-	-	-
Glycogen	-	-	-	-	-	-	W	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-
Inulin	-	-	-	-	-	-	-	-	-	-
Lactose	+	-	-	-	-	-	-	-	-	-
Maltose	+	+	-	W	-	-	+	W	-	-
Mannose	+	+	-	-	-	-	+	+	-	-
Mannitol	+	-	-	-	-	-	+	+	-	-
Raffinose	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	W	-	-	-	W	-	-
Salicin	+	-	-	-	-	-	W	W	-	-
Sorbitol	-	-	-	W	-	-	-	-	-	-
Starch	+	-	-	-	-	-	-	-	-	-
Sucrose	+	+	-	-	-	-	+	+	-	-
Trehalose	+	+	-	-	-	-	+	+	-	-
Xylose	+	+	W	W	-	-	+	W	-	-

Table 3.7 (Cont'd)

Tests	RS 11	RS 12	RS 13	RS 14	RS 15	RS 16	RS 17	RS 18	RS 19	RS 20
Reduction										
NO_3 - NO_2	-	-	-	-	-	+	+	-	-	-
NO_3 - NO	-	-	-	-	-	-	-	-	-	-
NO_3 - gas	-	-	-	+	-	-	-	-	-	+
NO_2 - NO	-	-	-	-	-	-	-	-	-	-
NO_2 - gas	-	-	-	+	-	-	-	-	-	+
Anaer. gas	-	-	-	NT	-	-	-	-	-	+
Acid Prod.										
Adonitol	-	-	-	-	-	-	-	W	-	-
Aesculin	-	-	-	-	-	-	-	-	-	-
Arabinose	W	-	-	W	-	+	+	W/-	-	+
Dextrin	-	W	-	W	W	+	-	-	-	+
Dulcitol	-	-	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-
Fructose	W	+	-	W	+	W	+	+	-	+
Galactose	-	W	W	W	-	-	W	-	-	W/-
Glucose	-	W	-	+	+	+	-	+	-	+
Glycerol	-	+	-	W	W	+	W/-	+	-	-
Glycogen	-	+	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	W	-	-
Inulin	-	+/-	-	-	-	-	-	-	-	+
Lactose	-	-	-	-	-	-	-	-	-	-
Maltose	-	+	-	+	+	+	-	+	-	+
Mannose	W	W	-	+	+	+	W	+	-	+
Mannitol	-	-	-	+	-	-	W	+	-	+
Raffinose	-	-/W	-	-	-	-	-	-	-	+
Rhamnose	-	-	-	W	-	-	W	-	-	W/-
Salicin	-	-	-	+	-	-	-	+	-	+
Sorbitol	-	-	-	+	-	-	-	+	-	-
Starch	-	+	-	-	-	-	-	-	-	-
Sucrose	-	+	W	+	-	-	W	+	-	+
Trehalose	-	+/-	-	+	+	-	-	+	-	+
Xylose	W	-	-	NT	+	+	W	-	-	+

Table 3.7 (Cont'd)

Tests	RS 21	RS 22	RS 23	RS 37	RS 42	RS 43	RS 44	RS 45	RS 47	RS 48
Reduction										
NO_3 - NO_2	-	-	-	-	-	-	+	+	-	+
NO_3 - NO	-	-	-	-	-	-	-	-	-	-
NO_3 - gas	-	-	-	-	-	-	-	-	-	-
NO_2 - NO	-	-	-	-	-	-	-	-	-	+
NO_2 - gas	-	-	-	-	-	-	-	-	-	-
Anaer. gas	-	-	-	-	-	-	-	-	-	-
Acid Prod.										
Adonitol	-	-	-	W	-	-	W	-	-	-
Aesculin	-	-	-	-	-	-	-	-	-	-
Arabinose	-	W	-	W	W	+	-	W	W/-	-
Dextrin	-	-	-	W	-	-	-	-	-	+
Dulcitol	-	+	-	-	-	-	-	-	-	-
Erythritol	-	W	-	+	-	-	-	-	-	-
Fructose	+	+	+/-	W	+/-	+/-	+	-	W	W
Galactose	-	-	-	W	W/-	W/-	W	-	-	-
Glucose	+	+	-	+	-	-	W	-	+	+
Glycerol	+	+	-	+/-	W	-	W/-	W/-	+	-
Glycogen	-	-	-	-	-	-	W	-	-	-
Inositol	+	-	-	+	-	-	VW	-	W	-
Inulin	-	-	-	+	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	W	-	-	-
Maltose	W	+	-	+	-	-	-	-	+	+
Mannose	VW	+	-	+	W	-	W	W/-	W	+
Mannitol	+	+	-	+	-	-	W	-	-	W/-
Raffinose	-	-	-	+	-	W/-	W	-	-	W/-
Rhamnose	W	+	W/-	W	W	W/-	W	-	W	-
Salicin	+	+	-	+	-	-	-	-	+	-
Sorbitol	+	+	-	+	-	-	W	-	+/-	-
Starch	-	-	-	-	-	-	-	-	-	W/-
Sucrose	+	+	-	W	-	-	+	-	+	+
Trehalose	+	+	-	+	-	W/-	+	-	+	+
Xylose	-	-	-	NT	W	W	W	-	NT	NT

Table 3.7 (Cont'd)

Tests	RS 49	RS 51	RS 53	RS 54	RS 56	RS 57	RS 58	RS 70	RS 80	RS 85
Reduction										
NO_3 - NO_2	+	-	-	+	-	-	-	-	-	-
NO_3 - NO	-	-	-	-	-	-	-	-	-	+
NO_3 - gas	-	+	-	-	+	-	-	-	+	-
NO_2 - NO	-	-	-	-	-	-	-	W	-	W
NO_2 - gas	-	+	-	-	+	-	-	-	+	-
Anaer. gas	-	-	-	-	NT	-	-	-	NT	-
Acid Prod.										
Adonitol	W	-	W	W	W	W/-	-	-	-	W
Aesculin	-	-	-	-	-	-	-	-	-	-
Arabinose	+	+	W	+	-	-	-	-	W	+
Dextrin	-	+	W	-	-	W	+	-	W	+
Dulcitol	W	-	+	-	-	-	-	-	-	-
Erythritol	-	-	+	-	-	-	VW	-	-	VW
Fructose	+	+/-	W	+	W	W	W	+	+	+
Galactose	W	W	-	W	+	W	W/-	-	W	-
Glucose	+	+	+	+	+	W	+	+	W	+
Glycerol	+	+	+	W	+	-	W	-	-	+
Glycogen	W/-	-	-	-	W/-	-	VW	-	W/-	+/-
Inositol	W	-	W	W	W	+	-	VW	W/-	W/-
Inulin	-	-	-	W	NT	-	W	-	-	-
Lactose	W	W	-	W	W	-	-	-	-	-
Maltose	-	+	+	-	+	+	+	W	W	+
Mannose	+	+	-	+	+	+	+	-	W	+
Mannitol	W	+	+	+	+	-	+	+	W	+
Raffinose	W	W	-	W	W	-	-	-	W	+/-
Rhamnose	+	W	W	W	W	-/W	-/W	-	-	-
Salicin	-	-	+	W	-	+	W/-	+	-	-
Sorbitol	+	W	+	+	+	+	-	+	+	+
Starch	W/-	+	-	-	-	-	+	-	-	+
Sucrose	+	+	+	+	+	W	+	+	-	+
Trehalose	+	+	+	+	+	+	W	W	+	+/-
Xylose	W	+	NT	W	+	NT	W	-	NT	+

Table 3.7 (Cont'd)

Tests	RS 86	RS 87	RS 98	RS 99	RS 155	RS 166	RS 168	RS 173	RS 174	RS 182
Reduction										
NO_3 - NO_2	-	+	-	-	-	W	-	-	-	-
NO_3 - NO	-	-	-	-	-	W	-	-	-	-
NO_3 - gas	+	-	-	-	-	W	+	+	+	-
NO_2 - NO	-	-	-	-	-	W	-	-	-	-
NO_2 - gas	+	+	-	-	-	+	+	+	+	-
Anaer. gas	-	NT	-	-	-	-	-	W	W	-
Acid Prod.										
Adonitol	-	-	W	W	W	-	-	-	-	-
Aesculin	-	-	-	-	-	-	-	-	-	-
Arabinose	+	+	-	W/-	W	+	W	+	+	W
Dextrin	+	+	W	W	+	+	+	+	+	-
Dulcitol	-	-	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	VW	-
Fructose	+	+	+	W	+	+	+/-	+	W	-
Galactose	-	W	-	W/-	+	W	-	W	W	-
Glucose	+	+	+	+	+	+	+	+	+	-
Glycerol	+	-	+	+	+	-	W	W	VW/+	W
Glycogen	+	-	-	-	-	-	-	W/-	+	-
Inositol	-	-	W	W	W	-	-	-	VW	W
Inulin	-	-	-	-	-	W/-	-	-	VW	-
Lactose	-	-	-	-	-	+	-	-	-	-
Maltose	+	+	-	+	+	+	W	+	+	-
Mannose	+	+	W	W/-	+/-	+	+	+	+	-
Mannitol	+	+	+	+	-	+	W	+	+	-
Raffinose	-	W	-/W	-	W	+	-	+	+	-
Rhamnose	-	-	-	W/-	-	-	+/-	W	VW	W
Salicin	-	+	W	-	+/-	+	W/-	W	NT	-
Sorbitol	+	VW	+	+	-	+	W	W	W/-	W/-
Starch	+	+	-	-	-	W	+	W	+	-
Sucrose	+	+	W	W	-	+	W	+	+	-
Trehalose	+/-	VW	+	+	+	+	+	+	VW/+	-
Xylose	+	NT	W	W	-	W	W	+	W	-

Table 3.7 (Cont'd)

Tests	RS 187	RS 203	RS 204	RS 205	RS 209A	RS 209B	RS 210	RS 211	RS 215	RS 216
Reduction										
NO_3 - NO_2	-	-	-	+	-	-	-	-	-	-
NO_3 - NO	-	-	-	-	-	-	-	-	+	-
NO_3 - gas	+	+	+	-	-	+	-	+	-	-
NO_2 - NO	-	-	-	+	-	-	-	-	+	-
NO_2 - gas	+	+	+	-	-	+	-	+	-	-
Anaer. gas	+	W	W	-	-	+	-	-	-	-
Acid Prod.										
Adonitol	-	-	-/VW	-	-	-	VW	-	-	-/W
Aesculin	-	-	-	-	-	-	-	-	-	-
Arabinose	W	VW	+	-	W/-	W/-	-	W	-	W
Dextrin	+	+	+	+	+	+	W	+	+	VW
Dulcitol	-	-	-	-	VW	-	-	-	-	-
Erythritol	-	-	-	-	VW	VW	W/-	-	-	VW
Fructose	-	-	W	W	W	-	+	VW	W	-
Galactose	W/-	+	+	VW	W	W	-	-	-/VW	-
Glucose	+	+	+	+	+	W	+	+	+	VW/-
Glycerol	W	W	+	W/-	W	W	+	-	+	W
Glycogen	VW	+/-	+	-	VW	VW	-	W	-	VW/-
Inositol	-	VW	-	-	W	VW	+	-	-	-
Inulin	-	VW	-	-	-	-	+	-	VW/-	-
Lactose	-	VW/-	-	-	-	-	-	-	-	-
Maltose	+	+	+	+/-	W	W	+/-	+	+	-
Mannose	+	+	+	W	-	+	+	+	+	W
Mannitol	+	+	+	+	+	W	+	+	+	-
Raffinose	-/W	-	W	-	VW	W	+	-	-	-
Rhamnose	-/VW	-/VW	VW	+	VW	W/-	-	-	-	W
Salicin	-	VW	VW	+	-	-	W	+	-	-
Sorbitol	VW	W	VW	+	+	VW	+	VW	W	-
Starch	+	+	+	-	-	+	-	+	VW/-	-
Sucrose	W	VW	W	+	+	+/VW	W	-	+	-
Trehalose	+	+	+	+	VW	+	+	+	+	-
Xylose	W	-/W	W	+	W	W	-/W	W	VW/-	W

Table 3.7 (Cont'd)

Tests	RS 217	RS 222	RS 236	RS 237	RS 238	RS 239	RS 240	RS 241	RS 242	RS 243
Reduction										
NO_3 - NO_2	-	-	+	+	+	-	-	-	-	+
NO_3 - NO	-	-	-	-	W	-	-	-	-	-
NO_3 - gas	-	-	-	-	W	-	-	-	-	-
NO_2 - NO	-	-	-	W	+	-	-	-	-	-
NO_2 - gas	-	-	-	-	-	-	-	-	-	-
Anaer. gas	-	-	-	-	-	-	-	-	-	NT
Acid Prod.										
Adonitol	W	-	W	-	W/-	-	-	-	-	W
Aesculin	-	-	-	-	-	-	-	-	-	-
Arabinose	-	-	W	W	+/-	+/-	-	-	-	+
Dextrin	-	+	W	+	+/-	-	-	-	-	W
Dulcitol	-	-	-	-	-	-	-	-	-	VW
Erythritol	W	-	W/-	-	-	-	-	-	-	-
Fructose	W	W	+	+	W/-	-	-	-	-	-
Galactose	-	W/-	+	+	W/-	W/-	W/-	VW	VW/-	+
Glucose	+	+	+	+	+	-	-	-	+	+
Glycerol	+	W	W	+	+	-	-	W	-	+
Glycogen	-	+	-	-	-	VW	-	-	-	W
Inositol	W	-	W/-	-	-	-	-	-	-	W
Inulin	-	VW/-	W	-	-	-	-	-	-	W
Lactose	-	-	+	W	VW/-	-	-	-	-	+
Maltose	+	+	W	+	+	-	-	-	W	+
Mannose	+	-	+	+	+	W	-	-	+	+
Mannitol	+	-	-	-	+/VW	-	-	-	-	W
Raffinose	-	W/-	+	-	+	-	W/-	-	VW	W
Rhamnose	VW/-	-	+	+	-	-	-	-	-	+
Salicin	+	-	+	+	+	-	-	-	-	+
Sorbitol	+	-	W/-	-	-	-	-	-	-	W
Starch	-/W	+	W	-	+/-	-	-	+/-	-	+
Sucrose	W	+	W	+	+	-	-	VW/+	+	W/-
Trehalose	+	+	+	+	+	-	-	W/-	+	+/-
Xylose	W	-	W/-	W	+	W	-	-	-	W

Table 3.7 (Cont'd)

Tests	RS 244	RS 245	RS 248	RS 249
Reduction				
NO_3 - NO_2	-	+	+	-
NO_3 - NO	-	-	-	-
NO_3 - gas	-	-	-	-
NO_2 - NO	-	-	-	-
NO_2 - gas	-	-	-	-
Anaer. gas	-	-	-	-
Acid Prod.				
Adonitol	-/W	-/W	-	W
Aesculin	-	NT	-	-
Arabinose	-	+	+	-
Dextrin	W	W	+	-
Dulcitol	-	-	-	+
Erythritol	W	-	-	-
Fructose	-	-	W	-
Galactose	-	W	-/W	-
Glucose	W	+	+	+
Glycerol	-	W	-	-/W
Glycogen	-	W	-	-
Inositol	W	+	-	W
Inulin	-	W	-	-
Lactose	-	W	-	-
Maltose	+	W	+/W	W
Mannose	W	+	-	W
Mannitol	+	+	W/-	+
Raffinose	-	W	-	-/W
Rhamnose	-	W	+	-
Salicin	+	+/-	-	+
Sorbitol	+	+	-	+
Starch	-	W	-	-
Sucrose	W	+	-	W
Trehalose	+	+	W	+
Xylose	-	W	+	-

Table 3.7 (Cont'd)

Duplicate tests on a random selection of strains to examine reproducibility

Tests	T60	RS 2	RS 16	RS 47	RS 42	RS 51	RS 210	RS 173
Reduction								
NO_3 - NO_2	W	-	+	-	-	-	-	-
NO_3 - NO	W	-	-	-	-	-	-	-
NO_3 - gas	-	+	-	-	-	+	-	+
NO_2 - NO	+	-	-	-	-	-	-	-
NO_2 - gas	-	+	-	-	-	+	-	+
Anaer. gas	-	+	-	-	-	-	-	W
Acid Prod.								
Adonitol	-	-	-	-	-	-	-	-
Aesculin	-	-	-	-	-	-	-	-
Arabinose	-	-	+	-	W	+	-	+
Dextrin	NT	+	+	-	-	+	W	+
Dulcitol	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-
Fructose	W	-	W	-	-	+	+	+
Galactose	-	-	-	-	-	W	-	W
Glucose	+	+	+	+	-	+	+	+
Glycerol	NT	-	+	+	W	+	+	W
Glycogen	-	-	-	-	-	W	-	-
Inositol	-	-	-	W	-	-	+	-
Inulin	-	-	-	NT	-	-	+	-
Lactose	-	-	-	-	-	-	-	-
Maltose	+	+	W	+	-	+	+	+
Mannose	+	+	+	+	W	+	+	+
Mannitol	+	-	-	W	-	+	+	+
Raffinose	-	-	-	-	-	W	+	+
Rhamnose	-	W	-	W	W	-	-	-
Salicin	W	-	-	NT	NT	-	W	W
Sorbitol	NT	-	-	+	-	W	+	-
Starch	+	-	-	-	-	W	-	W
Sucrose	+	+	-	+	-	+	W	+
Trehalose	+	+	-	NT	-	W	+	+
Xylose	W	+	+	+	+	W	W	+

Legend (Table 3.8)

Antibiotic sensitivity zone radius was recorded in mm. Clear zones with a hazy outer zone were recorded differentially (e.g. 5+3).
h, hazy zone; r.c., resistant colonies in a clear zone.

Te30, tetracycline 30 µg; E10, erythromycin 10 µg; PN10, ampicillin 10 µg; C30, chloramphenicol 30 µg; Na30, naladixic acid 30 µg; S25, streptomycin 25 µg; W5, trimethoprim 5 µg; CN10, gentamicin 10 µg.

Spore morphology

Shape: c, cylindrical; o, oval; s, spherical.
Position: c, central; t, terminal; s.t., sub-terminal.
Swelling: sl, slight.

Cell form

S, single cells; P, pairs of cells; CH, chains of cells; F, filaments
The cell size was recorded in µm.

Colony form

Elevation : Fl, flat; R, raised; C, convex; P, pulvinate.
Edge : En, entire; Er, erose; Un, undulate.
Surface : Sm, smooth/shiny; R, rough, M, smooth/matt.
Plan view : C, circular; I, irregular.

Spreading

V - No evidence of spreading after 16 h incubation at 60 ^{o}C
W - Dry colonies showing evidence of moist spreaders
X - A small number of colonies showing evidence of spreading from original colony.
Y - Most colonies producing spreading areas
Z - All of agar surface covered in spreading growth with no evidence of the original streak.

Table 3.8

Tests	NCA 1503 (901)	ATCC 12016 (902)	EP 136 (903)	EP 240 (904)	EP 262 (905)	RS 93 (906)	NW 10 (907)	tenax (908)	velox (909)	lyticus (910)
Temperature										
75 °C	+	W	-	-	+	-	+	+	+	+
70 °C	+	+	-	+	+	-	+	+	+	+
65 °C	+	+	+	+	+	+	+	+	+	+
42 °C	+	W	+	+	+	+	+	+	+	+
37 °C	-	-	+	-	NT	-	-	-	-	-
30 °C	-	-	-	-	-	-	-	-	-	-
25 °C	-	-	-	-	-	-	-	-	-	-
Sensitivity										
Ery. E10	11	11	4	10	12	7	11.5	19	18	14
Chlor. C30	10	8	9	8	8.5	9	30*	14	15	12.5
Tet. Te30	15	12	11	12	14	11	13	17	18	15
Nal. Na30	1	0	6	5	2	1	2	0	0	0
Strep. S25	8	8	10	7	8	10	8	18	13	13
Amp. PN10	19	23	17	20	21	17	25	27	30	23
Gent. CN10	10	11	9	8	9	11	10	15	17	11
Tri. W5	12	15	4	10	15	1	15	18	16	13
Spore										
Shape	o	o	o	o	o	o	o	o	o	o
Position	s.t.	s.t.	s.t.	s.t.	t	s.t.	s.t.	t	t	t
Swelling	+	+	+	+	+	+	+	+	+	+
Cell										
Motility	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Gram	+	+	-	W	-	W	-	+	-	W
Form	S	S	S	S.P.	S.P.	S	S	S.P.	S	S. Ch
Length	2-4	2-4	2-3	3-5	1.5-4	2-4	2-4	2-5	1.5-5	2-5
Diameter	1	0.75	0.5	1	0.5	0.75	1	1	0.75	1
Colony										
Elevation	Co	Co	Fl	Co	Co	Co	Co	Co	Co	Co
Edge	En	En	En	En	En	En	En	En	En	En
Surface	Sm	Sm	Sm	Sm	Sm	Sm	Sm	Sm	Sm	Sm
Plan View	C	C	C	C	C	C	C	C	C	C
Spreading	-	-	X	-	-	-	-	-	-	-
Pigment										
Pink	-	-	-	-	-	+	-	-	-	-
Yellow	-	-	-	-	-	-	-	-	-	-

Table 3.8 (Cont'd)

Tests	ATCC 12245 (911)	ATCC 8038 (912)	T22 (913)	T42 (914)	T60 (915)	T141 (916)	T210 (917)	T214 (918)	DSM 456 (919)	DSM 463 (920)
Temperature										
75 °C	-	-	+	+	NT	-	NT	NT	+	-
70 °C	-	-	+	+	+	-	+	+	+	-
65 °C	-	-	+	+	+	+	+	+	+	+
42 °C	+	+	+	+	+	+	+	+	+	+
37 °C	+	+	-	W	W	+	-	-	-	-
30 °C	+	+	-	-	-	-	-	-	-	-
25 °C	+	+	-	-	-	-	-	-	-	-
Sensitivity										
Ery. E10	11	12	13	12	9	12	13	8	15	18
Chlor. C30	9	10	9	8	7	11	9	16	12	14
Tet. Te30	12	14	14	14	10	14	15	12	17	17
Nal. Na30	0	0	0	0	0	6	0	1	1	0
Strep. S25	10	12	8	8	10	8	13	9	10	13
Amp. PN10	13	15	19	22	20	20	23	NT	25	21
Gent. CN10	11	14	11	10	10	10	10	8	10	15
Tri. W5	3	0	17	13	13	4	18	9	14	9
Spore										
Shape	NT	NT	c	o	o	o	o	o	o	s
Position	NT	NT	s.t.	s.t.	s.t.	c	s.t.	s.t.	s.t.	s.t.
Swelling	NT	NT	-	+	+	+	+	+	+	+
Cell										
Motility	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Gram	+	W	-	-	W	+	W	-	NT	-
Form	SF	F	SP	S	SP	S	SP	S	NT	SCF
Length	2-5*	12	3-5	2-6	2-4	2	3-5	2-4	NT	3-7
Diameter	0.5	0.75	1	0.75	1	0.5	1	1	NT	0.5
Colony										
Elevation	Co	NT	Fl	Co	Co	Fl	Co	Co	Co	Fl
Edge	En	NT	Un	En	En	En	En	En	En	En
Surface	M	NT	R	Sm	Sm	Sm	Sm	Sm	Sm	R
Plan View	C	NT	Ir	C	C	C	C	C	C	C/Ir
Spreading	-	-	-	-	-	X	-	-	-	-
Pigment										
Pink	-	-	-	-	-	-	-	-	-	-
Yellow	-	-	-	-	-	-	-	-	-	-

Table 3.8 (Cont'd)

Tests	DSM 730 (921)	DSM 465 (922)	DSM 466 (923)	ATCC 8005 (924)	ATCC 10149 (925)	NCIB 8919 (926)	NCTC 10003 (927)	LO_2 516 (928)
Temperature								
75 °C	NT	+	+	+	+	+	NT	-
70 °C	+	+	+	+	+	+	+	-
65 °C	+	+	+	+	+	+	+	-
42 °C	NT	+	+	+	+	+	+	+
37 °C	-	W	-	-	-	-	-	+
30 °C	-	-	-	-	-	-	-	+
25 °C	-	-	-	-	-	-	-	+
Sensitivity								
Ery. E10	13	5	11	15	11	16	11	7
Chlor. C30	11	8	9	12	8	15	9	7
Tet. Te30	16	12	10	16	13	17	12	8
Nal. Na30	1	0	0	0	3	1	2	0
Strep. S25	16	8	13	13	8	22	6	4
Amp. PN10	23	17	19	26	23	26	21	2-5
Gent. CN10	15	9	8	15	7	14	8	4
Tri. W5	16	15	15	19	10	18	11	6
Spore								
Shape	c	c	c	o	o	o	o	NT
Position	t	s.t.	s.t.	t	s.t.	s.t.	s.t.	NT
Swelling	+	sl	sl	+	+	+	+	NT
Cell								
Motility	NT	NT	NT	NT	NT	NT	NT	NT
Gram	-	+	-	W	+	-	-	-
Form	F	S	S	S.P. Ch	SP	S	S	S.P. Ch
Length	5-200	2-5	2-6	3-6	3-4	2-4	2-5	2-4
Diameter	0.5	1	0.75	0.75	1	1-1.25	1	0.75
Colony								
Elevation	Co	Fl	Fl	Co	Co	Co	Co	NT
Edge	En	Er	Er	En	En	En	En	NT
Surface	M	R	R	Sm/M	Sm	Sm	Sm	M
Plan View	C	Ir	Ir	C	C	C	C	NT
Spreading	-	-	-	-	-	-	-	NT
Pigment								
Pink	-	-	-	-	-	-	-	+
Yellow	-	-	-	-	-	-	-	-

Table 3.8 (Cont'd)

Tests	RS 1	RS 2	RS 3	RS 4	RS 5	RS 6	RS 7	RS 8	RS 9	RS 10
Temperature										
75 °C	NT	NT	-	-	-	-	NT	-	-	NT
70 °C	+	+	W	-	-	-	+	-	-	+
65 °C	+	+	+	-	-	-	+	-	+	+
42 °C	+	+	+	+	+	+	+	+	+	+
37 °C	+	+	+	+	-	-	+	+	+	+
30 °C	-	-	+	+	-	-	-	+	+	W
25 °C	-	-	-	+	-	-	-	+	-	-
Sensitivity										
Ery. E10	11	11	13	0	NT	15	14	0	0	1
Chlor. C30	9	8	9	1	12	14	10	0	9	1
Tet. Te30	11	15	10	8+2	19	13+5	11	8	10	9
Nal. Na30	2h	0	0	0	0	0	2	0	0	1
Strep. S25	8	7	5+5	5+3	3+7	10+3	9+3	8	4	7+3
Amp. PN10	17	20	16	0	22	23	28	0	16	17
Gent. CN10	7+3	10	6+4	5+3	12	8+4	10	6+3	6	9+4
Tri. W5	8h	16	8	7	0	0	13h	6	13h	8
Spore										
Shape	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Position	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Swelling	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Cell										
Motility	+	+	NT	+	+	+	+	+	+	NT
Gram	-	-	W	-	-	-	-	NT	-	-
Form	S	S.P	S	S	S	S	S.P.	NT	S	S.P. Ch
Length	4-5	3-4	2-6	3	NT	3	2-5	NT	3-4	2-4
Diameter	0.75	0.75	0.75	0.75	NT	0.5	0.75	NT	0.75	0.75
Colony										
Elevation	Fl	Co	Fl	Co	NT	NT	Co	R	Co	Fl
Edge	Er	En	Un	En	NT	NT	En	Er	En	En
Surface	R	Sm	Sm	Sm	NT	NT	Sm	Sm	Sm	Sm
Plan View	Ir	C	Ir	Ir	NT	NT	C	C	C	C
Spreading	-	-	Y	X	Z	Z	-	-	-	Y
Pigment										
Pink	-	-	-	-	-	-	-	-	-	-
Yellow	-	-	-	-	-	-	-	-	-	-

Table 3.8 (Cont'd)

Tests	RS 11	RS 12	RS 13	RS 14	RS 15	RS 16	RS 17	RS 18	RS 19	RS 20
Temperature										
75 °C	-	-	-	-	NT	NT	-	NT	-	NT
70 °C	-	W	-	-	+	+	-	+	W	+
65 °C	+	+	+	+	+	+	-	+	+	+
42 °C	+	+	+	+	NT	W	+	+	+	W
37 °C	+	+	+	+	-	-	+	+	+	-
30 °C	+	-	+	W	-	-	+	-	+	-
25 °C	+	-	-	-	-	-	+	-	-	-
Sensitivity										
Ery. E10	2h	2	1	5	16	15	7	7	12	15
Chlor. C30	8	8	1	5h	12	13	7	7	1	13
Tet. Te30	8	12	12	11	17	14	5	7	11	15
Nal. Na30	1	3	1	1	1	0	0	0	1	1
Strep. S25	7+3	7+2	9+3	8	11	12	4+3	7	6+3	10
Amp. PN10	14+5	17	15h	18	21	25	2	2	16	20
Gent. CN10	7	5+6	9+3	8	14	14	6+5	8	6+3	13
Tri. W5	8	9	8	6h	11	19	7h	6	7	14
Spore										
Shape	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Position	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Swelling	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Cell										
Motility	+	+	NT	NT	NT	+	+	+	+	NT
Gram	W	NT	-	-	-	-	-	-	-	+
Form	S	NT	S	S	S	S.P. Ch	S	S	S.P.Ch	SP
Length	2-3	NT	3-4	2-3	2-5	2-6	3-4	1.5-2.5	2-4	3-4
Diameter	0.75	NT	0.75	0.75	0.75	0.75	1	0.75	0.75	1
Colony										
Elevation	Fl	Fl	Co	Fl	Co	Co	Co	R	Co	Co
Edge	Un	Un	En	Fl	En	En	En	En	En	En
Surface	Sm	Sm	Sm	R	Sm	Sm	Sm	Sm	Sm	Sm
Plan View	Ir	Ir	C	Ir	C	C	C	C	C	C
Spreading	Y	Y	Y	W	-	-	-	-	Y	-
Pigment										
Pink	-	-	-	-	-	-	-	-	-	-
Yellow	-	-	-	-	-	-	-	-	-	-

Table 3.8 (Cont'd)

Tests	RS 21	RS 22	RS 23	RS 37	RS 42	RS 43	RS 44	RS 45	RS 47	RS 48
Temperature										
75 °C	NT	NT	-	NT	-	-	-	-	NT	NT
70 °C	+	+	-	+	-	W	-	-	+	+
65 °C	+	+	+	+	W	+	+	VW	+	+
42 °C	+	+	+	+	+	+	+	+	+	+
37 °C	+	+	+	+	+	+	+	+	+	+
30 °C	-	W	W	-	+	NT	+	+	+	+
25 °C	-	-	-	-	-	-	+	+	-	+
Sensitivity										
Ery. E10	10	3+7	13	7	1	2	9	7	11	10
Chlor. C30	9	12	9	10	42*	9	8	4.5	10	5
Tet. Te30	10	13	12	13	11	13	10	8.0	15	9+3
Nal. Na30	3h	0	1	5	0	0	0	0	5	0
Strep. S25	8	7	8+2	11	9	9	6	6.5rc	9	9
Amp. PN10	15	22	15	21	19	17	6	1.5	17	0
Gent. CN10	NT	9	9	14	10	10	8	8rc	11	10
Tri. W5	5	7	9	0	8	11	8	7.5	3+3	6
Spore										
Shape	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Position	NT	NT	NT	NT	NT	NT	NT	NT	NT	t
Swelling	NT	NT	NT	NT	NT	NT	NT	NT	NT	+
Cell										
Motility	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Gram	NT	NT	NT	+	-	-	W	NT	+	+
Form	NT	NT	NT	S	S	SCHF	SP	NT	S	S
Length	NT	NT	NT	2-3.5	2-3	2-4	2-3	NT	2-3	2-5
Diameter	NT	NT	NT	0.75	0.5	0.5	0.75	NT	0.75	0.75
Colony										
Elevation	R	Fl	Fl	R	Fl	Fl	R	R	Fl	C
Edge	En	En	En	En	En	En	Er	Er	En	En
Surface	Sm	Sm	Sm	Sm	Sm	Sm	R/Sm	Sm	Sm	Sm
Plan View	C	C	C	C	C	C	C	C	C	C
Spreading	-	-	Y	Y	Y	Y	NT	-	-	-
Pigment										
Pink	-	-	-	-	-	-	-	-	-	-
Yellow	-	-	-	-	-	-	-	-	-	-

Table 3.8 (Cont'd)

Tests	RS 49	RS 51	RS 53	RS 54	RS 56	RS 57	RS 58	RS 70	RS 80	RS 85
Temperature										
75 °C	-	NT	NT	-	NT	NT	NT	NT	-	NT
70 °C	-	+	+	-	+	+	+	+	NT	+
65 °C	W	+	+	VW	+	+	+	+	+	+
42 °C	+	W	+	+	W	+	+	+	+	NT
37 °C	+	-	+	+	-	+	W	+	+	-
30 °C	+	-	-	+	-	-	-	-	-	-
25 °C	+	-	-	+	-	-	-	-	-	-
Sensitivity										
Ery. E10	8	11	5h	1.5	12	NT	10	7	2	NT
Chlor. C30	5	10	10	5	11	0	11	9	3	NT
Tet. Te30	8	12	13	8.5	15	17	14	12	10	NT
Nal. Na30	2h	3h	0	0	0	6RC	0	3h	0	NT
Strep. S25	4	8	7	4	10	11RC	11	8	7	NT
Amp. PN10	4RC	18	19	3	22	24	21	16	16	NT
Gent. CN10	10	10	10	5	12	17	13	10	8	NT
Tri. W5	10	15	7	7h	10	NT	17	6	9	NT
Spore										
Shape	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Position	NT	NT	c/s.t.	NT	NT	NT	NT	NT	c	NT
Swelling	NT	NT	+	NT	NT	NT	NT	NT	NT	NT
Cell										
Motility	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Gram	+	-	+	+	-	+	-	+	+	NT
Form	SPCh	SPCh	S	SP	S	S	S	S	SP	NT
Length	2-4.5	5-6	2-3	2-3	4-5	2-3	2-3	2-3	2-4	NT
Diameter	0.75	0.75	0.75	0.75	0.75	0.75	1	0.75	0.75	NT
Colony										
Elevation	R	Fl	R	R	R	Fl	NT	Fl	Fl	C
Edge	Er	Er	En	Er	En	En	NT	En	En	En
Surface	Sm/R	R	Sm	Sm	Sm	Sm	NT	Sm	Sm	Sm
Plan View	C	Ir	C	C	C	C	NT	C	C	C
Spreading	-	-	-	-	-	-	NT	-	-	-
Pigment										
Pink	-	-	-	-	-	-	NT	-	-	-
Yellow	-	-	-	-	-	-	NT	-	-	-

Table 3.8 (Cont'd)

Tests	RS 86	RS 87	RS 98	RS 99	RS 155	RS 166	RS 168	RS 173	RS 174	RS 182
Temperature										
75 °C	NT	NT	NT	NT	-	NT	NT	NT	NT	-
70 °C	+	+	+	+	-	+	+	+	+	-
65 °C	+	+	+	+	-	+	+	+	+	+
42 °C	NT	-	+	+	+	W	NT	W	W	+
37 °C	-	-	+	W	+	-	-	-	-	+
30 °C	-	-	-	-	+	-	-	-	-	-
25 °C	-	-	-	-	-	-	-	-	-	-
Sensitivity										
Ery. E10	5	9	5	6	11	15	12.5	15	15	1.5
Chlor. C30	4	12	12	10	10	15	9	13	15	1
Tet. Te30	13	16	11	12	12	17	12	17	16	11
Nal. Na30	2	0	3	2	NT	0	0	0	0	0
Strep. S25	5	14	7	6	11	12	8.5	10.5	12	8h
Amp. PN10	17.5	25	18	17	18	24	19	25	26	15
Gent. CN10	7.5	16	10	10	14	16	10	14	17	9
Tri. W5	NT	17	5	4	NT	18	16	18	18	6
Spore										
Shape	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Position	s.t.	NT	s.t.	s.t.	NT	NT	NT	NT	NT	NT
Swelling	+	NT	+	+	NT	NT	NT	NT	NT	NT
Cell										
Motility	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Gram	+	+	+	+	+	-	NT	+	-	+
Form	S	S.P.Ch.F	S	S	S.P	S.P.Ch	NT	S.P. Ch	S.P.Ch	S
Length	2-3	5-8	2-3	2-3	2-5	2-5	NT	2-4	4	2-3
Diameter	0.75	1	0.75	0.75	0.75	0.75	NT	0.75	0.75	0.5
Colony										
Elevation	C	R	Fl	C	C	R	Fl	C	C	Fl
Edge	En	En	En	En	En	En	Er	En	En	En
Surface	Sm	Sm	Sm	Sm	Sm	Sm	R	Sm	Sm	Sm
Plan View	C	C	C	C	C	C	Ir	C	C	C
Spreading	-	-	-	-	-	-	-	-	-	Y
Pigment										
Pink	-	-	-	-	-	-	-	-	-	-
Yellow	-	-	-	-	-	-	-	-	-	-

Table 3.8 (Cont'd)

Tests	RS 187	RS 203	RS 204	RS 205	RS 209A	RS 209B	RS 210	RS 211	RS 215	RS 216
Temperature										
75 °C	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
70 °C	+	+	+	+	+	+	+	+	+	+
65 °C	+	+	+	+	+	+	+	+	+	+
42 °C	NT	+	+	W	+	W/+	+	+	+	+
37 °C	-	-	-	-	+	-	+	W	-	+
30 °C	-	-	-	-	-	-	NT	-	-	-
25 °C	-	-	-	-	-	-	-	-	-	-
Sensitivity										
Ery. E10	13	12	13rc	15	10	12	21	11	14	13
Chlor. C30	10	6	9	11.5	9	10	6	8	8.5	11
Tet. Te30	13.5	12	14	16	12	13	14	10	15	12
Nal. Na30	0	0	0	0	6rc	0	NT	NT	0	0
Strep. S25	8	8	7.5	9	9rc	8	8	7	9	8
Amp. PN10	20	19	20	23	17	20	20	19	23	16
Gent. CN10	10	9	10	13	9	10.5	10	8	21	9
Tri. W5	17	15	17	17	4	15	6	13	13	9
Spore										
Shape	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Position	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Swelling	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Cell										
Motility	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Gram	-	-	+	-	+	+	+	W	+	-
Form	S.P. Ch	S	S	S	S	S.P. Ch	S	S.P. Ch	S	S
Length	3-4	2-4	2-3	2-3	3-4	3-5	2-3	5-7	3-4	3
Diameter	0.5	0.5	0.75	0.5	0.5	0.75	0.5	0.5	0.75	0.75
Colony										
Elevation	Fl	Fl	NT	NT	C	Fl	C	Fl	Fl	Fl
Edge	Er	Er	NT	NT	En	Er	En	Er	Er	En
Surface	R	R	NT	NT	Sm	R	Sm	R	Sm	Sm
Plan View	Ir	Ir	NT	NT	C	Ir	C	Ir	Ir	C
Spreading	-	-	NT	NT	-	-	-	-	-	Y
Pigment										
Pink	-	-	-	-	-	-	-	-	-	-
Yellow	-	-	-	-	-	-	-	-	-	-

Table 3.8 (Cont'd)

Tests	RS 217	RS 222	RS 236	RS 237	RS 238	RS 239	RS 240	RS 241	RS 242	RS 243
Temperature										
75 °C	NT	-	-	-	NT	NT	NT	NT	NT	-
70 °C	+	VW	-	-	+	+	+	+	+	-
65 °C	+	+	-	-	+	+	+	+	+	-
42 °C	+	+	+	+	+	+	+	+	+	+
37 °C	+	+	+	+	-	+	+	+	+	+
30 °C	NT	-	+	+	-	-	-	+	-	+
25 °C	-	-	W	W	-	-	-	-	-	W
Sensitivity										
Ery. E10	7	10	13	16	NT	12	12	12	11	NT
Chlor. C30	9	7	7	10	NT	0	7+3	3h	10h	NT
Tet. Te30	11	11	NT	12	NT	2+2	12	12h	11	NT
Nal. Na30	4	3	NT	NT	NT	NT	NT	NT	NT	NT
Strep. S25	5	5	NT	10	NT	NT	NT	NT	8h	NT
Amp. PN10	16	15	18	20	NT	14+5	17	12+4	20	NT
Gent. CN10	8	6	NT	13	NT	10	11	9h	9+3h	NT
Tri. W5	5	12	NT	10	NT	8	11	7h	5+6	NT
Spore										
Shape	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Position	c	NT	NT	NT	NT	NT	NT	c/st	t	NT
Swelling	+	NT	NT	NT	NT	NT	NT	+	+	NT
Cell										
Motility	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Gram	+	W	+	+	-	-	W	+	+	+
Form	NT	S	S.P. Ch	S	S	S	NT	S	S	S.P.
Length	2-3	2-4	2-4	2-3	2-5	2-4	1.5-3	2-3	2-3	2-3
Diameter	0.75	0.75	NT	0.5	0.5	0.5	0.75	0.75	0.75	0.5
Colony										
Elevation	Fl	C	C	C	C	Fl	Fl	Fl	R	C
Edge	En	En	En	En	En	En	En	En	En	En
Surface	Sm	Sm	Sm	Sm	Sm	Sm	Sm	Sm	Sm	Sm
Plan View	Ir	C	C	C	C	C	C	C	C	C
Spreading	-	-	-	-	-	Y	Y	Y	-	-
Pigment										
Pink	-	-	-	-	-	-	-	-	-	-
Yellow	-	+	-	-	-	-	-	-	-	-

Table 3.8 (Cont'd)

Tests	RS 244	RS 245	RS 248	RS 249
Temperature				
75 °C	NT	NT	NT	NT
70 °C	+	+	+	+
65 °C	+	+	+	+
42 °C	+	+	W	+
37 °C	+	+	-	+
30 °C	-	+	-	W
25 °C	-	+	-	-
Sensitivity				
Ery. E10	5	8	14	8
Chlor. C30	6	8	14	10
Tet. Te30	12	8	15	12
Nal. Na30	4	NT	0	2
Strep. S25	6	4	14	8
Amp. PN10	19	2	24	17
Gent. CN10	12	9	12	12
Tri. W5	3h	5h	15	9
Spore				
Shape	NT	NT	NT	NT
Position	c/st	NT	NT	t/st
Swelling	NT	NT	NT	+
Cell				
Motility	NT	NT	NT	NT
Gram	+	+	W	+
Form	NT	S.P	S.P	NT
Length	2-3	2-5	3-5	2-3
Diameter	0.75	0.75	0.75	0.75
Colony				
Elevation	Fl	R	R	Fl
Edge	En	Er	En	En
Surface	Sm	Sm	Sm	Sm
Plan View	C	C	C	C
Spreading	-	-	-	-
Pigment				
Pink	-	-	-	-
Yellow	-	-	-	-

Table 3.8 (Cont'd)

Duplicate tests on a random selection of strains to examine reproducibility

Tests	T60	RS 2	RS 16	RS 47	RS 42	RS 51	RS 210	RS 173
Temperature								
75 °C	NT	NT	NT	NT	NT	NT	NT	NT
70 °C	+	+	+	+	-	+	NT	+
65 °C	+	+	+	+	+	+	+	+
42 °C	+	+	W	+	+	NT	+	W
37 °C	W	+	-	+	+	-	W	-
30 °C	-	-	-	NT	+	-	-	-
25 °C	-	-	-	-	-	-	-	-
Sensitivity								
Ery. E10	12	11	15	13	1	10	12	13
Chlor. C30	7	9	12	10	10	9	10	10
Tet. Te30	16	13	13	18	10	12	12	14
Nal. Na30	0	0	0	6	0	2	2	0
Strep. S25	7.5	7	9	NT	8	8	7	9
Amp. PN10	23	20	22	18	16	18	20	22
Gent. CN10	10	9	11	10.5	9	9.5	11.5	11
Tri. W5	14	13	NT	NT	NT	18	7	15
Spore								
Shape	NT	NT	NT	NT	NT	NT	NT	NT
Position	NT	NT	NT	NT	NT	NT	NT	NT
Swelling	NT	NT	NT	NT	NT	NT	NT	NT
Cell								
Motility	NT	NT	NT	NT	NT	NT	NT	NT
Gram	W	-	-	W	-	-	W	+
Form	SP	SP	SPC	S	SP	SPC	S	SPC
Length	2-4	3-4	2-6	2-3	2-3	5-6	2-3	2-5
Diameter	1	0.75	0.75	0.75	0.5	0.75	0.5	0.75
Colony								
Elevation	Co	Co	Co	F	F	F	Co	Co
Edge	En	En	En	En	En	Er	En	En
Surface	Sm	Sm	Sm	Sm	Sm	R	Sm	Sm
Plan View	C	C	C	C	C	Ir	C	C
Spreading	-	-	-	-	Y	-	-	-
Pigment								
Pink	-	-	-	-	-	-	-	-
Yellow	-	-	-	-	-	-	-	-

Fig 3.1

Similarity Matrix of 102 thermophilic strains of Bacillus based on the examination of 96 characters

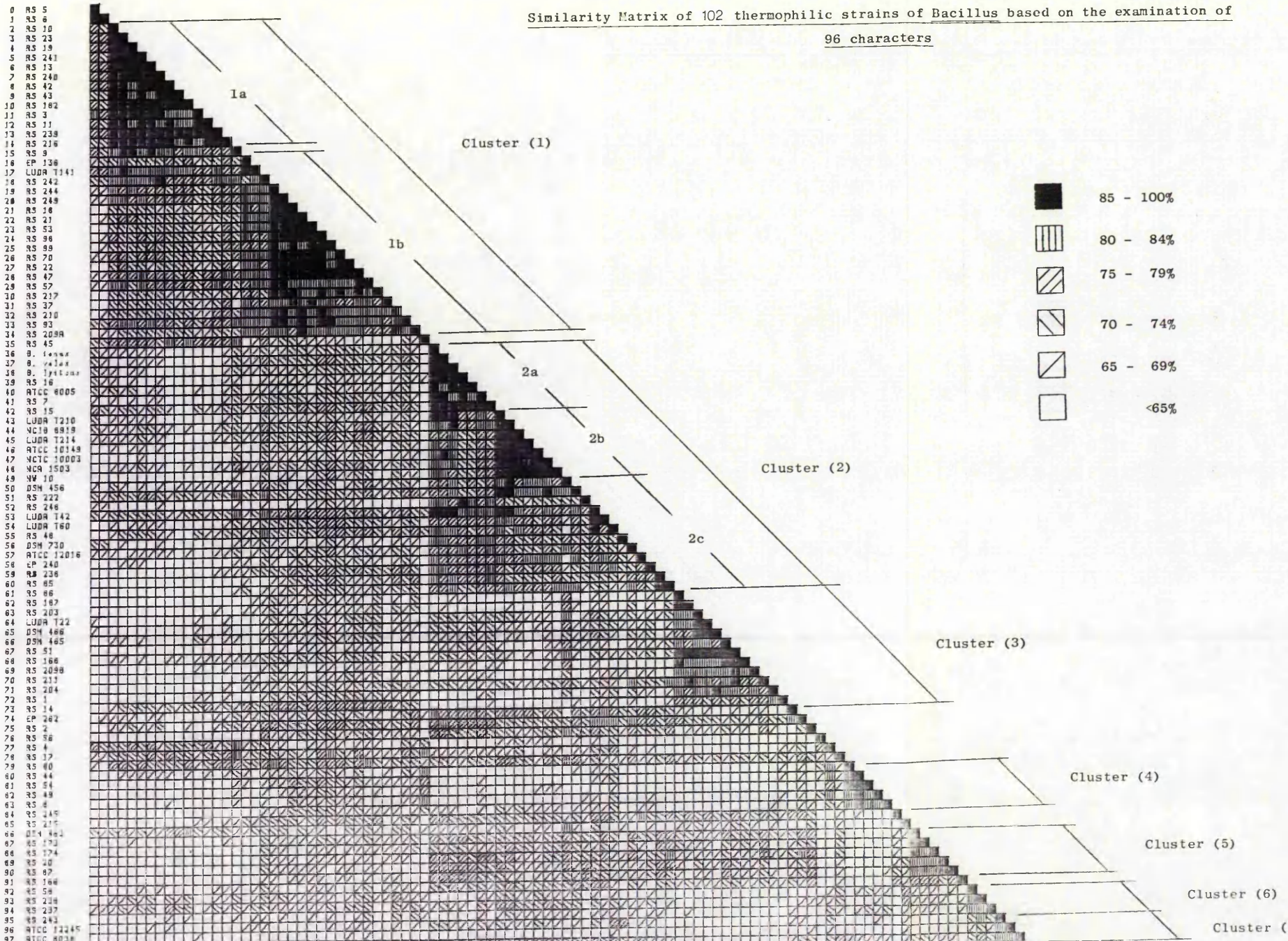
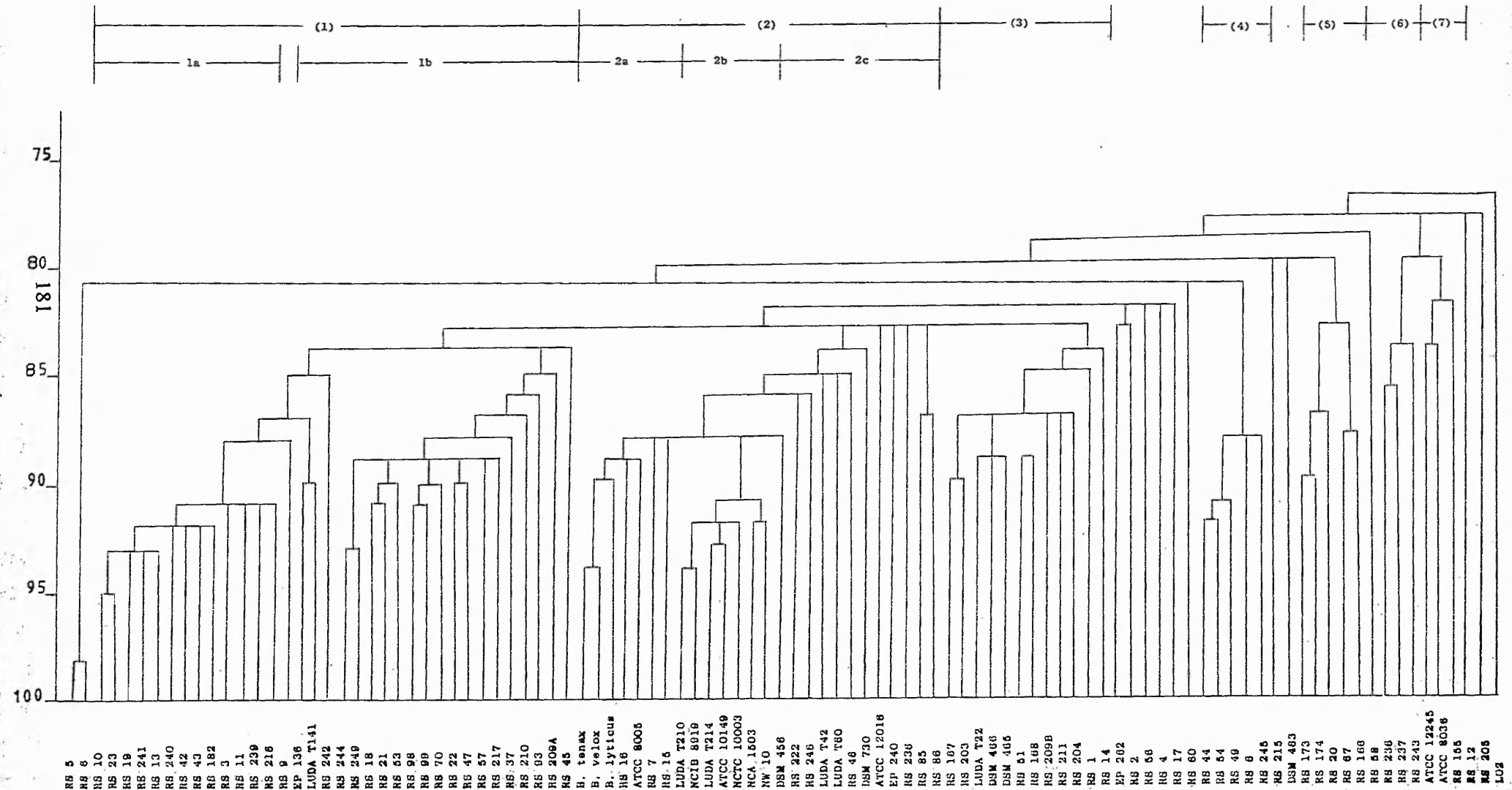


Figure 3.2

Single linkage cluster analysis of 102 thermophilic strains of Bacillus based on a study of 96 characters



Cluster (1) showed little evidence of starch hydrolysis and sub-group (1a) showed no evidence of starch hydrolysis (acid production in starch peptone broths was positive in 8% of strains). Proteolytic activity identified by activity on casein and gelatin was negligible. Both sub-groups showed growth in 5% (w/v) saline and at pH 6.5, 90% of the strains did not grow at pH 5.5. No strains in sub-group (1a) utilised citrate but 84% of strains in sub-group (1b) showed evidence of citrate utilisation. On Cal II minimal agar sub-group (1b) appeared prototrophic while only 23% of sub-group (1a) appeared prototrophic (a further 54% grew following the addition of casein hydrolysate).

All strains in cluster (1) were catalase and oxidase positive with little evidence of denitrifying ability. All strains grew at 65 ^{o}C and 97% grew at 37 ^{o}C.

Members of sub-group (1b) showed little evidence of spreading growth on TSBA plates while all members of group (1a) produced colonies with areas of growth spreading from the original colony. All members of this cluster produced shiny colonies. Sub-group (1a) showed little evidence of fermentation in peptone sugar broths, conversely all strains in group (1b) fermented glucose, sucrose and trehalose with acid production and more than 70% of strains fermented fructose, glycerol, inositol, maltose, mannose, mannitol, salicin, and sorbitol.

Two starch negative strains, RS 5 and RS 6 which had 98% similarity, clustered with sub-group (1a) at 75 - 80% similarity. They showed no evidence of sugar fermentation, were resistant to lysozyme and were typified by producing thick growth over the surface of the plate, obscuring any evidence of the original streak. Both strains grew at 42 ^{o}C but showed no evidence of growth at 37 ^{o}C or 65 ^{o}C.

Strain RS 9 clustered at 88% with three members of sub-group (1a) but was atypical of the group having strong amylolytic and proteolytic activity and being resistant to erythromycin. It showed no evidence of fermentative ability on peptone water sugars.

Strain RS 45 clustered between 80 and 85% with three members of sub-group (1a). It was atypical of this group having strong proteolytic and amylolytic activity, it was unable to grow at pH 6.5 in nutrient broth but was resistant to sodium azide. It showed strong growth at 25 ^{o}C and had little fermentative ability on peptone sugars. The colonies were erose resembling strains in cluster (4); strain RS 45 had 80 - 85% similarity with two members of this cluster (see cluster (4)).

Table 3.9

Characteristics of the clusters as percentages of positive reactions

Characters	Cluster (1)	Cluster (1a)	Cluster (1b)
Decomposition			
Starch	16	0	26
Casein	0	0	0
Gelatin	3	8	0
Tyrosine	3	0	5
Brown pigment	0	0	0
Phenyl alanine	0	0	0
Hippurate	3	0	6
Growth			
3% (w/v) NaCl	100	100	100
5% (w/v) NaCl	97	100	95
0.02% (w/v) Azide	16	8	21
Lysozyme	9	8	10.5
NB pH 6.5	90	75	100
NB pH 6.0	34	8	53
NB pH 5.5	9	8	10.5
Cal min	69	23	100
Cal + CA	91	77	100
SMS min	53	0	94
SMS + CA	81	77	94
TSBA anaerobic	81	61	95
Glu. anaer. acid	41	0	75
Production			
Catalase	100	100	100
Oxidase	100	100	100
Acid (MR)	0	0	0
Acetoin (VP)	8	25	0
Utilisation			
Citrate	50	0	84
Propionate	16	0	26

Table 3.9 (Cont'd)

Characters	Cluster (1)	Cluster (1a)	Cluster (1b)
Reduction			
NO_3 - NO_2	0	0	0
NO_3 - NO	0	0	0
NO_3 - gas	0	0	0
NO_2 - NO	3	0	6
NO_2 - gas	0	0	0
Anaer. NO_3 - gas	0	0	0
Acid Production			
Adonitol	34	8	58
Arabinose	41	46	37
Dextrin	34	8	47
Dulcitol	16	0	26
Erythritol	29	8	44
Fructose	62.5	31	84
Galactose	32	46	22
Glucose	59	0	100
Glycerol	56	31	74
Glycogen	6	8	5
Inositol	53	8	84
Inulin	9	0	16
Lactose	0	0	0
Maltose	56	10	89
Mannose	56	31	74
Mannitol	50	0	84
Raffinose	25	15	32
Rhamnose	45	38	50
Salicin	47	0	79
Sorbitol	56	8	90
Starch	6	8	5
Sucrose	66	15	100
Trehalose	66	15	100
Xylose	39	46	33

Table 3.9 (Cont'd)

Characters	Cluster (1)	Cluster (1a)	Cluster (1b)
Cell form and size			
Single cells	100	100	100
Cells in pairs	8	18	0
Cells in chains	12	27	0
Cells as filaments	4	9	0
Length <5 µm	97	92	100
Length 5 µm or >5 µm	3	8	0
Diameter <0.75 µm	28	33	24
Diameter >0.75 µm	75	73	76
Colony Morphology			
Elevation			
Flat	66	85	53
Raised	16	0	26
Convex	19	15	21
Pulvinate	0	0	0
Edge			
Entire	94	77	100
Erose	0	0	0
Undulate	6	15	0
Surface			
Smooth/Shiny	100	100	100
Smooth/Matt	0	0	0
Rough	0	0	0
Shape			
Circular	94	77	95
Irregular	9	15	5
Spreading			
V	50	0	84
X	6	0	10
Y	44	100	5
Z	0	0	0

Table 3.9 (Cont'd)

Characters	Cluster (1)	Cluster (1a)	Cluster (1b)
Temp. for growth			
75 °C	0	0	0
70 °C	75	61	84
65 °C	100	100	100
42 °C	100	100	100
37 °C	97	100	95
30 °C	41	73	12
25 °C	3	8	0
Antibiotic Sensitivity			
Erythromycin	100	100	100
Chloramphenicol	94	92	95
Tetracycline	100	100	100
Naladixic acid	70	50	82
Streptomycin	100	100	100
Ampicillin	100	100	100
Gentamicin	100	100	100
Trimethoprim	97	100	94

Legend

The number of strains in each cluster giving positive reactions was expressed as a percentage of the total number of strains in the cluster.

Weak reactions were regarded as positive. Starch hydrolysis was regarded as positive whether the reaction was diffuse or restricted. Antibiotic sensitivity was scored as positive when any signs of growth inhibition were evident.

3.2.3 Cluster (2)

Cluster (2) contains 26 strains of which 15 were marker strains. Two sub-groups were identified at the 85% similarity level. Sub-group (2a) was composed of seven strains including the three caldoactive bacteria reported by Heinen and Heinen (1972) and B. stearothermophilus ATCC 8005 originally named B. kaustophilus by Prickett (1928). Sub-group (2b) comprised seven strains including B. stearothermophilus LUDA T210 (NCA 26 the type strain), NCIB 8919, NCA 1503, LUDA T214, ATCC 10149, NW 10, NCTC 10003.

The remaining 12 strains in cluster (2) were not tightly clustered and had similarity coefficients between 75% and 85% with other members of cluster (2). This group was designated as sub-group (2c) and included B. stearothermophilus ATCC 12016, DSM 456, LUDA T42, LUDA T60, EP 240 and B. thermocatenulatus (DSM 730).

All strains in cluster (2) showed amylolytic activity and all members of sub-cluster (2b) showed a diffuse reaction on starch agar plates. All strains were proteolytic usually degrading both casein and gelatin. Hippurate was hydrolysed by all members of group (2a) but by only one member of group (2b). All strains grew at pH 6.5 but only 23% showed growth at pH 5.5, only 4% of strains grew in 5% (w/v) saline. Citrate was utilised by all members of group (2a) but none of group (2b). All strains appeared prototrophic on Cal II minimal agar. All members of group (2b) showed anaerobic growth on TSBA and acid production from anaerobic glucose peptone broth; cluster (2a) was positive in 71% and 49% of strains respectively. All of sub-group (2a) and (2c) were oxidase positive but only 57% of group (2b) were positive. Sub-groups (2a) and (2b) showed growth at 75 ^{o}C and 42 ^{o}C.

The cells in sub-group (2a) were larger than those in group (2b) with a cell length in excess of 5 µm, only 29% of group (2b) were greater than 5 µm in length.

The colony morphology of the majority of strains in cluster (2) was circular, entire, raised, shiny and showed no evidence of spreading. All strains in cluster (2) fermented glucose, fructose and maltose and the majority, including all of sub-group (2b) fermented dextrin, glycerol, mannose, sucrose and trehalose.

3.2.4 Cluster (3)

Cluster (3) contained 12 strains including the marker strains DSM 465 and DSM 466 identified as B. thermodenitrificans by Klaushofer and Hollaus (1970), and B. stearothermophilus LUDA T22 identified as a denitrifying strain by Walker and Wolf (1971).

Table 3.10

Characteristics of the clusters as percentages of positive reactions

Characters	Cluster (2)	Cluster (2a)	Cluster (2b)	Cluster (2c)
Decomposition				
Starch	100	100	100	100
Casein	81	100	100	58
Gelatin	81	14	71	83
Tyrosine	8	0	0	17
Brown pigment	38	43	14	50
Phenyl alanine	11	29	0	8
Hippurate	64	100	14	73
Growth				
3% (w/v) NaCl	32	14	29	45
5% (w/v) NaCl	4	0	0	8
0.02% (w/v) Azide	35	29	0	58
Lysozyme	11	0	0	25
NB pH 6.5	100	100	100	100
NB pH 6.0	69	100	71	50
NB pH 5.5	23	14	29	25
Cal min	100	100	100	100
Cal + CA	100	100	100	100
SMS min	48	57	14	64
SMS + CA	60	86	29	64
TSBA anaerobic	81	71	100	75
Glu. anaer. acid	76	43	100	82
Production				
Catalase	27	14	0	50
Oxidase	88	100	57	100
Acid (MR)	27	14	29	33
Acetoin (VP)	4	14	0	0
Utilisation				
Citrate	46	100	0	42
Propionate	4	0	0	8

Table 3.10 (Cont'd)

Characters	Cluster (2)	Cluster (2a)	Cluster (2b)	Cluster (2c)
Reduction				
NO_3 - NO_2	72	83	100	50
NO_3 - NO	16	0	0	33
NO_3 - gas	16	0	0	33
NO_2 - NO	42	0	71	50
NO_2 - gas	11	0	0	25
Anaer. NO_3 - gas	4	0	0	8
Acid Production				
Adonitol	8	0	0	18
Arabinose	28	14	14	46
Dextrin	92	100	100	80
Dulcitol	0	0	0	0
Erythritol	4	0	0	9
Fructose	100	100	100	100
Galactose	42	0	86	36
Glucose	100	100	100	100
Glycerol	82	86	100	73
Glycogen	56	29	100	45
Inositol	4	0	0	9
Inulin	0	0	0	0
Lactose	4	0	14	0
Maltose	100	100	100	100
Mannose	92	100	100	82
Mannitol	48	57	0	73
Raffinose	46	29	83	36
Rhamnose	5	0	0	11
Salicin	46	29	67	45
Sorbitol	12	0	17	18
Starch	64	29	100	64
Sucrose	76	57	100	73
Trehalose	84	57	100	91
Xylose	46	57	14	60

Table 3.10 (Cont'd)

Characters	Cluster (2)	Cluster (2a)	Cluster (2b)	Cluster (2c)
Cell form and size				
Single cells	96	100	100	90
Cells in pairs	37	57	29	30
Cells in chains	12	43	0	0
Cells as filaments	4	0	0	10
Length <5 µm	37	0	71	40
Length >5 µm	62	100	29	60
Diameter <0.75 µm	8	0	0	20
Diameter >0.75 µm	92	100	100	80
Colony Morphology				
Elevation				
Flat	0	0	0	0
Raised	4	0	0	9
Convex	96	100	100	91
Pulvinate	0	0	0	0
Edge				
Entire	100	100	100	100
Erose	0	0	0	0
Undulate	0	0	0	0
Surface				
Smooth/Shiny	96	100	100	91
Smooth/Matt	8	14	0	9
Rough	0	0	0	0
Shape				
Circular	100	100	100	100
Irregular	0	0	0	0
Spreading				
V	100	100	100	100
X	0	0	0	0
Y	0	0	0	0
Z	0	0	0	0

Table 3.10 (Cont'd)

Characters	Cluster (2)	Cluster (2a)	Cluster (2b)	Cluster (2c)
Temp. for growth				
75 °C	85	100	100	60
70 °C	100	100	100	100
65 °C	100	100	100	100
42 °C	100	100	100	100
37 °C	19	14	0	33
30 °C	4	0	0	8
25 °C	4	0	0	8
Antibiotic Sensitivity				
Erythromycin	100	100	100	100
Chloramphenicol	100	100	100	100
Tetracycline	100	100	100	100
Naladixic acid	54	29	86	50
Streptomycin	100	100	100	100
Ampicillin	96	100	100	90
Gentamicin	100	100	100	100
Trimethoprim	100	100	100	100

Legend

The number of strains in each cluster giving positive reactions was expressed as a percentage of the total number of strains in the cluster.

Weak reactions were regarded as positive. Starch hydrolysis was regarded as positive whether the reaction was diffuse or restricted. Antibiotic sensitivity was scored as positive when any signs of growth inhibition were evident.

The majority of strains in this group (92%) had amylolytic activity showing restricted hydrolysis on starch plates. None of the strains hydrolysed casein, but 42% hydrolysed gelatin. Most strains grew in nutrient broth with 3% (w/v) saline but only 42% grew in 5% (w/v) saline. All strains grew at pH 6.5 but none grew at pH 5.5, 83% of strains utilised citrate. All strains were prototrophic on Cal II minimal agar and were oxidase and catalase positive. Denitrification of nitrate and nitrite to gas was characteristic of this group and 73% of these strains reduced nitrate under anaerobic conditions. Most strains grew at 75 ^{o}C and 70 ^{o}C, only 33% grew at 37 ^{o}C. The colony morphology of this group was typified by flat, erose, irregular, rough colonies with little evidence of spreading. All strains produced acid from fermentation of dextrin, glucose, maltose, trehalose and xylose; 90% also fermented glycerol, mannose, mannitol and starch, neither dulcitol or adonitol were fermented.

3.2.5 Cluster (4)

Cluster (4) contained five strains none of which were marker strains. All strains had strong amylolytic and proteolytic activity, none hydrolysed tyrosine or hippurate. All of these strains grew in nutrient broth with 5% (w/v) saline and at pH 5.5; 80% of these strains grew in the presence of lysozyme and all were prototrophic on Cal II and SMS minimal agar. All strains grew anaerobically on TSBA and 80% produced acid from anaerobic glucose peptone broth. All strains were oxidase and catalase positive and produced acetoin. The colony morphology was typical of this group which produced raised, erose, shiny circular colonies following overnight incubation on TSBA; all strains grew at 25 ^{o}C on TSBA.

All strains produced acid from fermentation of galactose, glucose, mannose, mannitol, rhamnose, sucrose, trehalose and xylose and 80% fermented adonitol, arabinose, fructose, glycerol, inositol, lactose, raffinose and sorbitol. Erythritol was not fermented by any strain in this group.

Strains RS 45 and LO_2 had a high similarity (80 - 85% and 75 - 80% respectively) with two members of cluster (4). Both strains produced erose colonies, typical of members of cluster (4). Strain LO_2 was independently identified as a strain of B. licheniformis (Berkeley, pers. comm.). Gordon et al. (1973) reported citrate and propionate utilisation to be a distinguishing character of B. licheniformis; of the seven strains (members of cluster (4), RS 45 and LO_2) only LO_2 was found to utilise

Table 3.11

Characteristics of the clusters as percentages of positive reactions

Characters	Cluster (3)	Cluster (4)	Cluster (5)	Cluster (6)	Cluster (7)
Decomposition					
Starch	92	100	60	66	33
Casein	0	100	40	0	33
Gelatin	42	100	60	100	0
Tyrosine	25	0	100	0	0
Brown pigment	67	0	100	0	0
Phenyl alanine	8	0	20	33	0
Hippurate	42	0	80	0	0
Growth					
3% (w/v) NaCl	92	100	0	33	0
5% (w/v) NaCl	42	100	0	0	0
0.02% (w/v) Azide	50	100	80	33	100
Lysozyme	0	80	0	0	0
NB pH 6.5	100	100	20	66	100
NB pH 6.0	33	100	0	0	100
NB pH 5.5	0	100	0	0	100
Cal min	100	100	100	33	66
Cal + CA	100	100	100	33	100
SMS min	67	100	20	0	0
SMS + CA	100	100	40	33	100
TSBA anaerobic	82	100	100	66	100
Glu. anaer. acid	46	80	100	33	100
Production					
Catalase	100	100	100	100	100
Oxidase	100	100	80	66	0
Acid (MR)	0	0	50	0	66
Acetoin (VP)	0	100	0	0	66
Utilisation					
Citrate	83	0*	80	0	0
Propionate	0	0	0	0	0

Table 3.11 (Cont'd)

Characters	Cluster (3)	Cluster (4)	Cluster (5)	Cluster (6)	Cluster (7)
Reduction					
NO_3 - NO_2	0	100	40	100	33
NO_3 - NO	0	0	20	0	0
NO_3 - gas	100	0	80	0	0
NO_2 - NO	0	0	20	33	0
NO_2 - gas	100	0	100	0	0
Anaer. NO_3 - gas	73	0	75	0	0
Acid Production					
Adonitol	0	80	0	66	33
Arabinose	75	80	100	100	33
Dextrin	100	20	100	100	100
Dulcitol	0	20	0	33	0
Erythritol	8	0	20	33	0
Fructose	75	80	100	66	100
Galactose	50	100	100	100	100
Glucose	100	100	100	100	100
Glycerol	92	80	40	100	100
Glycogen	42	60	40	33	33
Inositol	20	80	20	66	33
Inulin	8	40	60	66	0
Lactose	17	80	20	100	66
Maltose	100	40	100	100	100
Mannose	92	100	100	100	100
Mannitol	92	100	100	33	33
Raffinose	42	80	100	66	33
Rhamnose	56	100	60	100	33
Salicin	67	60	100	100	100
Sorbitol	75	80	80	66	33
Starch	91	40	80	66	66
Sucrose	83	100	100	100	33
Trehalose	100	100	100	100	100
Xylose	100	100	80	100	66

Table 3.11 (Cont'd)

Characters	Cluster (3)	Cluster (4)	Cluster (5)	Cluster (6)	Cluster (7)
Cell form and size					
Single cells	100	100	100	100	66
Cells in pairs	45	100	100	66	33
Cells in chains	36	25	80	33	0
Cells in filaments	0	0	20	0	66
Length <5 µm	36	75	60	100	0
Length >5 µm	64	25	40	0	100
Diameter <0.75 µm	27	0	0	100	33
Diameter >0.75 µm	73	100	100	0	66
Colony Morphology					
Elevation					
Flat	100	0	0	0	0
Raised	0	100	40	0	0
Convex	0	0	60	100	100
Pulvinate	0	0	0	0	0
Edge					
Entire	0	0	100	100	100
Erose	82	100	0	0	0
Undulate	9	0	0	0	0
Surface					
Smooth/Shiny	0	100	100	100	50
Smooth/Matt	0	0	0	0	50
Rough	100	40	0	0	0
Shape					
Circular	0	100	100	100	100
Irregular	100	0	0	0	0
Spreading					
V	91	100	100	100	100
X	0	0	0	0	0
Y	0	0	0	0	0
Z	0	0	0	0	0

Table 3.11 (Cont'd)

Characters	Cluster (3)	Cluster (4)	Cluster (5)	Cluster (6)	Cluster (7)
Temp. for growth					
75 °C	75	0	X	0	0
70 °C	92	20	100	0	0
65 °C	100	80	100	0	0
42 °C	100	100	80	100	100
37 °C	33	100	0	100	100
30 °C	8	100	0	100	100
25 °C	0	100	0	100	66
Antibiotic Sensitivity					
Erythromycin	100	80	100	100	100
Chloramphenicol	100	80	100	100	100
Tetracycline	100	100	100	100	100
Naladixic acid	27	25	20	X	0
Streptomycin	100	100	100	100	100
Ampicillin	100	80	100	100	100
Gentamicin	100	100	100	100	100
Trimethoprim	100	100	100	100	50

Legend

The number of strains in each cluster giving positive reactions was expressed as a percentage of the total number of strains in the cluster.

Weak reactions were regarded as positive. Starch hydrolysis was regarded as positive whether the reaction was diffuse or restricted. Antibiotic sensitivity was scored as positive when any signs of growth inhibition were evident.

Table 3.12

Utilisation of citrate and propionate by members of cluster (4), strain LO_2 and RS 45

	Citrate utilisation							Propionate utilisation	
Strain	60 °C	57.5 °C	55 °C	54 °C	53 °C	50 °C	37 °C	52 °C	37 °C
RS 8	- (-)	- (-)	- (+)	- (+)	+ (+)	+ (+)	+ (+)	- (+)	+ (+)
RS 44	- (-)	- (-)	- (+)	- (+)	+ (+)	+ (+)	+ (+)	- (+)	+ (+)
RS 49	- (-)	- (-)	+ (+)	W (+)	- (+)	+ (+)	+ (+)	- (+)	+ (+)
RS 54	- (-)	- (-)	W (+)	W (+)	+ (+)	+ (+)	+ (+)	- (+)	+ (+)
RS 245	- (-)	- (-)	- (+)	- (+)	+ (+)	+ (+)	+ (+)	- (+)	+ (+)
LO_2	- (-)	- (+)	+ (+)	+ (+)	+ (+)	+ (+)	+ (+)	- (+)	+ (+)
RS 45	- (-)	- (W)	- (+)	+ (+)	+ (+)	+ (+)	+ (+)	NT NT	NT NT

Legend

Slopes of Koser's modified citrate agar, propionate agar and TSBA were inoculated from 16 h plate cultures. Slopes were incubated in thermostatically controlled water baths for 5 days. Results in parenthesis relate to growth on TSBA slopes. NT, not tested; W, weak positive reaction.

citrate at 55 °C. Following re-examination at 37 °C and 50 °C (Table 3.12), all seven strains utilised citrate and propionate; at 53 - 57.5 °C citrate utilisation was variable. Growth was positive on TSBA at 55 °C but negative at 60 °C; two members of cluster (4) had previously shown strong growth at 65 °C and two others previously grew weakly at 65 °C (Table 3.12).

3.2.6 Cluster (5)

Cluster (5) was composed of five cultures none of which were marker strains. Hydrolysis of starch, casein and gelatin was variable. All strains hydrolysed tyrosine producing a brown pigment and 80% hydrolysed hippurate. None of the cultures grew in 3% (w/v) saline and only one (20%) showed evidence of growth at pH 6.5. All cultures appeared prototrophic on Cal II minimal agar and grew anaerobically on TSBA and in glucose peptone broth. The group were all catalase positive and 80% were oxidase positive. Nitrite was reduced to gas by all strains and 75% reduced nitrate to gas under anaerobic conditions. All strains grew at 70 °C and none grew at 42 °C. The colony morphology was either raised or convex, entire, shiny and circular, with no evidence of spreading. All strains produced acid from fermentation of arabinose, dextrin, fructose, galactose, glucose, maltose, mannose, manitol, raffinose, salicin, sucrose and trehalose, and 80% of strains also fermented sorbitol, starch, and xylose.

3.2.7 Cluster (6)

Cluster (6) contained only three strains none of which were marker strains. Two hydrolysed starch, all three hydrolysed gelatin but none hydrolysed casein. None of the strains grew in 5% (w/v) saline or at pH 6.0 and citrate was not utilised. All three strains were catalase positive and two were oxidase positive. None grew at 65 °C but all three strains grew at 25 °C. Cells were small, less than 5 µm in length and 0.75 µm in diameter. Colonies were all convex, entire, shiny and circular showing no signs of spreading. All three strains in cluster (6) produced acid from arabinose, dextrin, galactose, glucose, glycerol, lactose, maltose, mannose, rhamnose, salicin, sucrose, trehalose and xylose.

3.2.8 Cluster (7)

Cluster (7) comprised only three strains, two of which were the marker strains, B. coagulans ATCC 8038 and ATCC 12245. One strain hydrolysed starch and casein but none grew in 3% (w/v) saline. All three strains grew at pH 5.5 and anaerobically on TSBA and in glucose

peptone broth. They were catalase positive and oxidase negative, did not grow at 65 °C but grew at 30 °C and two strains grew at 25 °C. Colonies were convex, entire, circular with either a shiny or matt surface and did not spread on TSBA plates. All three strains fermented dextrin, fructose, galactose, glucose, glycerol, maltose, mannose, salicin and trehalose.

3.2.9 Unassigned strains

Sixteen strains did not join any of the seven clusters. Four strains (RS 5, RS 6, RS 9 and RS 45) were discussed with cluster (1). Strain EP 262 had greatest similarity with cluster (2) and had a similarity of 80 - 85% with B. caldotenax. Strain RS 2 also showed 80 - 85% similarity with four members of cluster (2) and RS 56 had 80 - 85% similarity with one member of cluster (2). Strains RS 4, RS 17 and RS 60 all had greatest similarity with members of cluster (1). B. sphaericus (DSM 463) had little similarity with other strains except one member of cluster (1) and cluster (2). Strain RS 56 had 75 - 80% similarity with members of cluster (5). Strain RS 12 had some similarity with cluster (1) and RS 205 had some similarity with cluster (5). Strains LO_2 (AB 124) and RS 45 were considered with members of cluster (4). The major distinguishing characters of the seven clusters are presented in Table 3.13 and 3.14. A dichotomous key (Fig. 3.3) was devised using the data from this Table which enabled the preliminary identification of strains using no more than eight tests.

Table 3.13

The main distinguishing characteristics of the ten clusters and sub-clusters

	1a	1b	2a	2b	2c	3	4	5	6	7
Starch hydrolysis	-	v	+	+	+	+	+	v	v	v
Casein hydrolysis	-	-	+	+	v	-	+	v	v	v
Gelatin hydrolysis	-	-	-	v	+	v	+	v	+	-
Tyrosine hydrolysis	-	-	-	-	-	v	-	+	-	-
Hippurate hydrolysis	-	-	+	-	v	-	-	+	-	-
5% (w/v) saline	+	+	-	-	-	v	+	-	-	-
0.02% (w/v) azide	-	-	v	-	v	v	+	+	v	+
pH 5.5	-	-	-	v	v	-	+	-	-	+
Citrate utilisation	-	+	+	-	v	+	-	+	-	-
Anaer. TSBA	v	+	v	+	v	+	+	+	v	+
Anaer. glucose	-	v	v	+	+	-	+	+	v	+
Catalase prod.	+	+	-	-	v	+	+	+	+	+
Oxidase prod.	+	+	+	v	+	+	+	+	v	-
Acetoin prod.	v	-	-	-	-	-	+	-	-	v
NO_3 - gas	-	-	-	-	v	+	-	+	-	-
growth at 75 °C	-	-	+	+	v	v	-	NT	-	-
growth at 65 °C	+	+	+	+	+	+	v	+	-	-
growth at 25 °C	-	-	-	-	-	-	+	-	+	v
Colonies dry	-	-	-	-	-	+	-	-	-	-
Colonies erose	-	-	-	-	-	+	+	-	-	-

Legend

\+ >80% strains +ve

\- <20% strains +ve

v 20 - 80% strains positive

NT Not tested

Table 3.14

Sugar fermentations of the ten clusters and sub-clusters

	1a	1b	2a	2b	2c	3	4	5	6	7
Adonitol	-	v	-	-	-	-	+	-	v	v
Arabinose	v	v	v	-	v	v	+	+	+	v
Dextrin	-	v	+	+	+	+	-	+	+	+
Dulcitol	-	v	-	-	-	-	v	-	v	-
Erythritol	-	v	-	-	-	-	-	v	v	-
Fructose	v	+	+	+	+	v	+	+	v	+
Galactose	v	v	v	+	v	v	+	+	+	+
Glucose	-	+	+	+	+	+	+	+	+	+
Glycerol	v	v	+	+	v	+	+	v	+	+
Glycogen	-	-	v	+	v	v	v	v	v	v
Inositol	-	+	-	-	-	v	+	v	v	v
Inulin	-	-	-	-	-	-	v	v	v	-
Lactose	-	-	-	-	-	-	+	v	+	v
Maltose	-	+	+	+	+	+	v	+	+	+
Mannose	v	v	+	+	+	+	+	+	+	+
Mannitol	-	+	v	-	v	+	+	+	v	v
Raffinose	-	v	v	+	v	v	+	+	v	v
Rhamnose	v	v	-	-	-	v	+	v	+	v
Salicin	-	v	v	v	v	v	v	+	+	+
Sorbitol	-	+	-	-	-	v	+	+	v	v
Starch	-	+	v	+	v	+	v	+	v	v
Sucrose	-	+	v	+	v	+	+	+	+	v
Trehalose	-	+	+	+	+	+	+	+	+	+
Xylose	v	v	v	-	v	+	+	+	+	v

Legend

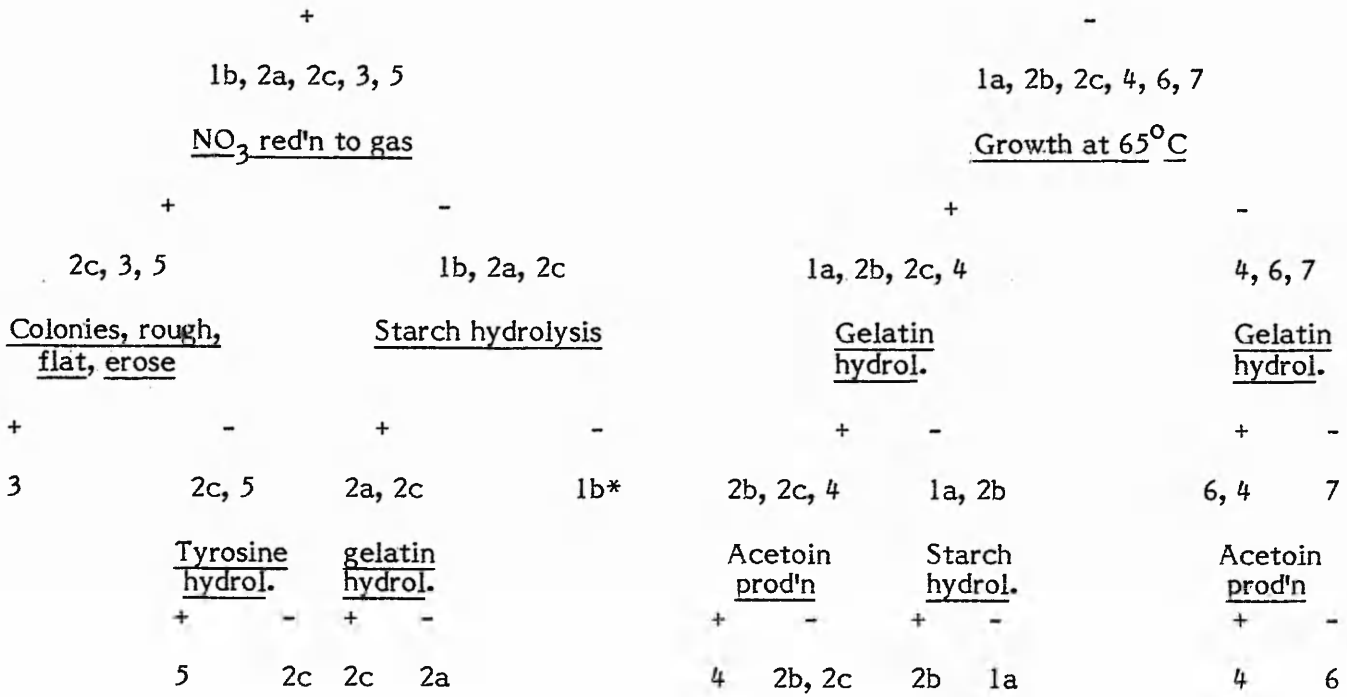
\+ >80% strains +ve

\- <20% strains +ve

v 20 - 80% strains positive

Figure 3.3

Dichotomous key based on the results presented in Table 3.13



Legend

The key is based on the taxonomic data obtained from a study of 102 thermophilic aerobic sporeformers. A positive character was defined as, greater than 80% of strains in group giving a positive reaction. A negative character was defined as less than 20% of strains in group giving a positive reaction. Characters with positive reactions between 20% and 80% were considered to be variable within the group. *26% of group (1b) hydrolyse starch.

3.3 API ZYM ANALYSIS OF 12 THERMOPHILIC STRAINS OF BACILLUS

API ZYM analysis data is presented in Table 3.15. The data was examined with reference to the clusters established by numerical taxonomy. Strains EP 136 and RS 93 (from cluster (1b)) showed similar low levels of constitutive enzyme production. B. caldotenax, B. caldovelox, and B. caldolyticus all produced similar levels of extracellular enzymes; however, B. stearothermophilus NCA 1503 and strain NW 10 (from cluster (2b)) did not show close similarity in their levels of enzyme production.

3.4 ESTERASE ENZYME ANALYSIS

Examination of 12 strains by esterase analysis (Table 3.16) indicated B. caldotenax and B. caldovelox to have similar esterase patterns; B. caldolyticus had two similar bands although one exhibited a slightly different mobility; B. stearothermophilus EP 262 also had two similar bands but had also an additional weaker band. B. stearothermophilus NCA 1503 and NW 10 (cluster (2b)) showed similar mobilities as did RS 93 and EP 136 (cluster (1b)). B. stearothermophilus ATCC 12016 indicated two strongly staining bands and one weaker band, these appeared different to the bands produced by other strains.

3.5 PYROLYSIS MASS SPECTROMETRY

The data obtained from 12 thermophiles by Dr. C. Gutteridge is presented in Fig. 3.4 using average linkage cluster analysis. The strains were examined as triplet or quadruplet samples. Strain NW 10 and NCA 1503 showed close similarity at the level of >98%, B. caldotenax and B. caldovelox similarly indicated >97% similarity. The strains of B. coagulans ATCC 12245 and ATCC 8038 did not show very close similarity with each other and appeared distinct from the other strains examined. Strains RS 93 and EP 136 indicated similarity at 91% and similarity with the main group of obligate thermophiles at 85%. The B. caldovelox and B. caldotenax group appeared linked at 95% similarity to strains NCA 1503 and NW 10, and ATCC 12016. Strains EP 240 and EP 262 were linked to this group at 90% similarity.

Table 3.15

Constitutive enzyme production from thermophiles using the API ZYM system

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
B. stearothermophilus EP 136	0	2	2	2	1	1	2	1	1	3	3	2	0	0	0	1	1	0	0	0
B. stearothermophilus RS 93	0	1	1	1	1	0	0	0	0	0	2	3	0	0	0	1	0	0	0	0
B. caldotenax	0	5	3	4	3	3	3	2	3	3	4	4	4	1	1	4	3	1	0	0
B. caldovelox	0	5	4	4	3	2	2	1	2	2	4	4	4	2	3	4	4	2	1	0
B. caldolyticus	0	4	3	3	3	2	1	1	2	2	3	3	3	2	3	3	4	1	1	0
B. stearothermophilus NCA 1503	0	4	3	4	3	2	1	1	2	2	2	4	4	2	2	1	0	0	0	0
B. stearothermophilus NW 10	0	1	2	2	1	0	0	0	0	0	3	2	3	1	2	3	1	0	0	0
B. stearothermophilus ATCC 12016	0	2	2	3	2	1	0	0	1	0	2	4	4	3	2	2	1	0	0	0
B. stearothermophilus EP 240	0	2	2	3	2	2	1	1	1	4	2	2	0	1	1	3	2	1	0	0
B. stearothermophilus EP 262	0	1	1	1	1	1	0	0	0	0	1	2	0	0	2	2	1	1	0	0

Legend

Bacteria were grown for 16 h at 60 °C on TSBA. Suspensions of cells were prepared in distilled water to give an OD of 1.5 at 450 nm and 60 µl added to each reaction cupule. The strips were incubated at 60 °C for 10 h before reading the results. Enzyme production was qualitatively assessed on a scale 0 - 5. 0 indicated no enzyme production, 5 indicated high enzyme production. 1, 2, 3, 4, correspond to intermediate levels.

Enzymes detected in reaction cupules 1 - 20.

(1) Negative control, (2) Phosphatase alkaline, (3) Esterase (C4), (4) Esterase Lipase (C8), (5) Lipase (C14), (6) Leucine arylamidase, (7) Valine arylamidase, (8) Cystine arylamidase, (9) Trypsin, (10) Chymotrypsin, (11) Phosphatase acid, (12) Phosphamidase, (13) galactosidase, (14) galactosidase, (15) glucuronidase, (16) glucosidase, (17) glucosidase, (18) N-acetyl glucosaminidase, (19) mannosidase, (20) fucosidase.

Table 3.16

Electrophoretic mobility of esterases in cell extracts of thermophilic Bacillus

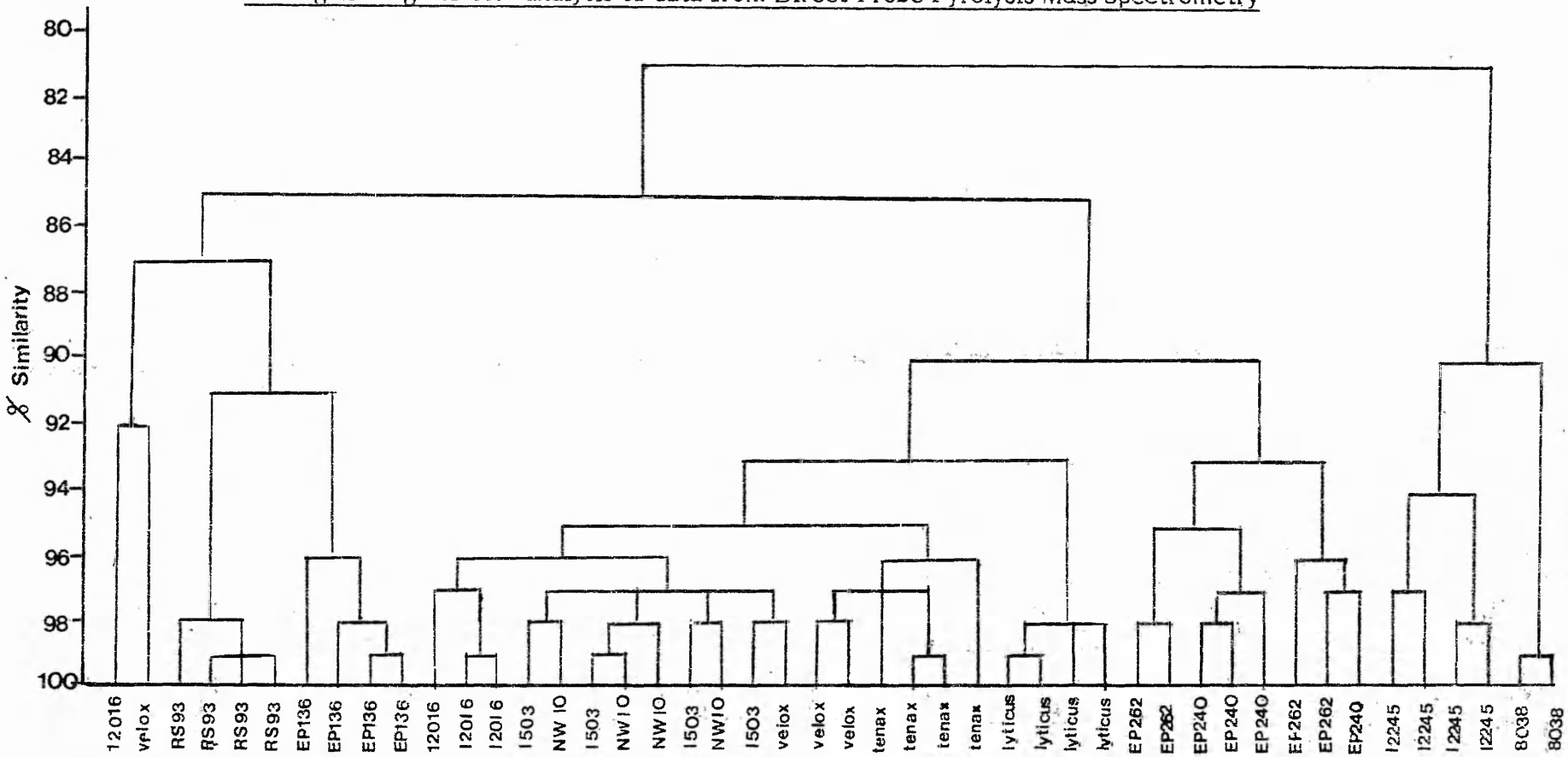
Strain		Rf values
B. stearothermophilus RS 93		0.30(s)
B. stearothermophilus EP 136		0.30(s)
B. caldotenax	(i)	0.18(s)
	(ii)	0.37(s)
B. caldovelox	(i)	0.18(s)
	(ii)	0.37(s)
B. caldolyticus	(i)	0.18(s)
	(ii)	0.30(s)
B. stearothermophilus EP 262	(i)	0.06(w)
	(ii)	0.18(s)
	(iii)	0.37(s)
B. stearothermophilus NCA 1503		0.20(s)
B. stearothermophilus NW 10		0.21(s)
B. stearothermophilus EP 240		0.14(s)
B. stearothermophilus ATCC 12016	(i)	0.13(w)
	(ii)	0.30(s)
	(iii)	0.67(s)

Legend

Conditions were as described in Materials and Methods. Rf values were determined with respect to bromophenol blue. The letter in parenthesis denotes whether the band was strong (s) or weak (w).

Figure 3.4

Average linkage cluster analysis of data from Direct Probe Pyrolysis Mass Spectrometry



Legend

Data from Dr. C. Gutteridge.

3.6 BACTERIOCIN TYPING OF BACILLUS THERMOPHILES

3.6.1 Selection of typing procedures

Bacteriocin typing was initially examined by replica plating the bacteriocin producers and overseeding with the test strains (2.9.1). This technique, although relatively simple, was hampered by the inability of chloroform to kill all the bacteriocin producing cells; this resulted in the spread of bacteriocin producers away from the original area of growth and confused the results (Table 3.17). Bacteriocin producers which normally produced spreading colonies could not be used in this technique.

Removal of the bacteriocin producing colonies from the plate, before exposure to chloroform, reduced (but did not eliminate) the number of remaining viable cells and resulted in the zones of inhibition being much clearer and easier to record.

Growth of the bacteriocin producing colonies on cellophane, made removal of all viable cells from the plate very simple and observation of the zones in the seeded layer was much easier. Build up of condensation on the cellophane was a problem and resulted in some strains which were normally discrete colonies spreading over the surface of the cellophane. Incubation at 60 ^{o}C also resulted in the shrinkage and wrinkling of the cellophane sheets.

Typing with preparations of crude bacteriocins (2.9.2) using a well diffusion system, avoided problems of killing the cells, and permitted the use of bacteriocin producers with normal form spreading colonies.

3.6.2 Bacteriocin typing

The results obtained from typing 100 strains with 30 different preparations of crude bacteriocin are shown in Table 3.18.

One of the first facts to become apparent was the insensitivity of 38 strains to any of the bacteriocins. This group appear to comprise the majority of the starch negative strains, 20 out of 29 of the starch negative strains were insensitive. The bacteriocins which appeared to show the widest range of activity were NU 10 (59 strains out of 81 strains showing bacteriocin activity), RS 98 (53 strains), RS 93 (44 strains), RS 249 (34 strains), RS 70 (31 strains), RS 53 (25 strains), NU 1620 (20 strains), DSM 465 (16 strains), ATCC 12976 (15 strains), NU 234 (12 strains).

Table 3.17

Bacteriocin typing of strains using replica plating of bacteriocin producers followed by seeding with test strains

Bacteriocin Producing Strains	Test strains									
	EP 262	RS 93	LUDA T22	LUDA T60	LUDA T210	LUDA T214	DSM 463	DSM 465	DSM 466	ATCC 10149
RS 7	*	W+	*	D	0	0	0	*	W	D
RS 46	*	WD	+	D	0	*	+	*	D	*
RS 53	D	D	D	D	0	*	*	D	D	D
RS 58	D	0	D	0	+	*	*	D	D	0
RS 70	D	D	D	D	D	+	D	D	D	D
RS 77	*	0	D	D	+	*	D	D	D	D
RS 80	D	W	*	0	0	*	+	*	D	*
RS 82	D	D	D	D	D	+	D	D	D	D
RS 83	D	D	D	D	D	+	D	D	D	D
RS 88	0	0	0	*	+	*	+	0	0	D
RS 91	*	0	*	*	0	*	D	D	D	D
RS 93	D	0	D	D	+	D	D	D	D	D
RS 98	*	0	D	+	D	D	D	D	*	D
RS 249	D	WD	D	D	D	D	D	D	D	D
NCTC 10003	+	+	D	+	0	*	+	D	D	D
ATCC 10149	0	+	WD	+	0	+	+	D	D	0
ATCC 12976	D	W	D	+	0	D	+	D	D	D
NCIB 8919	D	+	D	D	0	*	D	D	D	D
ATCC 8005	0	0	0	0	0	*	0	0	0	0
LUDA T210	*	0	*	D	0	0	0	*	0	D
DSM 456	+	W	D	0	*	0	0	D	D	0
DSM 459	0	0	0	D	0	0	0	0	0	0
DSM 460	0	0	0	0	0	0	0	0	0	0
DSM 463	*	0	*	0	0	*	*	0	0	0
DSM 465	*	W	*	*	0	*	*	0	0	0
DSM 466	*	W	0	+	0	*	*	0	0	0
DSM 730	0	0	0	0	0	0	0	0	0	*

Table 3.17 (Cont'd)

Bacteriocin Producing Strains	Test strains										
	NCIB 8919	NCTC 10003	RS 33	RS 56	RS 70	RS 77	RS 80	RS 98	RS 249	RS 28	RS 32
RS 7	0	+	0	0	0	0	0	*	0	0	*
RS 46	*	D	+	*	*	0	D	D	D	D	D
RS 53	*	D	*	*	D	D	D	D	D	D	D
RS 58	*	*	*	0	0	0	0	0	0	0	0
RS 70	*	D	D	D	0	0	D	D	D	D	D
RS 77	*	D	D	D	+	0	D	+	0	0	D
RS 80	*	*	*	*	0	WD	D	D	D	*	0
RS 82	*	D	D	0	D	D	D	D	D	D	D
RS 83	*	D	D	0	D	D	D	D	D	D	D
RS 88	*	0	0	*	0	0	0	0	0	0	0
RS 91	*	*	0	*	*	0	0	0	0	*	0
RS 93	*	D	D	D	D	0	D	D	+	D	D
RS 98	*	D	D	D	WD	0	D	0	0	D	D
RS 249	*	D	D	D	D	D	D	0	0	D	D
NCTC 10003	+	D	+	*	0	+	+	0	W	+	+
ATCC 10149	+	0	0	*	+	+	+	+	+	+	+
ATCC 12976	0	D	+	0	+	+	+	+	+	+	+
NCIB 8919	0	D	+	0	D	+	+	+	+	+	D
ATCC 8005	0	*	0	*	0	0	0	0	0	0	0
LUDA T210	*	0	0	*	*	0	*	0	0	0	0
DSM 456	D	0	0	0	0	0	0	0	W	+	+
DSM 459	D	0	0	0	0	0	0	0	0	0	0
DSM 460	0	0	0	0	0	0	0	0	0	0	0
DSM 463	*	0	0	*	*	0	0	0	0	0	0
DSM 465	*	0	0	*	W	0	0	+	WD	0	0
DSM 466	*	0	*	*	W	0	0	0	0	0	0
DSM 730	0	0	0	0	0	0	0	0	0	0	0

Table 3.17 (Cont'd)

Bacteriocin Producing Strains	Test strains										
	RS 46	RS 53	RS 82	RS 83	RS 85	RS 88	RS 91	DSM 456	DSM 459	DSM 460	ATCC 12976
RS 7	0	0	0	0	*	*	*	0	0	D	*
RS 46	0	WD	D	WD	*	*	0	0	*	0	*
RS 53	0	WD	D	D	*	*	*	*	D	D	*
RS 58	0	0	0	0	*	*	*	0	0	0	*
RS 70	0	0	D	D	D	D	D	D	D	D	0
RS 77	0	0	0	0	D	D	D	+	D	D	*
RS 80	0	0	D	D	*	*	D	0	W	D	*
RS 82	0	D	D	D	D	D	D	D	D	D	*
RS 83	0	D	D	D	*	D	D	D	D	D	*
RS 88	0	0	0	0	0	*	*	*	0	0	*
RS 91	0	0	0	0	0	*	*	*	D	D	*
RS 93	W	D	WD	0	D	D	D	D	D	D	D
RS 98	0	0	0	0	D	D	D	+	D	D	*
RS 249	VW	WD	0	0	D	D	D	D	D	D	*
NCTC 10003	0	0	D	W	*	*	*	D	WD	+	*
ATCC 10149	0	WD	+	+	*	*	*	0	0	+	*
ATCC 12976	0	0	+	+	*	0	*	+	D	D	*
NCIB 8919	0	0	+	+	*	0	+	D	D	D	*
ATCC 8005	0	0	0	*	*	*	*	0	0	0	*
LUDA T210	0	0	0	0	*	*	*	0	0	0	*
DSM 456	0	0	W	W	*	*	*	0	0	+	*
DSM 459	0	0	0	0	0	D	*	0	0	0	*
DSM 460	VW	0	0	0	*	*	*	0	0	W	*
DSM 463	VW	0	0	0	0	0	0	0	0	0	*
DSM 465	0	0	0	*	0	0	0	0	0	0	*
DSM 466	W	0	0	*	0	0	0	0	0	0	*
DSM 730	0	0	0	0	*	*	*	0	0	0	*

Legend (Table 3.17)

Zone sizes are recorded as:

+ - zones of 1 - 2 mm in the immediate vicinity of the colony.

D - for wide or diffuse zones of inhibition.

WD - weak diffuse zones were intermediate between + and D.

0 - for no evidence of inhibition.

* - Not tested or result inconclusive due to contamination or spreading of colonies due to inefficient killing of colonies by chloroform.

Legend (Table 3.18)

Crude thermocin preparations (0.1 ml) were added to wells cut into seeded TSBA bio-assay plates. The plates were incubated at 60 ^{o}C for 16 h and zones of inhibition recorded in mm. h, hazy zone; vh, very hazy zone; sa, signs of bacteriocin activity.

Table 3.18

Bacteriocin typing using crude filtered bacteriocin preparation

Bacteriocin Producing Strains	Test strains									
	NCA 1503	ATCC 12016	EP 136	EP 240	EP 262	RS 93	NW 10	tenax	velcx	lyticus
RS 6	0	*	0	0	0	0	0	0	1h	2sa
RS 33	0	*	0	0	0	0	0	0	0	0
RS 53	0	*	0	0	2h	0	0	2.5	1.5	2h
RS 70	0	*	0	0	3h	0	0	3	3.0	4h
RS 77	0	*	0	0	.5h	0	0	*	0	1h
RS 80	0	*	0	0	.5h	0	0	0	0	*
RS 82	5h	2.5	0	2	2	0	5h	4	5	0
RS 85	0	*	0	0	0	0	0	0	0	0
RS 86	0	*	0	0	0	0	0	0	0	0
RS 88	0	*	0	0	0	0	0	0	4	0
RS 93	1	1	0	1	3h	0	2	5	1.5	5
RS 98	3h	*	0	1.5	6h	0	3.5	6	5	5
RS 210	0	*	0	0	2	0	0	3	0	.5h
RS 212	0	*	0	0	0	0	0	2	2h	0
RS 249	4vh	*	0	1	3	0	4vh	3	3h	4h
NCTC 10003	0	*	0	0	.5h	0	0	0	0	0
ATCC 10149	0	*	0	0	1h	0	0	4	2	0
ATCC 12976	0	*	0	0	2h	0	0	0	2	0
NCIB 8919	0	*	0	0	0	0	0	2	0	0
DSM 463	0	*	0	0	0	0	0	2	2	0
DSM 465	0	*	0	0	1.5	0	0	0	3	0
NU 3	0	*	0	0	5	0	0	0	0	1.5h
NU 4	0	*	0	0	1.5	0	0	0	0	0
NU 10	5	*	0	5	6h	0	6	5	5	7
NU 12	0	*	0	0	0	0	0	0	0	0
NU 234	0	*	0	0	3	0	0	0	0	0
NU 1490	0	*	0	0	0	0	0	0	0	0
NU 1503	0	*	0	0	0	0	0	0	0	0
NU 1620	0	*	0	0	3	0	0	2.5h	4	0
NCA 2184	0	*	0	0	0	0	0	0	0	0

Table 3.18 (Cont'd)

Bacteriocin Producing Strains	Test strains									
	ATCC 12245	ATCC 8038	T22	T42	T60	T141	T210	T214	DSM 456	DSM 463
RS 6	0	0	0	0	1h	0	0	0	0	0
RS 33	0	0	0	0	0	0	0	0	0	0
RS 53	0	0	0	0	1	0	0	0	2	1
RS 70	0	0	0	0	2h	0	0	3sa	2h	1
RS 77	0	0	0	0	1	0	0	0	0	0
RS 80	0	0	0	0	0	0	0	0	0	0
RS 82	0	0	0	1	3h	0	3	0	4	4
RS 85	0	0	0	0	6	0	0	*	0	0
RS 86	0	0	0	0	0	0	0	*	0	0
RS 88	0	0	0	0	3h	0	0	*	0	0
RS 93	0	0	0	0	1	0	1.5	0	4	5*
RS 98	0	0	3	1.5	4h	0	2	0	5	3sa
RS 210	0	0	0	0	0	0	0	0	0	0
RS 212	0	0	0	0	6h	0	0	0	0	0
RS 249	0	0	0	0	2h	0	2h	0	*	0
NCTC 10003	0	0	0	0	0	0	0	0	0	0
ATCC 10149	0	0	0	0	3h	0	0	0	0	0
ATCC 12976	0	0	0	0	4h	0	0	0	0	0
NCIB 8919	0	0	0	0	0	0	0	0	0	0
DSM 463	0	0	0	0	0	0	0	.5h	0	0
DSM 465	0	0	0	0	4h	0	0	0	0	0
NU 3	0	0	0	0	0	0	1	4h	4	0
NU 4	0	0	0	0	0	0	0	6sa	0	0
NU 10	3	1.5	0	3	3	0	4	6	6	3sah
NU 12	0	0	0	0	0	0	0	0	0	0
NU 234	0	*	0	0	0	0	0	0	0	0
NU 1490	0	0	0	0	0	0	0	0	0	0
NU 1503	0	0	0	0	0	0	3	3sa	5	3h
NU 1620	0	0	0	0	0	0	0	0	0	0
NCA 2184	0	1	0	0	0	0	0	0	0	0

Table 3.18 (Cont'd)

Bacteriocin Producing Strains	Test strains									
	DSM 730	DSM 465	DSM 466	ATCC 8005	ATCC 10149	NCIB 8919	NCTC 10003	LO_2 516	RS 1	RS 2
RS 6	*	3h	0	0	6h	4	2	0	0	0
RS 33	0	0	0	0	0	0	0	0	0	0
RS 53	3	0	0	1	0	0	0	0	.5h	0
RS 70	4*	1.6	3h	2	0	3h	2	0	.5h	0
RS 77	0	0	0	0	0	0	0	0	0	0
RS 80	0	0	0	0	0	0	0	0	0	0
RS 82	3	3	2h	3	4	4h	5	0	2.5	2
RS 85	0	0	0	0	0	0	0	0	0	0
RS 86	0	0	0	0	0	0	0	0	0	0
RS 88	1.5	0	0	0	0	0	0	0	0	0
RS 93	1	5h	2h	3	2	3h	2	0	.5h	2
RS 98	7	2	2h	3	4	5h	5	0	2h	2
RS 210	0	0	0	0	0	0	0	0	0	0
RS 212	2h	0	0	0	0	0	5h	0	0	0
RS 249	3h	1h	3h	3	2	0	3	0	1.5h	0
NCTC 10003	0	0	0	0	0	1	0	0	0	0
ATCC 10149	0	0	3h	0	0	0	0	0	0	0
ATCC 12976	1	0	0	0	0	0	0	0	0	0
NCIB 8919	0	0	0	0	0	0	0	0	0	0
DSM 463	0	0	0	0	0	0	0	0	0	0
DSM 465	1.5h	0	0	0	0	0	0	0	0	0
NU 3	1h	0	0	0	0	1h	0	0	.5	0
NU 4	0	0	0	0	0	0	0	0	0	0
NU 10	4h	2	1	4	5	5h	5	0	3	5
NU 12	0	0	0	0	0	.5h	0	0	0	0
NU 234	1	0	0	0	0	0	0	0	0	0
NU 1490	0	0	0	0	0	0	0	0	0	0
NU 1503	0	3h	0	0	0	0	0	0	4h	.5
NU 1620	5	0	0	0	0	0	0	0	0	0
NCA 2184	0	0	0	0	0	0	0	0	0	0

Table 3.18 (Cont'd)

Bacteriocin Producing Strains	Test strains									
	RS 3	RS 4	RS 5	RS 6	RS 7	RS 8	RS 9	RS 10	RS 11	RS 12
RS 6	0	0	0	0	3	0	0	0	0	0
RS 33	0	0	0	0	0	0	0	0	0	0
RS 53	0	0	0	0	3h	0	0	0	0	0
RS 70	0	0	0	0	3h	4	0	0	0	0
RS 77	0	0	0	0	1h	0	0	0	0	0
RS 80	0	0	0	0	1h	0	0	0	0	0
RS 82	0	0	0	0	5h	3	0	0	0	0
RS 85	0	0	0	0	0	0	0	4vh	0	0
RS 86	0	0	0	0	1h	0	0	0	0	0
RS 88	0	0	0	0	0	0	0	0	0	0
RS 93	0	0	0	0	4	4	0	0	0	0
RS 98	0	0	0	1.5	3	4	0	0	0	0
RS 210	0	0	0	0	0	2	0	0	0	0
RS 212	0	0	0	0	0	0	0	0	0	0
RS 249	0	0	0	0	0	0	0	0	0	0
NCTC 10003	0	0	0	0	0	0	0	0	0	0
ATCC 10149	0	0	0	0	0	0	0	0	0	0
ATCC 12976	0	0	0	0	0	0	0	0	0	0
NCIB 8919	0	0	0	0	3h	0	0	0	0	0
DSM 463	0	0	0	0	3h	0	0	0	0	0
DSM 465	0	0	0	0	1	0	0	0	0	0
NU 3	0	0	0	0	1	0	0	0	0	0
NU 4	0	0	0	0	6	0	0	0	0	0
NU 10	0	0	0	0	5	5	0	2	0	0
NU 12	0	0	0	0	4vh	0	0	0	0	0
NU 234	0	0	0	0	1	0	0	0	0	0
NU 1490	0	0	0	0	1vh	0	0	0	0	.5
NU 1503	0	0	0	0	1.5h	0	0	0	0	0
NU 1620	0	0	0	0	1h	0	0	0	0	0
NCA 2184	0	0	0	0	1h	0	0	0	0	0

Table 3.18 (Cont'd)

Bacteriocin Producing Strains	Test strains									
	RS 13	RS 14	RS 15	RS 16	RS 17	RS 18	RS 19	RS 20	RS 21	RS 22
RS 6	0	0	0	0	0	0	0	3	0	0
RS 33	0	0	0	0	0	0	0	0	0	0
RS 53	0	0	1.5	2	0	0	0	0	0	0
RS 70	0	0	2h	3h	0	0	0	4	0	0
RS 77	0	0	0	0	0	0	0	4	0	0
RS 80	0	0	0	0	0	0	0	5	0	0
RS 82	0	0	4	4	0	0	0	2	0	0
RS 85	0	0	1	0	0	*	0	0	0	0
RS 86	0	0	0	0	0	0	0	0	0	0
RS 88	0	0	0	.5h	0	0	0	0	0	0
RS 93	1	0	1.5	1.5	0	0	0	3	0	0
RS 98	.5	0	3.5h	5	0	0	0	5	0	0
RS 210	0	0	0	0	0	0	0	0	0	0
RS 212	0	0	2h	0	0	0	0	0	0	0
RS 249	0	0	0	4h	0	0	0	.5	0	0
NCTC 10003	0	0	0	2h	0	0	0	0	0	0
ATCC 10149	0	0	0	0	0	0	0	0	0	0
ATCC 12976	0	0	0	1h	0	0	0	0	0	0
NCIB 8919	0	0	0	0	0	0	0	0	0	0
DSM 463	0	0	0	0	0	0	0	0	0	0
DSM 465	0	0	1.5	.5h	0	0	0	0	0	0
NU 3	0	0	0	0	0	0	0	0	0	0
NU 4	0	0	0	0	0	*	0	0	0	0
NU 10	3	0	6	0	0	3	0	3	0	0
NU 12	0	0	0	0	0	0	0	0	0	0
NU 234	0	0	0	0	0	0	0	0	0	0
NU 1490	0	0	0	0	0	0	0	0	0	0
NU 1503	0	0	3	3	0	0	0	0	0	0
NU 1620	0	0	1.5	1.5h	0	4h	0	0	0	0
NCA 2184	0	0	0	0	0	0	0	0	0	0

Table 3.18 (Cont'd)

Bacteriocin Producing Strains	Test strains									
	RS 23	RS 37	RS 42	RS 43	RS 44	RS 45	RS 47	RS 48	RS 49	RS 51
RS 6	0	0	0	0	0	0	0	5h	0	0
RS 33	0	0	0	0	2h	0	0	0	0	0
RS 53	0	0	0	0	0	0	0	0	0	0
RS 70	0	0	0	0	0	0	0	0	0	0
RS 77	0	0	0	0	0	0	0	0	0	0
RS 80	0	0	0	0	0	0	0	0	0	0
RS 82	0	0	0	0	0	0	0	.5h	0	2
RS 85	0	0	0	0	0	0	0	0	0	0
RS 86	0	0	0	0	0	0	0	0	0	0
RS 88	0	0	0	0	0	0	0	0	0	0
RS 93	0	0	0	0	0	0	0	0	0	0
RS 98	0	0	0	0	0	0	0	1.5	0	2
RS 210	0	0	0	0	3vh	0	0	0	0	0
RS 212	0	0	0	0	0	0	0	0	0	1h
RS 249	0	0	0	0	0	0	0	0	0	.5h
NCTC 10003	0	0	0	0	0	0	0	0	0	0
ATCC 10149	0	0	0	0	0	0	0	0	0	0
ATCC 12976	0	0	0	0	0	0	0	0	0	0
NCIB 8919	0	0	0	0	0	0	0	0	0	0
DSM 463	0	0	0	0	0	0	0	0	0	0
DSM 465	0	0	0	0	0	0	0	0	0	0
NU 3	0	0	0	0	0	0	0	0	0	0
NU 4	0	0	0	0	0	0	0	0	0	0
NU 10	0	4	0	0	0	0	0	3	0	1.5
NU 12	0	0	0	0	0	0	0	0	0	0
NU 234	0	0	0	0	0	0	0	1h	0	0
NU 1490	0	0	0	0	0	0	0	0	0	0
NU 1503	0	0	0	0	0	0	0	0	0	1h
NU 1620	0	0	0	0	0	0	0	0	0	0
NCA 2184	0	0	0	0	0	0	0	0	0	0

Table 3.18 (Cont'd)

Bacteriocin Producing Strains	Test strains									
	53	RS 57	RS 70	RS 80	RS 85	RS 86	RS 87	RS 98	RS 99	RS 155
RS 6	0	0	0	0	1h	2	*	0	0	0
RS 33	0	0	0	0	0	0	*	0	0	0
RS 53	0	0	0	0	2h	2	1h	0	0	0
RS 70	0	0	0	0	10	3	3	0	0	0
RS 77	0	0	0	0	0	0	2	0	0	0
RS 80	0	0	0	0	0	0	0	0	0	0
RS 82	0	.5h	0	0	3	3	5	0	0	0
RS 85	0	0	*	0	0	0	*	0	0	.5h
RS 86	0	0	0	0	0	0	0	0	0	1
RS 88	0	0	0	0	.5	1	0	0	0	0
RS 93	0	0	0	0	0	0	4	0	0	0
RS 98	0	1.5h	0	3h	4h	4h	5	0	0	0
RS 210	0	0	0	0	0	0	3	0	0	0
RS 212	0	0	0	0	2	1	0	0	0	0
RS 249	0	0	0	0	3h	4h	0	0	0	0
NCTC 10003	0	0	0	0	.5	0	0	0	0	0
ATCC 10149	0	0	0	0	.5	0	0	0	0	0
ATCC 12976	0	0	0	0	.5	1.5	3	0	0	0
NCIB 8919	0	0	0	0	0	0	0	0	0	0
DSM 463	0	0	0	0	0	0	3	0	0	0
DSM 465	0	0	0	0	0	.5h	0	0	0	0
NU 3	0	0	0	0	0	0	0	0	0	0
NU 4	0	0	0	0	0	0	0	0	0	0
NU 10	0	1	0	2	4h	4	6	0	0	2.5
NU 12	0	0	0	0	0	0	0	0	0	0
NU 234	0	0	0	0	0	0	0	0	0	0
NU 1490	0	0	0	0	0	0	0	0	0	0
NU 1503	0	0	0	0	2	0	3	0	0	0
NU 1620	0	0	0	0	0	2	2	0	0	0
NCA 2184	0	0	0	0	1h	0	1	0	0	0

Table 3.18 (Cont'd)

Bacteriocin Producing Strains	Test strains									
	RS 166	RS 168	RS 173	RS 174	RS 182	RS 187	RS 203	RS 204	RS 205	RS 209A
RS 6	5sah	3h	2h	4gh	0	0	0	0	0	0
RS 33	0	0	0	0	0	0	0	0	1	0
RS 53	3h	0	3	2	0	1h	0	0	1	0
RS 70	5h	2h	3	3.5	0	0	0	0	0	0
RS 77	0	0	0	0	0	0	0	0	0	0
RS 80	0	0	5	0	0	0	0	0	0	0
RS 82	0	2h	4	3	0	1.5h	*	1	0	0
RS 85	0	0	1.5	1	0	*	*	6	1	0
RS 86	0	0	0	0	0	*	2h	.5	*	0
RS 88	3	2h	3h	2	0	1h	0	0	0	0
RS 93	2	2h	.5h	2	0	3h	.5h	.5	3	0
RS 98	6h	3h	5	4	0	4h	5	2	3	0
RS 210	0	0	0	0	0	0	*	0	0	0
RS 212	0	0	0	2	0	3h	*	0	0	0
RS 249	4h	10h	3	4	0	4h	2h	1.5h	*	0
NCTC 10003	0	0	0	0	0	*	0	0	0	0
ATCC 10149	0	10h	1h	1	0	1h	0	.5	0	0
ATCC 12976	1.5h	0	1h	1	0	1.5h	1h	0	0	0
NCIB 8919	0	2h	0	0	0	0	*	0	0	0
DSM 463	3h	6h	0	1h	0	3h	*	1h	0	0
DSM 465	4h	0	1h	1	0	3h	2h	2.5h	0	0
NU 3	1	0	0	0	0	2h	1h	0	0	0
NU 4	2	0	2	0	0	3h	1sah	0	0	0
NU 10	4	2	4	5	0	3h	3sah	1.5	2	0
NU 12	0	5h	0	0	0	0	0	0	1	0
NU 234	4h	1h	0	0	0	.5h	0	0	*	0
NU 1490	3h	5	0	0	0	0	5h	0	0	0
NU 1503	0	8	3	2	0	.5h	1h	1h	5	0
NU 1620	4h	5	2h	2	0	3h	1h	1h	*	0
NCA 2184	0	0	0	0	0	0	3h	0	0	0

Table 3.18 (Cont'd)

Bacteriocin Producing Strains	Test strains									
	RS 209B	RS 210	RS 211	RS 215	RS 216	RS 217	RS 222	RS 236	RS 237	RS 238
RS 6	0	0	0	0	0	0	*	5sa	0	1h
RS 33	0	0	0	0	0	0	0	0	0	0
RS 53	.5h	0	0	0	0	0	0	1.5	0	0
RS 70	1	*	0	0	0	0	0	0	0	0
RS 77	0	0	0	0	0	0	0	0	0	1h
RS 80	0	0	0	0	0	0	0	0	0	1h
RS 82	1.5	0	2	3h	0	0	6	1.5	0	3
RS 85	*	0	.5	0	0	0	3	.5	*	0
RS 86	0	0	0	0	0	0	0	0	0	.5
RS 88	2h	1h	0	0	0	0	0	0	0	0
RS 93	2h	0	1	2	0	0	1.5	*	3h	2
RS 98	7h	0	2	3	0	0	2	3	2h	2
RS 210	0	0	0	0	0	0	0	0	0	0
RS 212	*	0	0	0	0	0	0	2	0	0
RS 249	3	0	1	2h	0	0	0	0	1h	1
NCTC 10003	3	0	0	0	0	0	3	0	0	.5h
ATCC 10149	3	0	0	0	0	0	.5	0	0	0
ATCC 12976	1	0	0	0	0	0	0	0	0	0
NCIB 8919	.5h	0	0	0	0	0	0	0	0	0
DSM 463	1	0	*	0	0	0	0	0	0	1h
DSM 465	3	0	*	0	0	0	0	1h	0	1h
NU 3	1	0	0	0	0	0	3.5	0	0	0
NU 4	6	0	0	0	0	0	1h	0	0	0
NU 10	2	0	4	5	0	4	4	*	4	5
NU 12	.5	0	0	0	0	0	0	0	0	0
NU 234	3	0	1	0	0	0	0	3	0	2h
NU 1490	0	0	1.5	0	0	0	0	0	0	0
NU 1503	3	0	0	0	0	0	4	0	0	1.5
NU 1620	1	0	0	0	0	0	0	2h	0	0
NCA 2184	0	0	0	0	0	0	0	0	0	0

Table 3.18 (Cont'd)

Bacteriocin Producing Strains	Test strains								
	RS 239	RS 240	RS 241	RS 242	RS 243	RS 244	RS 245	RS 246	RS 248
RS 6	0	0	0	4	0	0	0	0	1sa
RS 33	0	0	0	0	0	0	5vh	0	0
RS 53	0	0	0	0	0	0	5vh	0	3
RS 70	0	0	0	0	0	0	4vh	0	4
RS 77	0	0	0	0	0	0	3vh	0	1
RS 80	0	0	0	0	0	0	0	0	0
RS 82	0	0	0	3h	0	0	0	0	4
RS 85	0	0	0	0	0	0	0	0	0
RS 86	0	0	0	0	0	0	0	0	0
RS 88	0	0	0	0	0	0	0	0	4
RS 93	0	0	0	1	0	0	0	0	3
RS 98	0	0	0	0	0	0	0	0	6
RS 210	0	0	0	0	0	0	0	0	0
RS 212	0	0	0	0	0	0	0	0	0
RS 249	0	0	0	0	0	0	0	0	4
NCTC 10003	0	0	0	0	0	0	0	0	0
ATCC 10149	0	0	0	0	0	0	0	0	0
ATCC 12976	0	0	0	0	0	0	0	0	4
NCIB 8919	0	0	0	0	0	0	0	0	0
DSM 463	0	0	0	0	0	0	0	0	1
DSM 465	0	0	0	0	0	0	0	0	3
NU 3	0	0	0	0	0	0	0	0	2
NU 4	0	0	0	0	0	0	0	0	1h
NU 10	0	0	0	4	0	3.5	0	0	6
NU 12	0	0	0	0	0	0	0	0	0
NU 234	0	0	0	0	0	0	0	0	2h
NU 1490	0	0	0	0	0	0	0	0	0
NU 1503	0	0	0	2h	0	0	0	0	0
NU 1620	0	0	0	0	0	0	0	0	3.5
NCA 2184	0	0	0	0	0	0	0	6	0

The clusters which were identified from numerical taxonomic studies were examined for sensitivity to the 11 bacteriocins with the greatest activity. The percentage of strains within each cluster showing sensitivity to each bacteriocin was calculated (Table 3.19). Any evidence of growth inhibition was recorded as positive sensitivity. The results indicated a lack of sensitivity in the strains from cluster (1) to all bacteriocins except NU 10. Cluster (2) was sensitive to all of the bacteriocins to a varied extent; sub-group (2a) was 100% sensitive to RS 53, RS 70, RS 93 and RS 98; sub-group (2b) was 100% sensitive to NU 10. Cluster (3) was 100% sensitive to RS 98. Cluster (4) had two members which were insensitive to all bacteriocins used. Cluster (5) had high sensitivity to all bacteriocins used except NU 234. Only two members of cluster (6) were examined. The three strains in cluster (7) were only sensitive to bacteriocin NU 10.

3.7 BACTERIOPHAGE TYPING OF THERMOPHILIC STRAINS OF BACILLUS

Table 3.20 presents the results of bacteriophage typing of 15 thermophilic strains of Bacillus using eight bacteriophages. Phage JS 004 infected two members of cluster (1) but did not infect any members of cluster (2) or cluster (7) which were examined. Phage JS 013 infected two members of sub-group (2b) and showed a very low level of infectivity on strain RS 93 from sub-group (1b), no other strains were infected. Phages JS 017, JS 020, JS 025, TP 1C, TP 84 and ATCC 12016 had a high level of infectivity on members of sub-group (2a) which were examined, other strains in cluster (2) showed varying levels of infection. Strain RS 93 also showed varied levels of infectivity with this group of phages.

The evidence suggests considerable group specificity for phage infection although strain RS 93 from sub-group (1b) appears sensitive to several phages harvested from B. caldotenax which is a member of sub-group (2a).

Table 3.19

The percentage of strains sensitive to bacteriocin within the taxonomic clusters established by numerical taxonomy

Taxonomic clusters	1	1a	1b	2	2a	2b	2c	3	4	5	6	7
Number of strains	32	13	19	26	7	7	12	10	5	5	2	3
Thermocin												
RS 53	0	0	0	50	100	0	50	20	20	80	50	0
RS 70	0	0	0	65	100	57	50	50	40	100	0	0
RS 93	3	0	5	80	100	85	66	70	40	100	50	0
RS 98	6	15	0	92	100	85	92	100	40	100	100	0
NU 10	28	15	37	92	85	100	92	90	40	100	50	100
RS 82	0	0	6	88	85	85	92	80	40	80	50	0
RS 249	0	0	0	65	71	71	58	90	20	80	50	0
NU 1620	3	0	5	31	71	0	25	40	0	80	50	0
NU 234	0	0	0	15	0	0	33	30	0	20	50	0
ATCC 12976	0	0	0	27	29	0	42	20	0	80	0	0
DSM 465	0	0	0	31	43	0	42	40	0	80	50	0

Legend

The taxonomic clusters identified by numerical taxonomy were examined for evidence of similar groupings based on sensitivity to bacteriocins. The number of strains sensitive to each of the 11 bacteriocins were determined and the percentage of sensitive strains within each cluster was calculated. Any evidence of growth inhibition was recorded as sensitivity to the bacteriocin.

Table 3.20

Phage sensitivity of thermophilic Bacillus cultures

Strains	JS 004	JS 013	JS 017	JS 020	JS 025	TP 1C	TP 84	ATCC 12016B
EP 136	-	-	-	+	-	-	-	-
RS 93	++++	+	*	+++	++++	+	+	-
RS 239	+++	-	-	-	-	-	-	-
B. caldotenax	-	-	++++	++++	++++	++++	++++	++++
B. caldovelox	-	-	++++	++++	++++	++++	++++	++++
B. caldolyticus	-	-	+++	+++	+++	+++	+++	++
B. stearothermophilus ATCC 8005	-	*	+++	*	*	*	*	*
B. thermocatenulatus DSM 730	-	*	+++	*	*	*	*	*
B. stearothermophilus NCA 1503	-	++++	-	-	-	+	++	-
B. stearothermophilus NW 10	-	++++	-	-	-	+	++	-
B. stearothermophilus EP 240	-	-	-	+	-	-	+++	++++
B. stearothermophilus ATCC 12016	-	-	+++	-	-	-	++++	++++
B. stearothermophilus EP 262	-	-	++	-	-	+	++	+
B. coagulans ATCC 8038	-	-	-	-	-	-	-	-
B. coagulans ATCC 12245	-	-	-	-	-	-	-	-

Legend

Bacteriophage suspensions (10^6 p.f.u. ml^{-1}) and dilutions were plated with 0.1 ml of 6 h bacterial cultures and incubated at 55°C for 18 h. The results are expressed as efficiences of plating; ++++, e.o.p. of 1 to 0.1; +++, e.o.p. of 0.1 to 0.01; ++, e.o.p. of 0.01 to 0.0001; +, low level of infection; - no visible evidence of phage infection; * not tested. Phage JS 004 suspension was prepared on strain RS 93, phage JS 013 suspension was prepared on strain NCA 1503, phage JS 017, JS 020, JS 025, TP 1C and TP 84 suspensions were prepared on B. caldotenax, phage ATCC 12016B suspension was prepared on strain ATCC 12016.

3.8 DETERMINATION OF Tm AND % G+C OF DNA

Twenty-eight thermophilic and three mesophilic strains of Bacillus were examined to determine their DNA melting temperature and mole % G+C. The % G+C values of the thermophilic strains ranged from 42 -71% with the facultative thermophilic strains at the lower end of the range. To examine the sensitivity and reproducibility of the system Tm determinations were made using reference DNA samples which covered the range of % G+C found within the Bacillus group. The DNA samples used comprised Clostridium perfringens (Tm 80.5 °C), Calf thymus (Tm 87 °C), Salmon sperm (Tm 87.5 °C), E. coli (Tm 90.6 °C) and Micrococcus lysodeikticus (Tm 99.5 °C). The results of repeated determinations are presented in Table 3.21. The standard error in the determination of the Tm values was calculated using data obtained from repeated testing of reference DNA (Sigma) from E. coli and salmon sperm (Table 3.22). Standard error values of 0.29 for E. coli and 0.3 for Salmon sperm were calculated. Using this system 95% of the results obtained could be expected to lay within two standard errors, i.e. approximately 0.6 °C or 1.5% G+C.

Tm determinations carried out in SSC and 0.1 x SSC differ by 15.4 °C (Marmur & Doty, 1961). Tm values of E. coli and salmon sperm DNA were examined in a range of concentrations of saline citrate buffer and showed the expected decrease in melting temperature with decreasing ionic strength (Fig. 3.5). It was essential to observe the changes in absorbance at temperatures below the boiling point of the sample, hence strains with Tm values above 95 °C were examined in 0.1 x SSC. The results, however, did not follow the expected pattern; Tm values in SSC and 0.1 SSC appeared to be similar, or differed by only a few degrees, (Table 3.23) DNA from strain LO_2 (B. licheniformis) however, exhibited Tm values of 89.5 °C in SSC and 74.3 °C in 0.1 x SSC (differing by 15.2 °C).

Since stock DNA was maintained frozen in SSC, it was important to determine if "stabilisation" of the DNA was occurring in this buffer which then persisted when DNA was diluted to 0.1 x SSC. Samples of DNA were precipitated in ethanol and then redissolved directly to 0.1 x SSC. The Tm values of those samples did not show any appreciable difference to those diluted from SSC to 0.1 x SSC.

The reason for the unexpectedly high Tm values in 0.1 x SSC is not clear and the % G+C values calculated were all based on Tm in SSC.

Table 3.21

Tm values in standard saline citrate (SSC) buffer and % G+C of DNA from thermophilic and mesophilic strains of Bacillus

Strains	Tm value in SSC	% G+C
Obligate thermophiles		
thermophile RS 1	98.6 (10)	71.5
B. thermocatenulatus DSM 730	98 (5)	70
thermophile RS 222	97.6 (3)	69
B. stearothermophilus LUDA T60	96.8 (1)	67
B. caldovelox	96 (4)	65
B. caldolyticus	95.1 (5)	63
B. caldotenax	95.1 (3)	63
B. stearothermophilus EP 262	95.1 (2)	63
B. stearothermophilus LUDA T22	95.1 (4)	63
thermophile RS 85	95.1 (2)	63
B. stearothermophilus EP 240	94.3 (4)	61
B. stearothermophilus ATCC 12016	94.3 (2)	61
B. stearothermophilus ATCC 8005	93.9 (3)	60
B. stearothermophilus NW 10	93.5 (2)	59
B. thermodenitrificans DSM 465	93.5 (2)	59
B. stearothermophilus LUDA T214	93.1 (2)	58
B. stearothermophilus LUDA T210	93.1 (3)	58
B. stearothermophilus NCA 1503	93.1 (2)	58
thermophile RS 57	92.7 (5)	57
thermophile RS 5	91.0 (4)	53
B. stearothermophilus LUDA T42	90.2 (6)	51
B. stearothermophilus EP 136	88.2 (7)	46
B. stearothermophilus RS 93	87.3 (5)	44
B. stearothermophilus LUDA T141	86.5 (3)	42
Facultative thermophiles		
B. coagulans ATCC 12245	88.6 (2)	47
B. coagulans ATCC 8038	89.5 (2)	48
B. sphericus DSM 463	87.3 (4)	44
B. licheniformis LO2	86.9 (3)	43

Table 3.21 (Cont'd)

Strains	Tm value in SSC	% G+C
Mesophiles		
B. megaterium ATCC 4531	86.5 (3)	42.4
B. subtilis NCTC 3610	88.5 (2)	46.8
B. pumilus NCIB 9369	85 (3)	38.3

Legend

Tm values were determined by measurement of the hyperchromic shift at 260 nm in a Pye Unicam SP8 100 spectrophotometer with heated cell block. Tm was taken as the mid-point in the hyperchromic shift. Numbers in parenthesis are the number of tests. % G+C was calculated from the method of Marmur and Doty (1961).

Table 3.22

Tm values in SSC buffer and % G+C of DNA standards

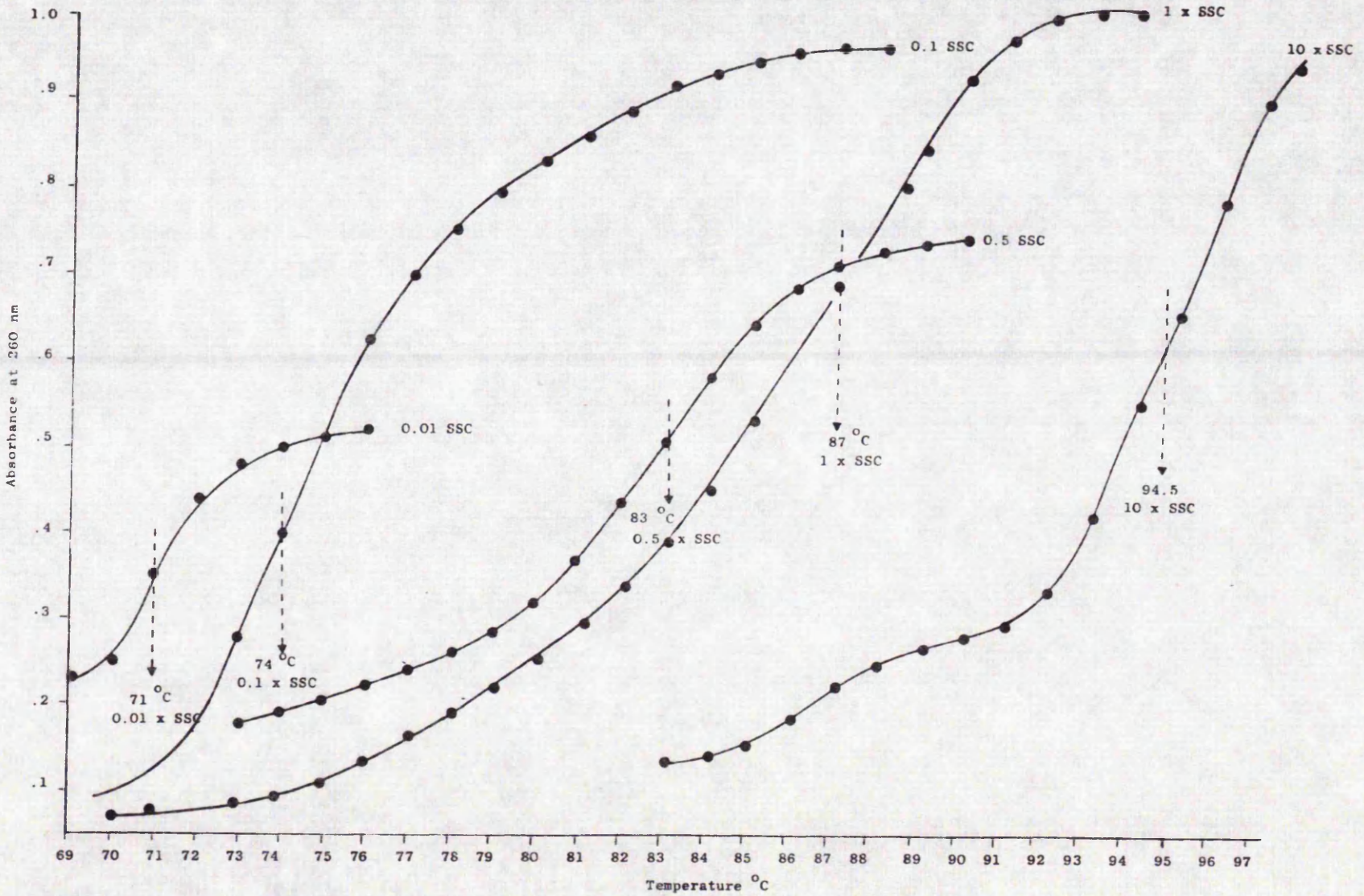
Strains	Tm (ref)	Tm (recorded)	% G+C (ref)	% G+C (calculated)
Clostridium perfringens	80.5	80 (4)	26.5	26.1
Escherichia coli	90.6	90.5 (11)	50	51.7
Micrococcus lysodeikticus	99.5	98.1 (4)	72	70.2
Salmon sperm	87.5	87.49 (8)	43	43
Calf thymus	87	87 (3)	42	42

Legend

The numbers in parenthesis refer to the number of tests carried out. Tm and % G+C reference data from Marmur and Doty (1962) and Sigma Chemical Company data sheets.

Figure 3.5

The effect of ionic strength of SSC buffer on the Tm of salmon sperm DNA



Legend

Tm values were determined using a Pye-Unicam SP8 100 spectrophotometer. The sample temperature was increased by 1 $^{o}C\ min^{-1}$. Values were transposed from a direct print out of changing absorbance values.

Table 3.23

A comparison of Tm values in various ionic strengths of saline citrate buffer

Strain	Standard saline citrate concentrations					
	10x	1x	0.5x	0.1	0.05	0.01
E. coli	-	90.5	-	77.2	-	-
Salmon sperm	94	87.5	81	73.3	72	71
B. licheniformis (LO_2)	-	89.5	-	74.3	-	-
B. thermocatenulatus	-	98	-	96.5	-	-
thermophile RS 1	-	98.6	-	93.8	-	-
B. caldovelox	-	96	-	95	-	-
B. caldolyticus	-	95	-	96	-	-
B. stearothermophilus (ATCC 8005)	-	94	-	96	-	-

3.9 DNA HOMOLOGY AMONG MESOPHILIC AND THERMOPHILIC STRAINS OF BACILLUS

The results of DNA hybridisation studies with 12 thermophiles are presented in Table 3.24. They indicate a close similarity between B. caldotenax and B. caldovelox (88% hybridisation) and to a lesser extent with EP 262 (66% hybridisation). DNA from B. stearothermophilus NCA 1503 showed a high degree of hybridisation with DNA from strains EP 240 (92%), NW 10 (85%), RS 93 (77%), ATCC 12016 (74%) and B. caldolyticus (53%). The two strains of B. coagulans showed a high level of hybridisation (88%) between themselves and to a lesser extent hybridisation with strain EP 136 (66%). All mesophilic strains examined indicated a low level of DNA hybridisation with all the thermophilic strains examined.

Table 3.24

DNA homology among mesophilic and thermophilic strains of Bacillus

Total unlabelled DNA from	Percentage of reassociation with (P^{32}) DNA from:			
	B. stearothermophilus NCA 1503	*B. caldotenax*	*B. subtilis* 168, I^-	*B. coagulans* ATCC 8038
B. stearothermophilus NCA 1503	100	23	17	11
B. caldotenax	22	100	9	9
B. subtilis 168 I^-	19	11	100	10
B. coagulans ATCC 8038	9	7	16	100
B. stearothermophilus NW 10	85	19	16	12
B. stearothermophilus EP 240	92	21	14	15
B. stearothermophilus ATCC 12016	74	25	8	16
B. stearothermophilus RS 93	77	23	9	15
B. caldolyticus	53	38	11	9
B. stearothermophilus EP 262	28	66	7	11
B. caldovelox	23	88	5	8
B. stearothermophilus EP 136	12	11	10	66
B. coagulans ATCC 12245	18	6	11	88
B. amyloliquefaciens HI	5	8	19	10
B. megaterium KM	9	4	8	10

Legend

Nitrocellulose filters (25 mm diam) were loaded with 25 µg of total denatured DNA and reassociation was performed with 0.2 µg of sonicated, denatured (P^{32}) DNA in 6 x SSC at 65 °C for 16h under paraffin oil. Reassociation values (α) were defined as:

$$\alpha = 100 \times \frac{(\text{Counts bound to Heterol. filters} - \text{counts bound to } E.\ coli \text{ filters})}{(\text{Counts bound to Homol. filters} - \text{counts bound to } E.\ coli \text{ filters})}$$

CHAPTER 4

ISOLATION OF PLASMIDS FROM THERMOPHILIC MICROORGANISMS

4. ISOLATION OF PLASMIDS FROM THERMOPHILIC MICROORGANISMS

Three strains of Thermus aquaticus, four strains of B. stearothermophilus and the three caldoactive bacteria of Heinen and Heinen (1972) were examined for the presence of plasmid DNA. The method (Section 2.13.2) is based on the separation of plasmid DNA from chromosomal DNA in caesium chloride density gradients by the inclusion of ethidium bromide which alters the buoyant density of plasmid DNA (Radloff et al., 1967). Two bands appeared in the gradient when visualised under u.v. light, a main chromosomal DNA band and a lower, smaller plasmid DNA band. Incorporation of ^{3}H-thymidine into the DNA (Guerry et al., 1973) aids the detection of low levels of plasmid DNA that cannot be seen visually. The ^{3}H profiles of the density gradients for each of the strains examined are shown in Fig. 4.1 - 4.10.

Thermus aquaticus strain B and Thermus aquaticus strain XI both had satelite bands of DNA when visualised under u.v.; an examination of their ^{3}H profiles also indicated the presence of plasmid DNA. The only Bacillus strain to show evidence of plasmid DNA from its ^{3}H profile was B. caldolyticus; from visual examination only one band was evident. Examination of this pseudo-satelite band using restriction enzymes EcoRI and HindIII, did not confirm the presence of plasmid DNA (Bingham, 1980). Only heterogeneous sized fragments were obtained which were more characteristic of chromosomal DNA.

Twenty of the RS strains of thermophilic Bacillus which showed antibiotic resistance, or bacteriocin production, were also screened by Bingham (1980) using this method. Only strain RS 93 which was a strong bacteriocin producer, showed evidence of a plasmid (Sharp et al., 1979) in its ^{3}H profile; there was no evidence of a satelite band from visual examination under u.v. This plasmid was further characterised by Bingham (1980).

Figure 4.1 - 4.10

^{3}H thymidine profiles of labelled DNA from thermophilic microorganisms in caesium chloride density gradients

Legend

DNA was labelled with ^{3}H thymine by incorporating ^{3}H-thymidine in the growth medium. Cleared lysates were prepared and subjected to ethidium bromide-caesium chloride density gradient centrifugation as described in Section 2.13.2.

Figure 4.1

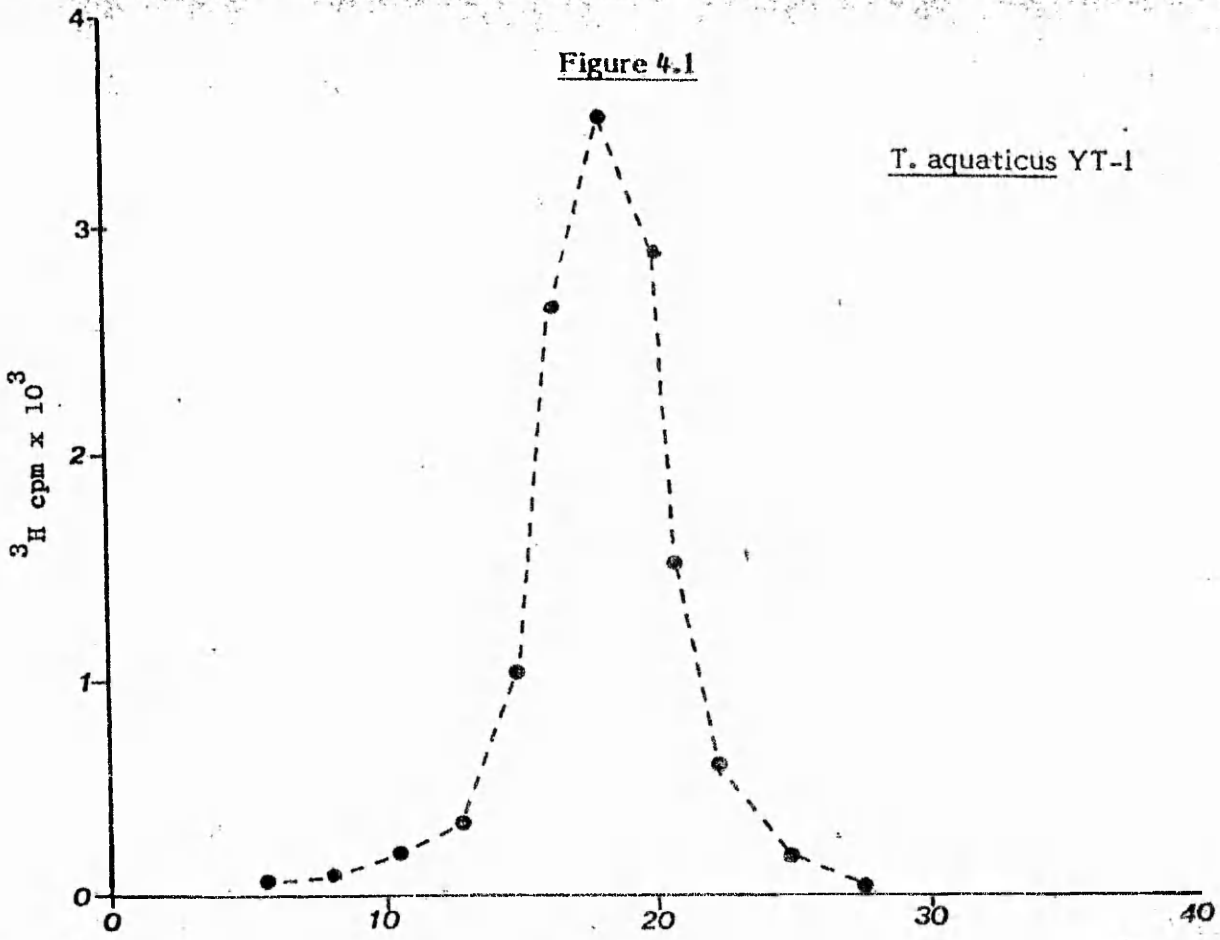
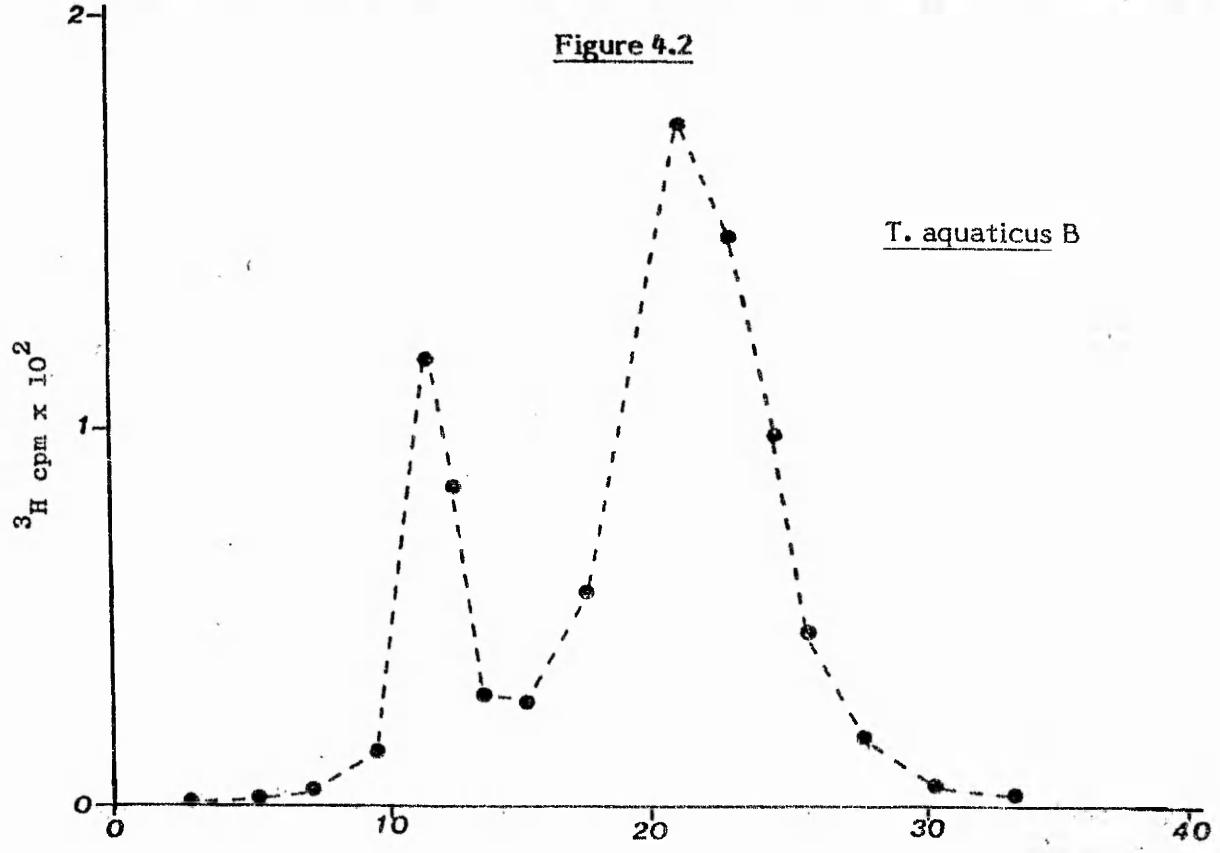


Figure 4.2



Fraction

Figure 4.3

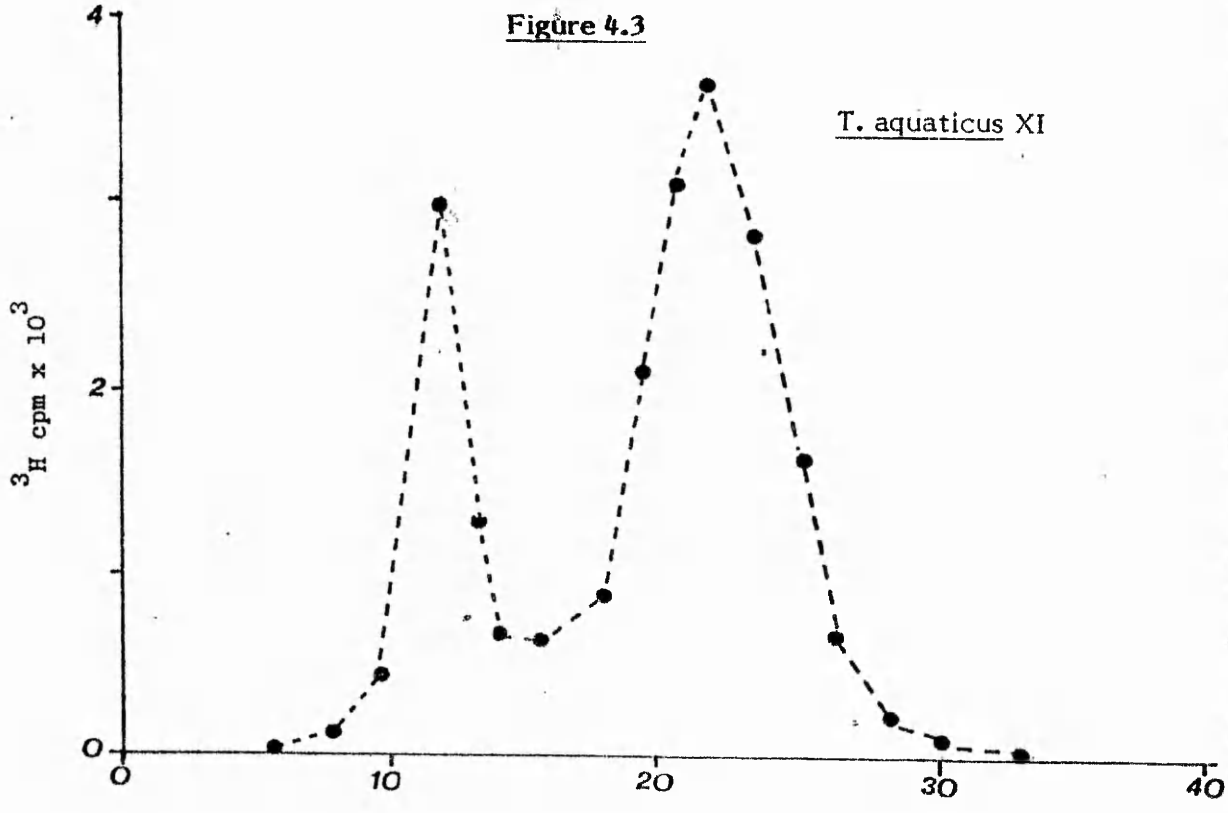


Figure 4.4

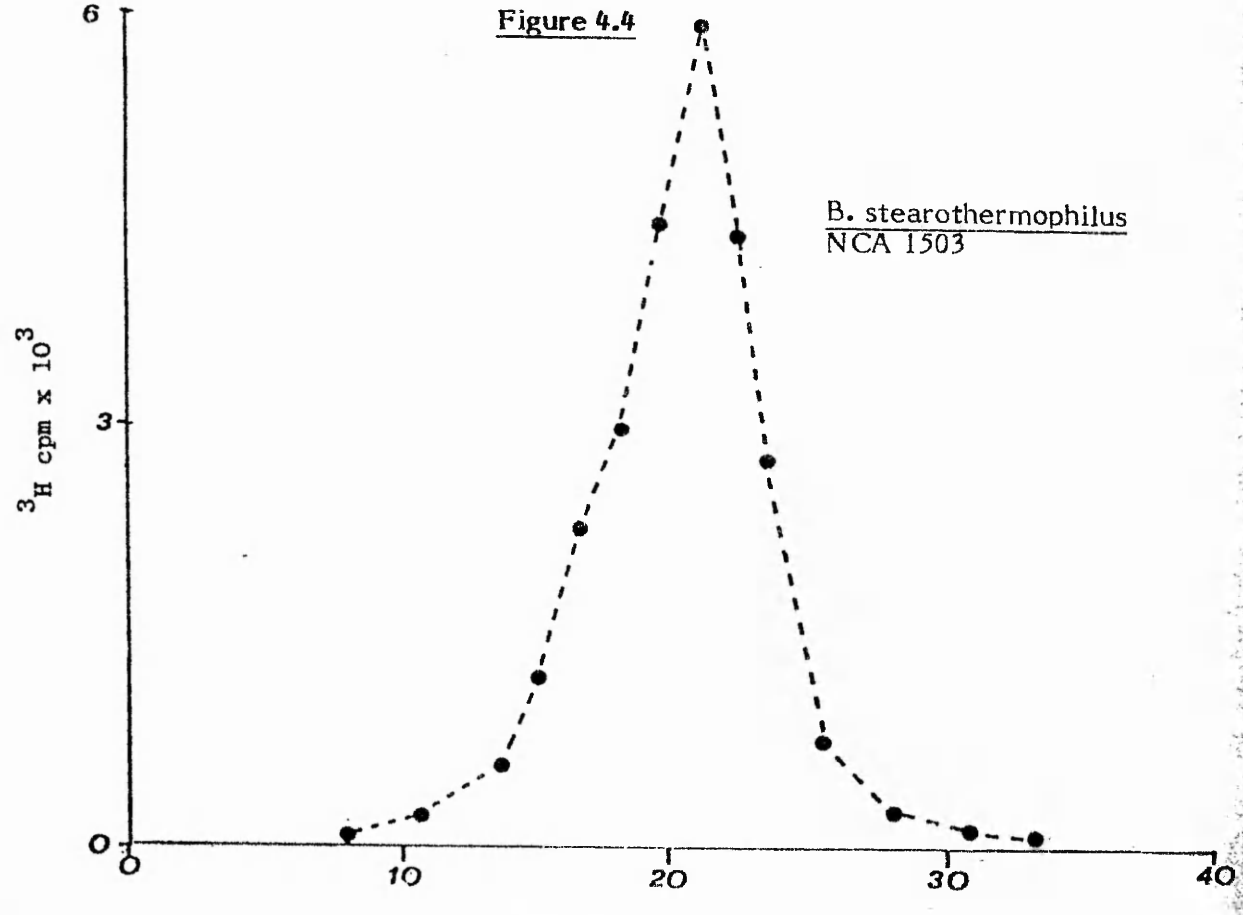


Figure 4.5

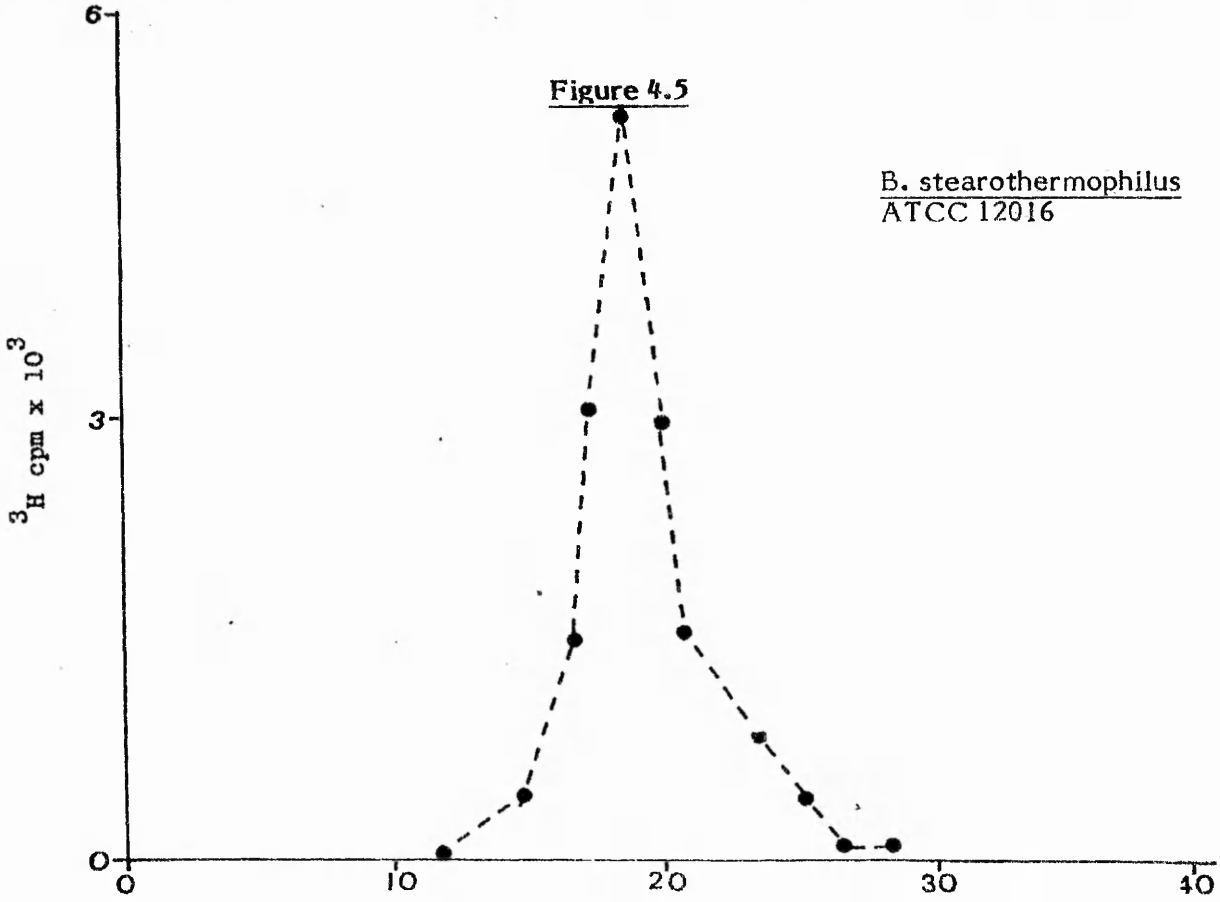


Figure 4.6

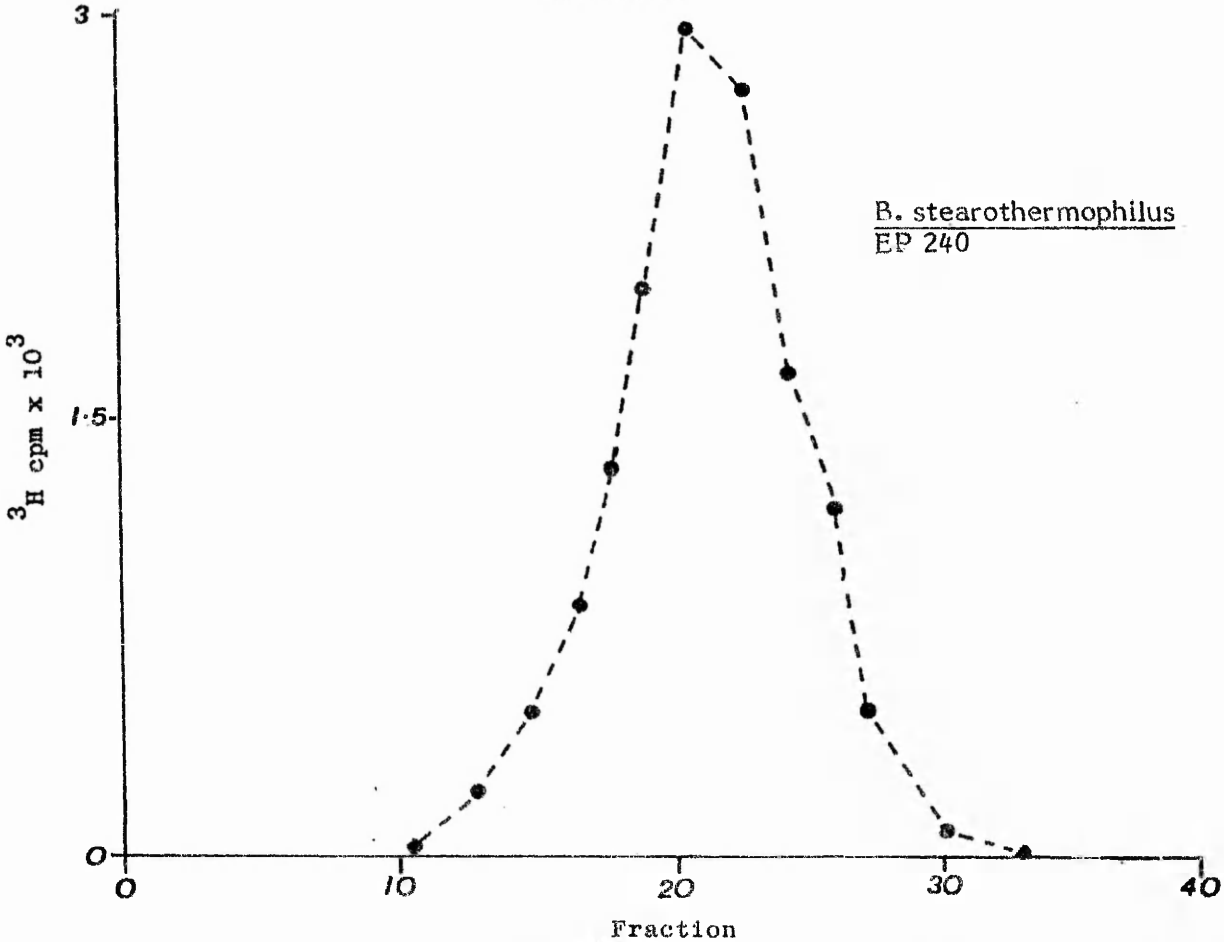


Figure 4.7

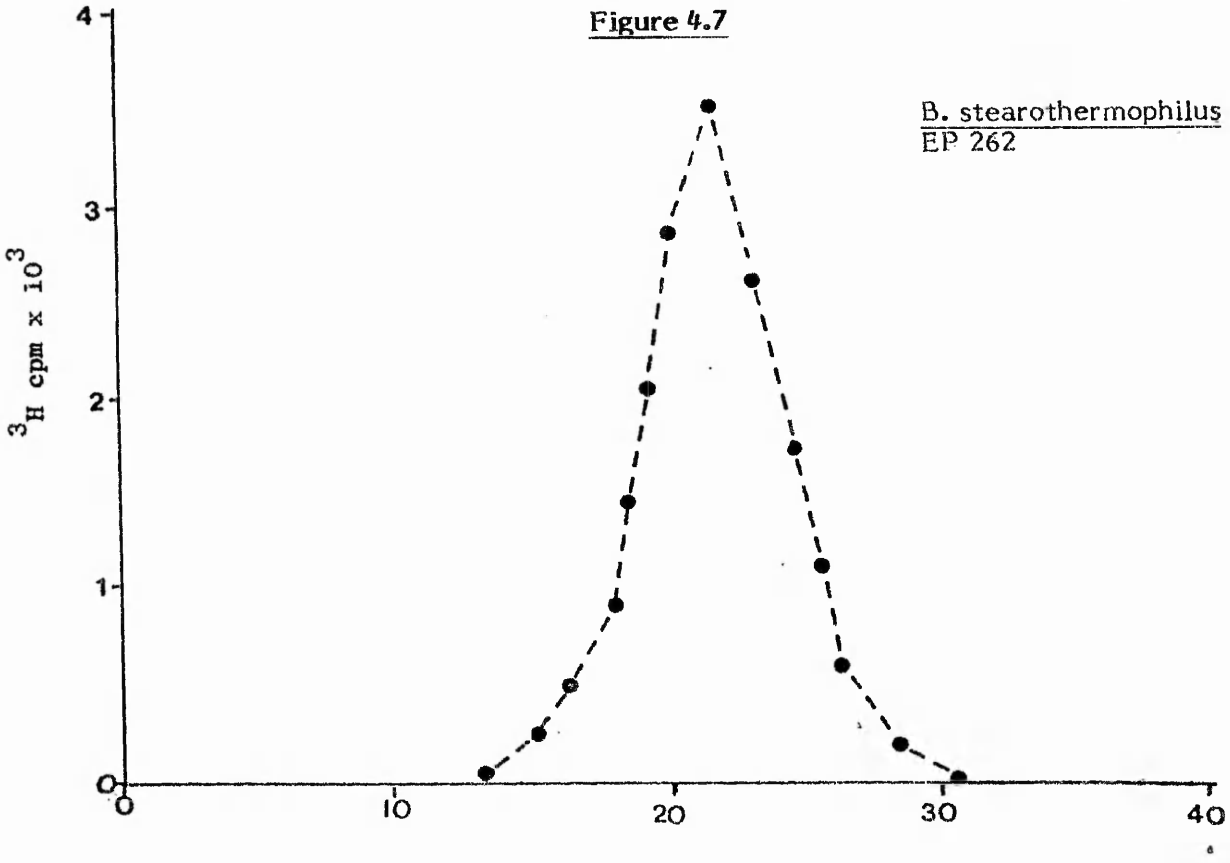


Figure 4.8

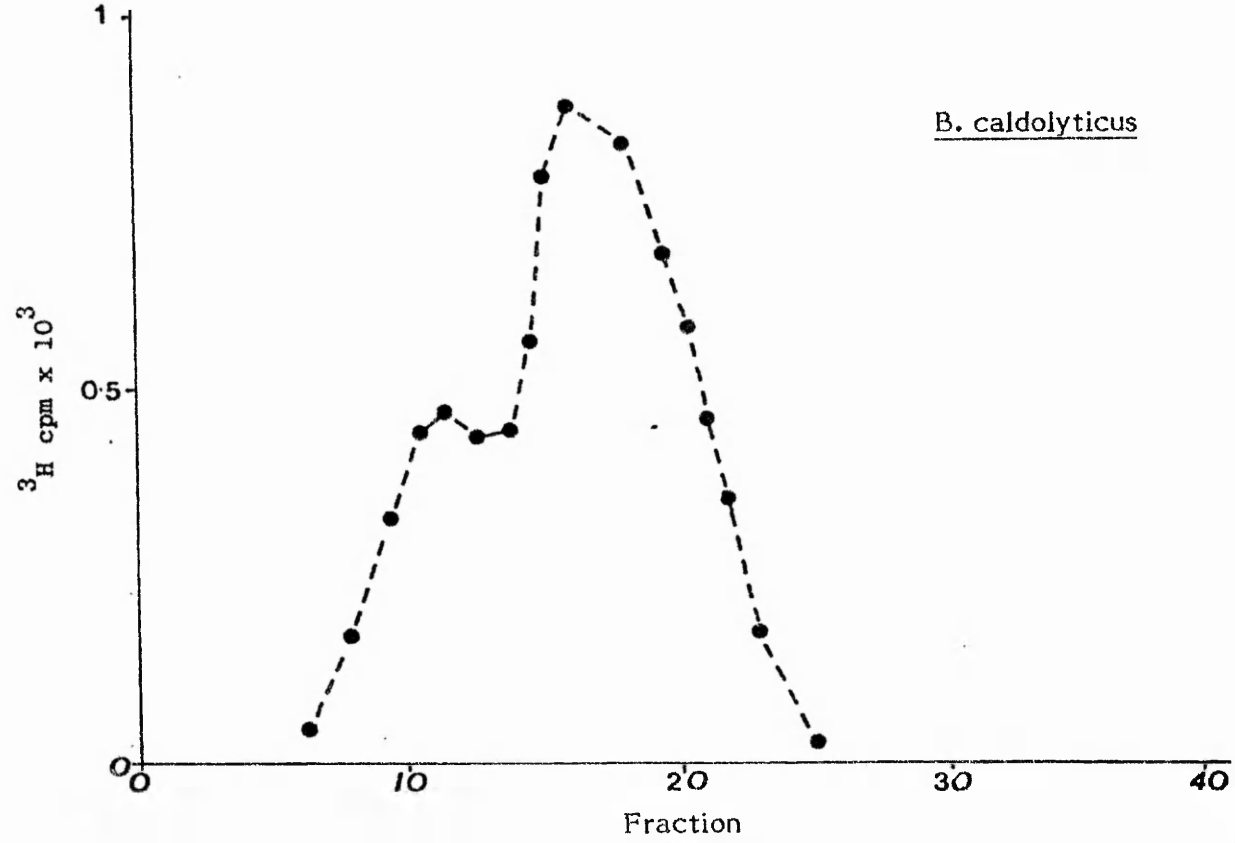


Figure 4.9

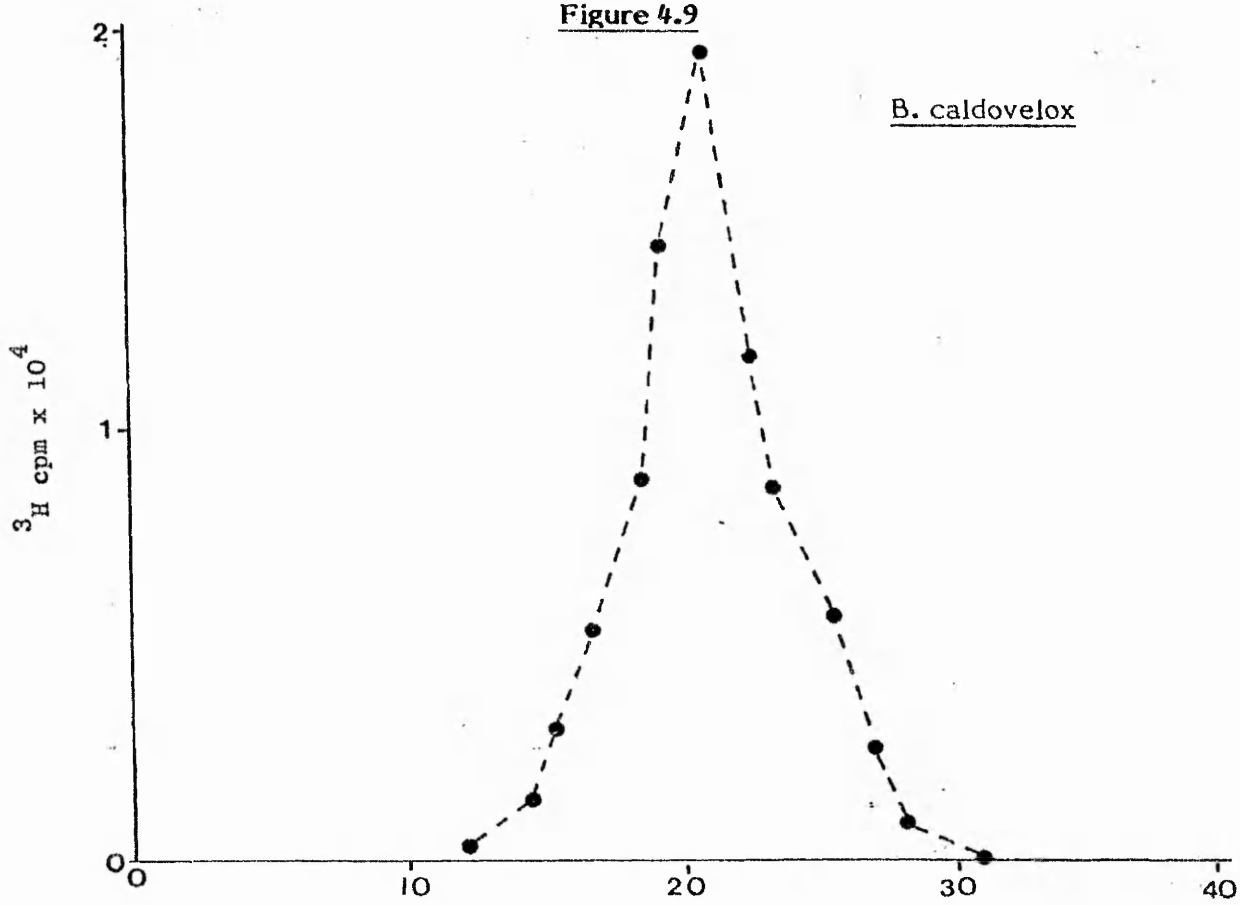
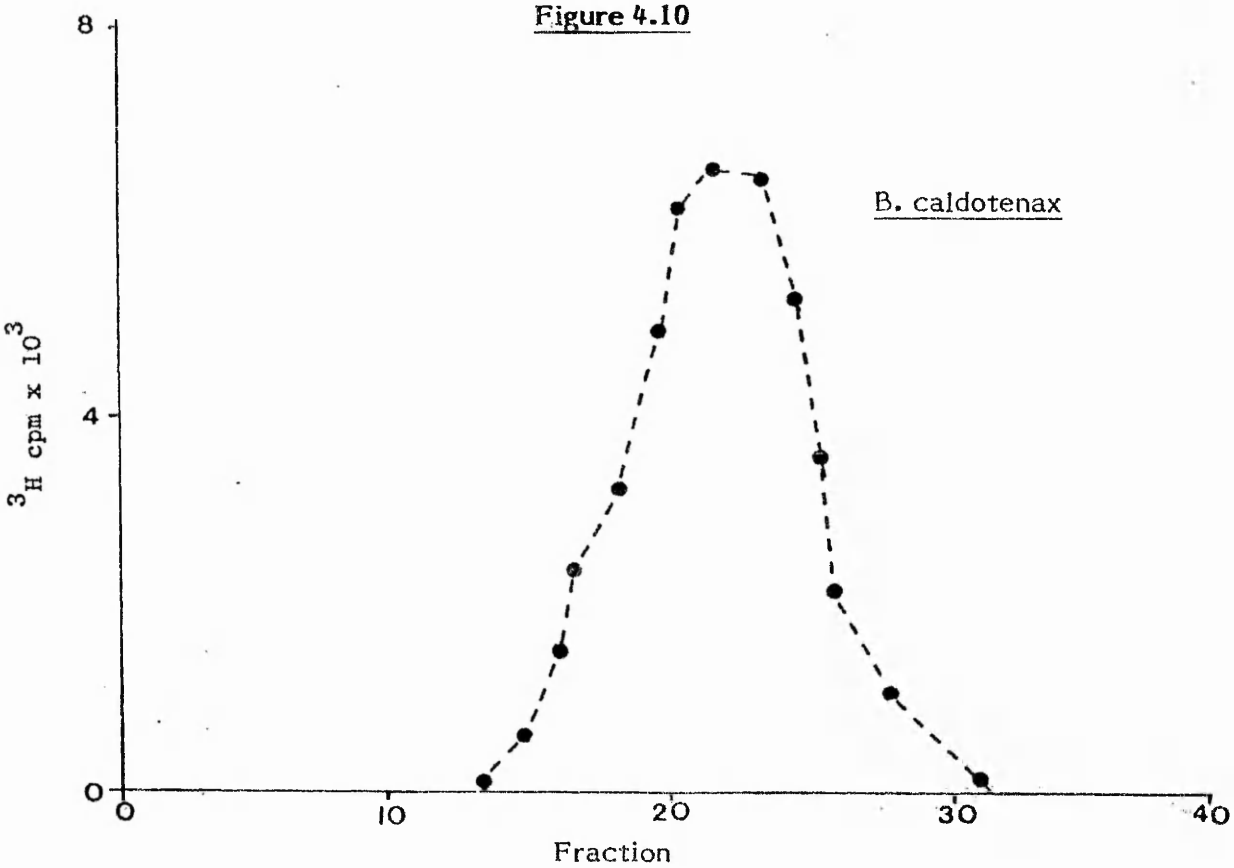


Figure 4.10



CHAPTER 5

BACTERIOPHAGE ISOLATION AND CHARACTERISATION

5. BACTERIOPHAGE ISOLATION AND CHARACTERISATION

5.1 BACTERIOPHAGE MAINTENANCE, CONCENTRATION AND ISOLATION

5.1.1 Bacteriophage maintenance

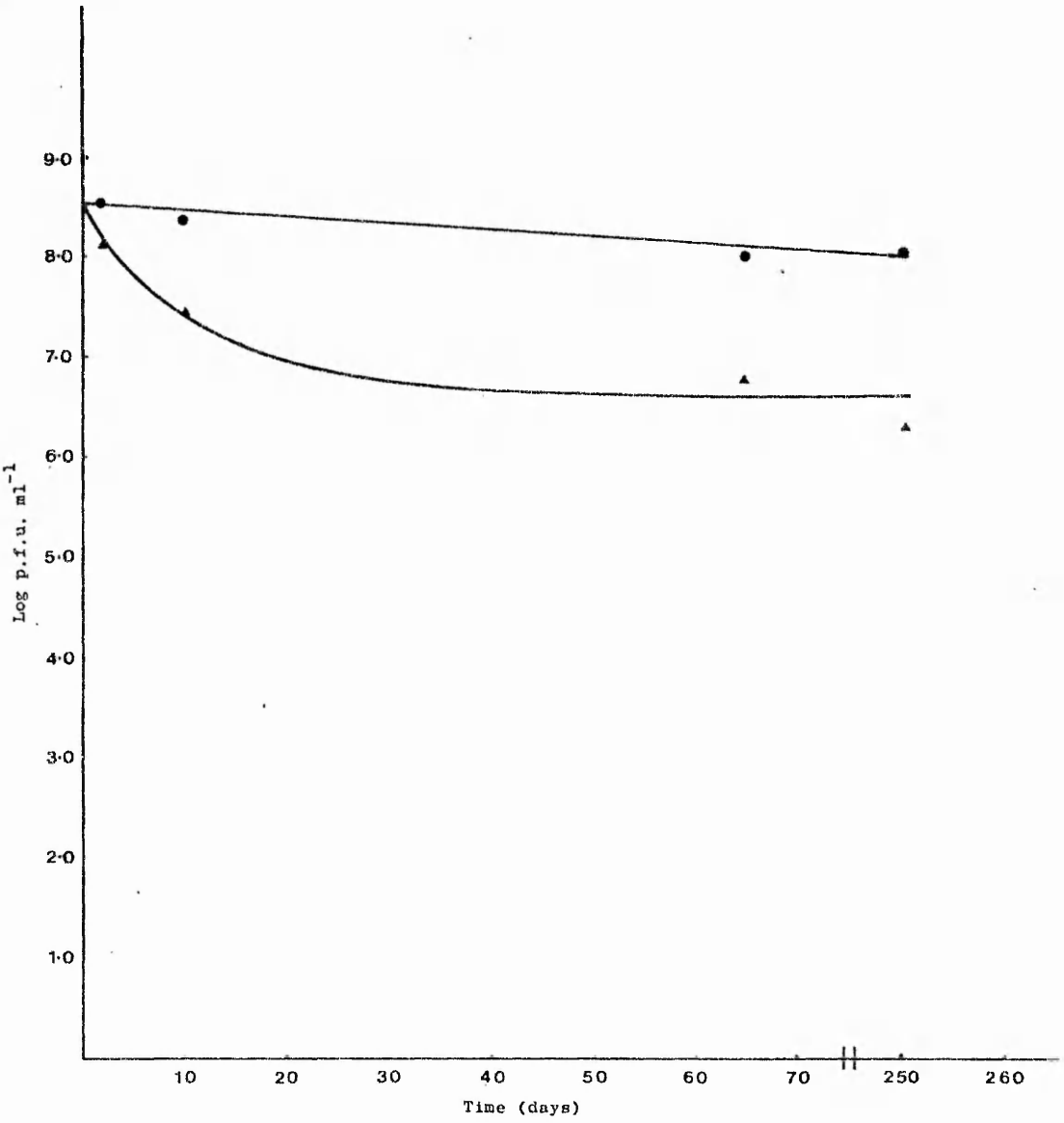
Bacteriophage suspensions were stored at 4 °C in 1 oz or 4 oz glass screw capped bottles with either a few drops of chloroform added (approx. 1%), or as filter sterilised preparations. A number of putative thermophilic phage isolates were lost when chloroform was used to sterilise the preparations. Studies on the long term survival of JS 017 in PMN buffer with chloroform indicated a ten fold reduction in viability over 10 - 20 days (Fig. 5.1) with little further reduction over the next 200 days. The control sample showed a slight (two to three fold) decrease in viability over 250 days. Examination of other suspensions of JS 017 in filtered culture supernatant without chloroform showed no loss in p.f.u. after one year at 4 °C. Plates with plaques of phages JS 004 - JS 015 were sealed with tape and stored at 4 °C. Viable phages were isolated from plaques following storage for three years. Storage of phage JS 017 in liquid nitrogen following addition of 10% (w/v) glycerol to filtered culture supernatant was successful in retaining viability for one year but the level of viability was not measured. R. Yeo (pers. comm.) successfully stored high titre coliphage concentrates at -20 °C with the addition of 50% (w/v) glycerol for periods of three to five years. Sterilisation with chloroform was unsuccessful in killing spores, particularly in preparations harvested from B. stearothermophilus RS 93. Sterile filtration of small volumes was successfully achieved using Millipore millex 0.22 µm and 0.45 µm disposable filters. (With some preparations the 0.22 µm filter blocked after 2 - 3 ml had passed and the 0.45 µm filter was generally used.) Loss of p.f.u. following filtration with the 0.22 µm filter was higher (approx. 25% from 10^8 p.f.u. ml^{-1} preparation) than with the 0.45 µm filter (approx. 10% from 10^8 p.f.u. ml^{-1} preparation).

5.1.2 Concentration of bacteriophage with hydroxylapatite

Attempts were made to find a simple means of concentrating phage particles from 200 ml of culture supernatant, before examination for the presence of small numbers of spontaneously induced bacteriophages. Hydroxylapatite columns (Section 2.14.3) were loaded with

Figure 5.1

The effect of 1% (v/v) of chloroform on phage JS 017



Legend

A phage suspension of JS 017 was divided and to one aliquot was added 1% (v/v) chloroform. The two samples were assayed at intervals for viable phage. ● , control; ▲ , 1% (v/v) chloroform added to sample.

Table 5.1

Bacteriophages isolated

Collection number	Major host	Method of isolation	Source
JS 001	B. caldotenax	Induction	NCA 1503
JS 002	B. caldotenax	Induction	RS 93
JS 003	B. caldotenax	Induction	RS 108
JS 004	RS 239	Str^r	Silage (Wilts.)
JS 005	RS 239	Str^r	Rotting straw (Wilts.)
JS 006	RS 239	Str^r	Compost (Wilts.)
JS 007	RS 240	Str^r	Silage (Wilts.)
JS 008	RS 241	Str^r	Rotting straw (Wilts.)
JS 009	RS 243	Str^r	Stable manure (Wilts.)
JS 010	RS 243	Str^r	Compost (Wilts.)
JS 011	RS 239	Str^r	Silage (Wilts.)
JS 012	RS 239	Str^r	Compost (Wilts.)
JS 013	NCA 1503	Str^r	Soil (Salisbury Plain)
JS 014	NCA 1503	Str^r	Rotting straw (Wilts.)
JS 015	NCA 1503	Str^r	Compost (Wilts.)
JS 016	B. caldotenax	use of indicator strains	Compost (Wilts.)
JS 017	B. caldotenax	use of indicator strains	Compost (Wilts.)

Table 5.1 (Cont'd)

Collection number	Major host	Method of isolation	Source
JS 018	B. caldotenax	use of indicator strains	Rotting vegetation (Wilts.)
JS 019	B. caldotenax	use of indicator strains	Rotting vegetation (Wilts.)
JS 020	B. caldotenax	use of indicator strains	Rotting vegetation (Wilts.)
JS 021	B. caldotenax	use of indicator strains	Rotting vegetation (Wilts.)
JS 022	B. caldotenax	use of indicator strains	Compost (Wilts.)
JS 023	B. caldotenax	use of indicator strains	Compost (Wilts.)
JS 024	B. caldotenax	Use of indicator strains	Compost (Wilts.)
JS 025	B. caldotenax	use of indicator strains	Compost (Wilts.)
JS 026	B. caldotenax	use of indicator strains	Compost (Wilts.)
JS 027	RS 241	Str^r	Compost (Wilts.)
JS 028	B. thermo-	use of indicator strains	Compost (Wilts.)

Legend

Bacteriophages were isolated by phage induction using mitomycin C and u.v. irradiation and from soil, compost and water samples.

200 ml of TP 1C phage suspension (10^6 p.f.u. ml^{-1}). Using a 10 ml column of hydroxylapatite, prepared by the method of Atkinson et al. (1973) with a flow rate of 15 ml h^{-1}, phages were concentrated four-fold with a recovery of 40%. No phages were isolated from the column eluate and it appeared the residual 60% of phages remained bound to the column.

Using spheroidal hydroxylapatite (BDH) with a flow rate of 60 ml h^{-1} phages were concentrated ten fold with recoveries of 80%. Approximately 1% of the phage was recovered from the eluate indicating that it did not bind to the column.

The use of spheroidal hydroxylapatite columns proved a useful method for the concentration of bacteriophage but required 3 - 5 h for adsorption to the column followed by 2 - 3 h for elution and washing, etc. Batch adsorption appeared to be a more rapid alternative when a large number of samples were involved. Batch adsorption of TP 1C (10^3 - 10^5 p.f.u. ml^{-1}) to hydroxylapatite resulted in recoveries of 50 - 70% with concentration between twelve and fifteen fold.

5.1.3 Bacteriophage isolation

Ten samples of organic material including compost, soil and rotting vegetation were examined as potential sources of thermophilic phages using the method detailed in Section 2.14.1. Ten indicator strains were used to detect the presence of phages with varied success. B. stearothermophilus RS 93 indicated the presence of phage in eight of the samples; B. caldovelox and B. caldotenax indicated phage in five samples; NCA 1503 three samples; EP 240 two samples; ATCC 12016 and B. caldolyticus one sample; strain NW 10 four samples, while EP 262 showed no evidence of phage in any of the samples. Many of the phage plaques, evident in the initial isolation, were lost on transfer to fresh media. All phage isolates able to infect strains NCA 1503 and EP 240 were lost together with a number able to infect RS 93, B. caldotenax and B. caldovelox. Some phage isolates were lost following the addition of chloroform to the suspensions. Phages JS 016 - JS 026, whose main hosts were B. caldotenax and B. caldovelox, were maintained for future study (Table 5.1).

Thirteen bacteriophages, JS 004 - JS 015 and JS 027, were isolated using the method of Romig and Brodetsky (1961) detailed in Section 2.14.2. Seven source materials were examined (compost, silage, rotting straw, river sludge, stable manure, soil and digested sludge) for the presence of phage using five Str^r strains of B. stearothermophilus

Table 5.2

Detection of spontaneously induced lysogenic phage from thermophilic strains of Bacillus

Culture supernatant	Indicator strains														
	NCA 1503	ATCC 12016	EP 240	EP 262	RS 93	B. tenax	B. velox	B. lyticus	RS 15	RS 23	RS 38	RS 108	RS 125	RS 161	RS 176
RS 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	+	+	-	+	-	-	+	+
6	-	-	-	-	-	-	-	+	-	-	+	+	-	+	-
7	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 5.2 (Cont'd)

Culture supernatant		Indicator strains														
		NCA 1503	ATCC 12016	EP 240	EP 262	RS 93	*B. tenax*	*B. velox*	*B. lyticus*	RS 15	RS 23	RS 38	RS 108	RS 125	RS 161	RS 176
RS	17	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+
	18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
	19	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
	20	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-
	21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	22	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
	23	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-
	24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
	27	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	28	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+
	29	-	-	+	-	+	-	-	-	-	+	-	-	-	-	+
	30	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+
	31	+	-	-	-	-	-	-	-	+	-	-	-	-	-	+
	32	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+

Table 5.2 (Cont'd)

Culture supernatant	Indicator strains														
	NCA 1503	ATCC 12016	EP 240	EP 262	RS 93	*B. tenax*	*B. velox*	*B. lyticus*	RS 15	RS 23	RS 38	RS 108	RS 125	RS 161	RS 176
RS 33	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+
34	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
35	-	-	+	-	-	-	-	-	-	+	+	-	-	-	+
36	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+
37	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
38	-	-	-	-	+	-	-	-	+	+	-	-	-	-	+
39	-	-	+	+	-	-	-	-	-	-	+	-	-	-	+
40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
41	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
42	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+
43	-	-	-	+	+	-	+	-	-	-	-	-	-	-	+
44	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
45	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
46	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
47	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
48	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+

Table 5.2 (Cont'd)

Culture supernatant		Indicator strains															
		NCA 1503	ATCC 12016	EP 240	EP 262	RS 93	*B. tenax*	*B. velox*	*B. lyticus*	RS 15	RS 23	RS 38	RS 108	RS 125	RS 161	RS 176
RS	49	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	50	+	-	+	+	-	+	+	-	+	+	-	-	+	-	-
	51	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-
	52	+	-	-	+	-	-	+	-	+	+	-	-	-	-	-
	53	+	+	-	+	+	+	+	+	+	-	-	-	-	-	-
	54	+	-	-	+	-	+	-	+	-	+	-	-	-	-	-
	55	+	+	-	+	-	+	+	+	+	-	-	-	+	-	-
	56	-	+	-	-	-	-	-	-	+	-	-	+	-	-	-
	57	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	58	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-
	59	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-
	60	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	61	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	62	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-
	63	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	64	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	65	-	+	-	-	-	+	-	+	+	-	-	-	+	-	-
	66	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 5.2 (Cont'd)

Culture supernatant		Indicator strains														
		NCA 1503	ATCC 12016	EP 240	EP 262	RS 93	*B. tenax*	*B. velox*	*B. lyticus*	RS 15	RS 23	RS 38	RS 108	RS 125	RS 161	RS 176
RS	67	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-
	68	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-
	69	-	-	-	+	-	-	+	+	-	-	-	+	-	-	-
	70	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-
	71	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	72	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
	73	-	-	-	-	+	-	-	-	-	+	-	+	-	-	-
	74	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	75	+	-	-	+	-	+	-	-	+	-	-	-	-	-	-
	76	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
	77	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-
	78	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
	79	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
	80	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	81	-	-	+	-	-	-	-	-	-	-	-	-	+	-	+
	82	+	-	+	+	-	-	-	-	+	+	-	-	-	-	-
	83	-	-	-	+	-	+	+	+	+	+	-	+	-	-	-
	84															

Table 5.2 (Cont'd)

Culture supernatant		Indicator strains															
		NCA 1503	ATCC 12016	EP 240	EP 262	RS 93	B. tenax	B. velox	B. lyticus	RS 15	RS 23	RS 38	RS 108	RS 125	RS 161	RS 1
RS	85	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	86	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	87	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
	88	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	89	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	90	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	91	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-
	92	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
	93	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	94	-	-	+	-	+	-	-	-	+	-	-	-	-	-	-
	95	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
	96	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-
	97	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	98	+	-	-	+	+	-	-	+	-	-	-	-	-	-	-
	99	-	+	+	+	-	+	+	+	+	+	-	+	-	-	-
	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

(NCA 1503, RS 239, RS 240, RS 241, RS 242). No phages were isolated from samples of river sludge or digested sludge. One type of phage was isolated from stable manure, one from the soil sample, three from silage, four from rotting straw and six from compost.

Examination of the culture supernatants from 98 thermophilic strains of Bacillus (Table 5.2) for the presence of spontaneously induced phage resulted in 62 showing evidence of phage plaques when plated on one or more of 15 indicator strains. Attempts to sub-culture phage plaques were only successful from 14 of the 62 putative lysogens initially indicated. All 14 strains were isolated from samples of soil collected from St Jean De Monts and Ile De Re (La Rochelle) in France. Four strains, RS 51, RS 53, RS 56 and RS 58, were included in the taxonomic study and were found to be members of three different taxonomic groups. The plaques produced from the culture supernatant from other strains, which did not sub-culture, may have been artifacts, defective phages, bacteriocin producers or phages sensitive to prolonged incubation at 55 - 60 ^{o}C. Thirteen of these phages which were isolated and successfully sub-cultured are listed in Table 5.3 together with their major host and brief descriptions of plaque size and morphology. This group of phages has not been characterised or examined for the ability to act as transducing phages.

5.1.4 Bacteriophage induction

Five bacterial strains (RS 5, RS 6, RS 29, RS 30 and RS 31) showing spontaneous phage induction, were examined for the enhancement of lysogenic induction following exposure to mitomycin C and u.v. radiation. Strain RS 93 (which was a strong bacteriocin producer) and strain RS 176 (when used as an indicator for the detection of spontaneous lysogens this strain indicated the presence of phage in many of the samples) were also examined (Table 5.2). B. stearothermophilus NCA 1503 was also examined following the observations of R. Yeo and C.G.T. Evans (pers. comm.); during their studies of glycerokinase production from NCA 1503 in continuous culture, they observed intermittent foaming corresponding with a loss in viability. When dilutions of the culture supernatant were plated with B. caldotenax and B. stearothermophilus ATCC 12016 in soft agar a small number of irregular hazy areas of growth were observed. These were picked off and replated with the same host strain and after three successive transfers a number of clear plaques became evident.

Table 5.3

Thermophilic bacteriophages isolated from culture supernatant

Strain of thermophilic Bacillus	Host	Plaque size and morphology
RS 50	B. caldotenax	1.5 - 3 mm circular, clear with hazy edge
RS 51	RS 108	1 - 4 mm circular, clear
RS 52	B. caldovelox	1 - 2.5 mm circular, clear with hazy edges
RS 53	B. caldotenax	1.5 - 5 mm circular, clear with turbid centre
RS 55	B. caldotenax	1.5 - 4 mm circular, clear with colony at centre
RS 56	B. stearo. 12016	1 - 4 mm circular, clear with hazy edges
RS 58	B. stearo. 12016	0.5 - 3 mm circular, clear with hazy edge turbid centre
RS 59	B. stearo. 12016	1 - 3.5 mm circular, clear with hazy edge
RS 62	RS 23	<1 mm circular, hazy
RS 65	B. caldovelox	1 - 2.5 mm circular, clear with colonies in zone
RS 67	B. caldotenax	1 - 3 mm circular, clear
RS 68	B. caldotenax	0.5 - 4 mm circular, clear with hazy edge
RS 69	B. caldotenax	0.5 - 3 mm circular, clear

Legend

Bacteriophages were isolated from culture supernatant following spontaneous lysogenic induction.

There was no evidence of bacteriophage induction from strains RS 5, RS 6, RS 29, RS 30, RS 31 and RS 176 following exposure to mitomycin C or u.v. radiation. Bacteriophages were isolated from culture supernatant from B. stearothermophilus NCA 1503 and RS 93.

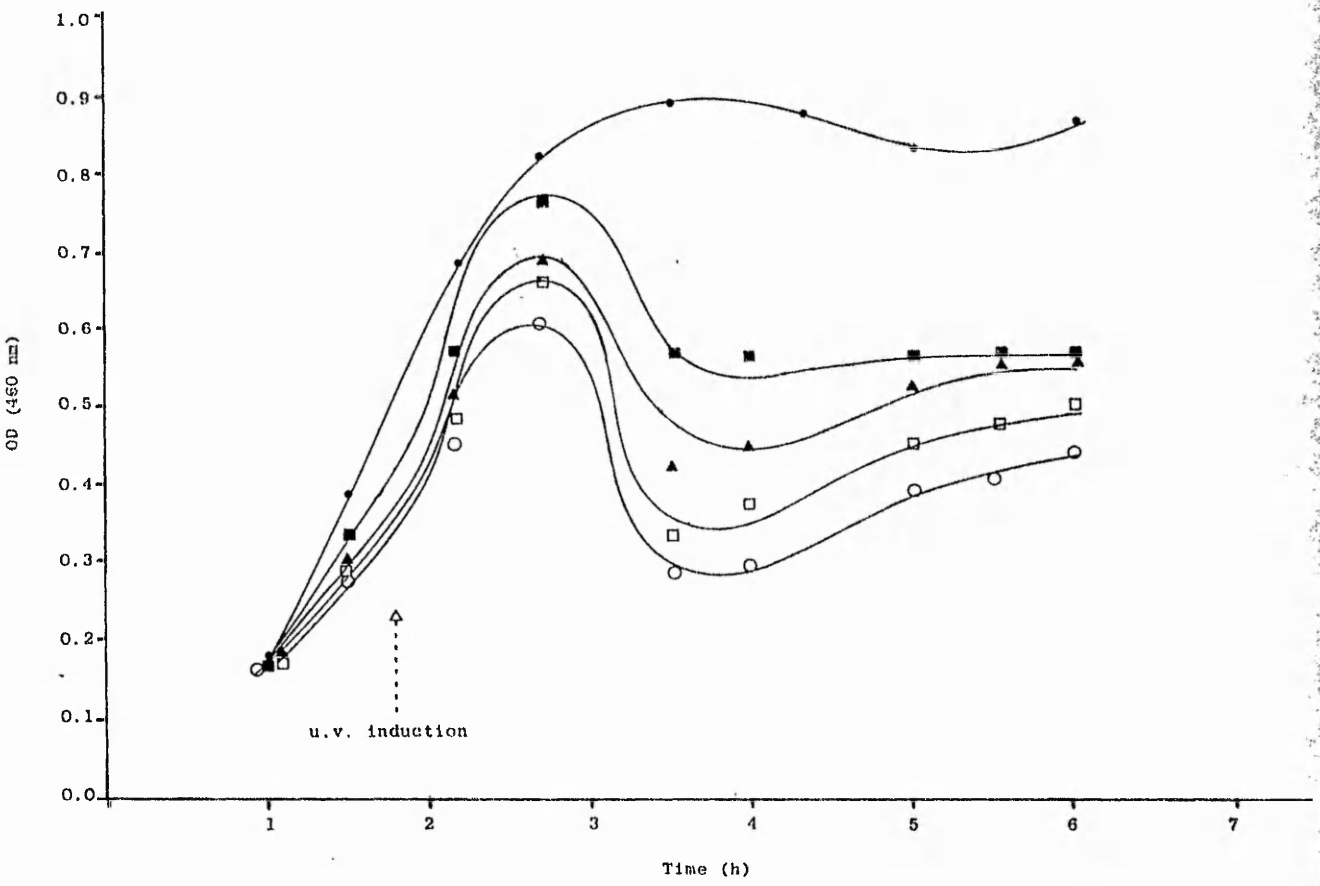
The exposure of NCA 1503 to u.v. radiation (186 - 372 J m^{-2}) or exposure to mitomycin C (0.05 - 0.1 µg ml^{-1}) resulted in a rapid decline in OD (Fig. 5.2 and 5.3). Examination of the indicator plates showed little evidence of the formation of typical phage plaques but a number of plates showed areas of irregular growth with hazy and moist areas on the plate. When the cells from these areas were picked off and shaken in 5 ml of PMN buffer, centrifuged to remove cells and the supernatant replated back to the original indicator strains, clear plaques were observed on strains ATCC 12016, 4S and B. caldotenax. Strains NCA 1503 and EP 240 still showed the same irregular areas of weaker cell growth on the plates and RS 15 gave no evidence for the presence of phage. One plaque was purified and designated as JS 001.

The exposure of strain RS 93 to u.v. radiation did not result in the isolation of bacteriophage from the culture supernatant. Exposure to mitomycin C (0.1 - 0.15 µg ml^{-1}) resulted in a rapid fall in OD after 40 min (Fig. 5.4) and the isolation of phage from the supernatant. Plating the supernatant with 12 indicator strains did not show any obvious plaques, but many of the plates showed areas of patchy growth or zones with a single colony at the centre. (These were assumed to be colonies of RS 93 producing bacteriocin.) The patchy areas of growth were resuspended in buffer, the cells removed by centrifugation and the supernatant replated to the original indicator strains. B. caldotenax and B. stearothermophilus ATCC 12016 indicated the presence of clear plaques in the lawn of cells. One plaque was successively reselected and replated three times to ensure phage purity and designated as JS 002.

During the course of these studies strain RS 108 was used as an indicator and repeatedly showed evidence of phage plaques on the indicator control plates (i.e. RS 108 suspension added to soft agar and plated, no culture supernatant added). One of these plaques was picked off and purified by repeated sub-culture and designated as JS 003. It appears that a small percentage of the RS 108 population are lysogenic and are spontaneously induced, releasing phages able to infect the rest of the population.

Figure 5.2

The effect of u.v. irradiation on a growing culture of B. stearothermophilus NCA 1503

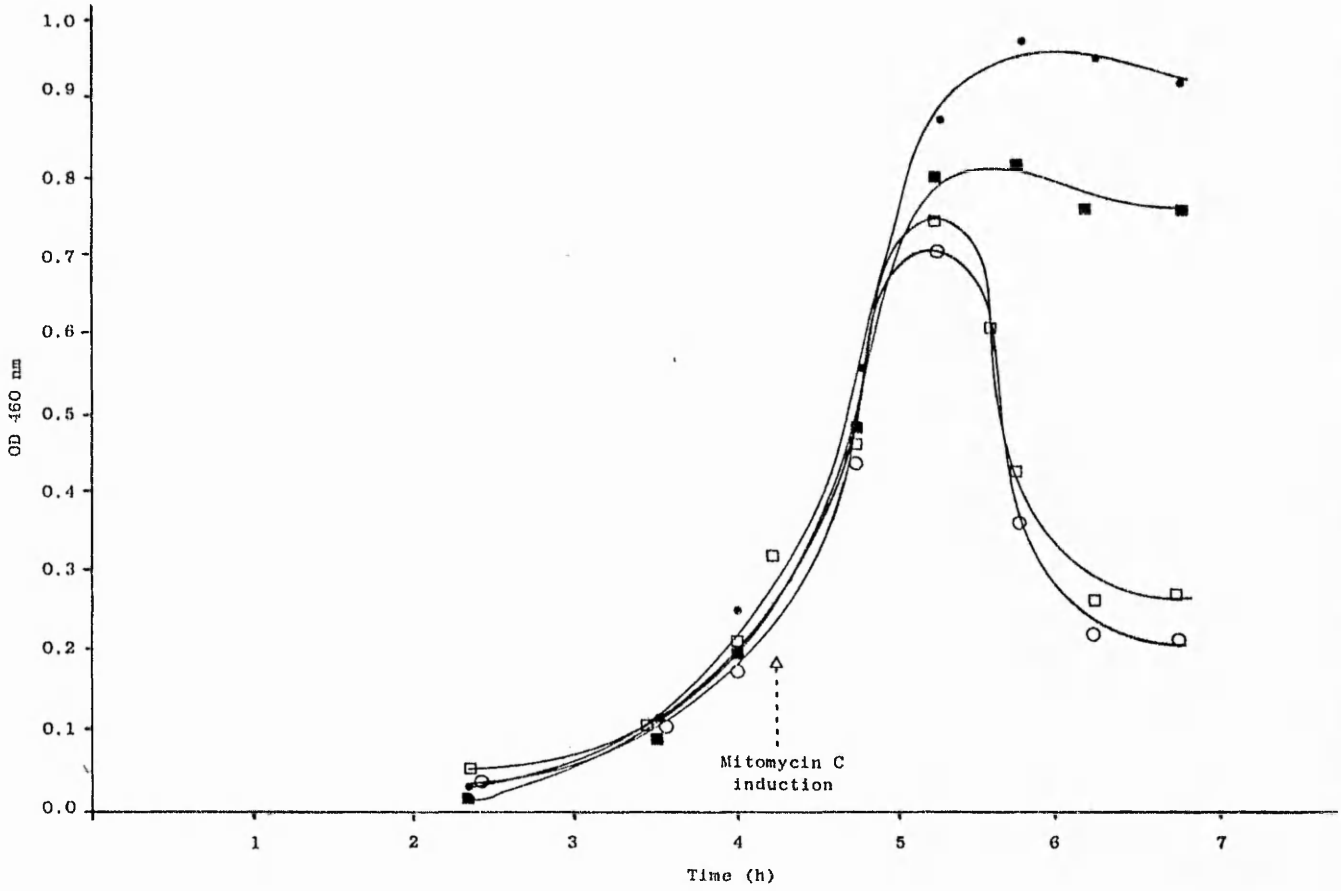


Legend

The culture was grown in T tubes in BS medium at 55 °C in a Gallenkamp shaking water bath. Mid log phase cells were exposed to u.v. irradiation.
●, control; ■, 186 J m^{-2} s^{-1}; ▲ 248 J m^{-2} s^{-1}; □, 310 J m^{-2} s^{-1}; ○, 372 J m^{-2} s^{-1}.

Figure 5.3

The effect of mitomycin C on a growing culture of B. stearothermophilus NCA 1503

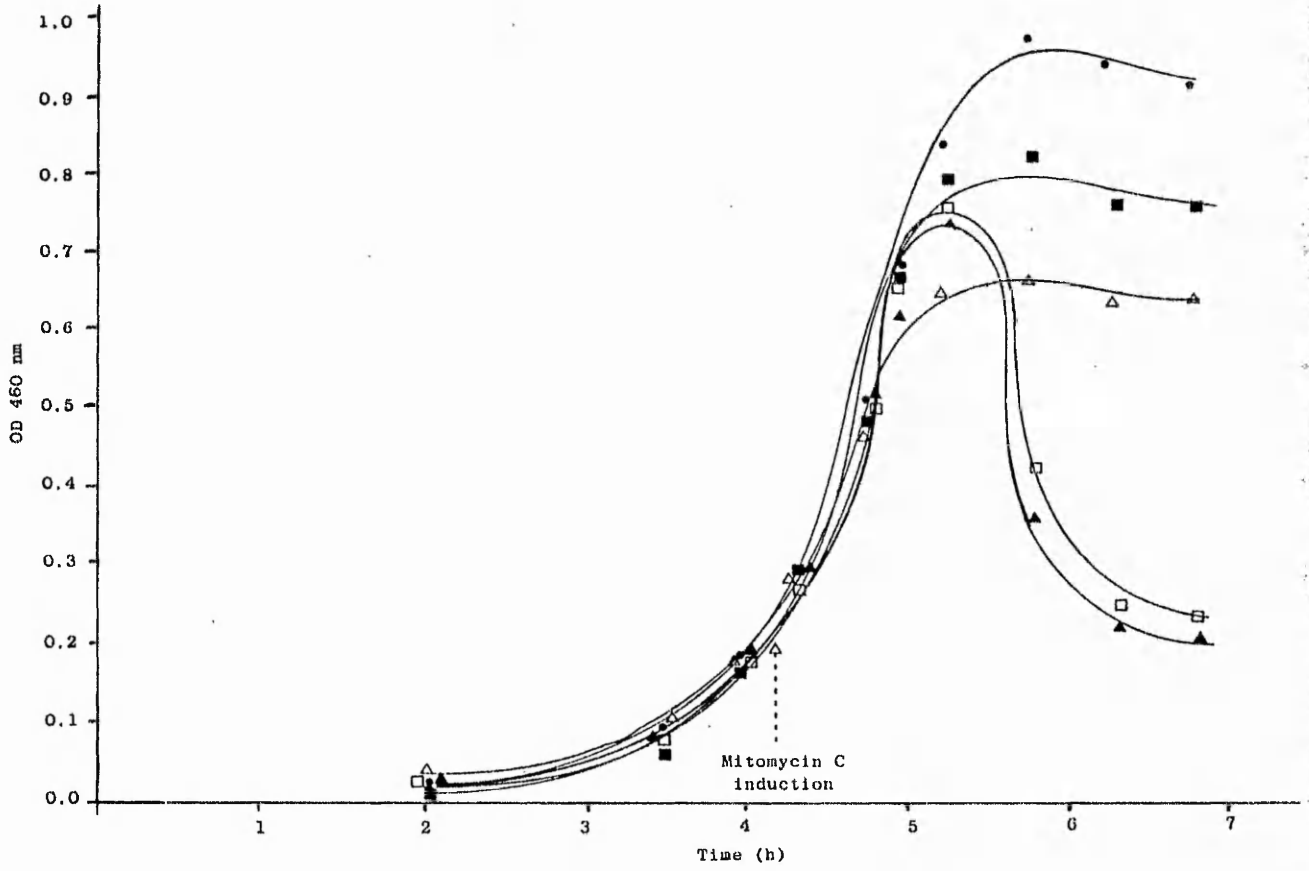


Legend

The culture was grown in T tubes in BS medium at 55 °C in a Gallenkamp shaking water bath. Early log phase cells were treated with mitomycin C. ●, control l; ■, 0.01 μg ml^{-1}; □, 0.1 μg ml^{-1}; ○, 0.05 μg ml^{-1}.

Figure 5.4

The effect of mitomycin C on a growing culture of B. stearothermophilus RS 93



Legend

The culture was grown in T tubes in BS medium at 55 °C in a Gallenkamp shaking water bath. Early log phase cells were treated with mitomycin C.
●, control; ■, 0.01 μg ml^{-1}; ▲, 0.5 μg ml^{-1}; □, 0.1 μg ml^{-1}; ○, 0.15 μg ml^{-1}.

Table 5.4

Examination of culture supernatant from B. stearothermophilus NCA 1503 following exposure to u.v. radiation and mitomycin C

Indicator strains	Mitomycin C μg ml^{-1}				u.v. radiation J m^{-2}				
	Control	0.1 μg	0.05 μg	0.01 μg	Control	186	248	310	370
B. caldotenax	0	?	?	0	0	0	0	?	?
B. stearo. NCA 1503	0	0	0	0	0	0	0	0	0
B. stearo. NW 4S	0	?	?	0	0	0	?	?	?
B. stearo. EP 240	0	0	0	0	0	0	0	?	?
B. stearo. ATCC 12016	0	?	?	0	0	?	?	+	?
B. stearo. RS 15	0	0	0	0	0	0	?	?	?

Legend

Supernatant from cultures of B. stearothermophilus NCA 1503 exposed to mitomycin C or u.v. radiation were examined for evidence of bacteriophage induction. Culture supernatant was plated with a range of indicator strains using the soft agar overlayer technique. Plates were incubated overnight at 60 °C and observed for evidence of bacteriophage infection. Plaques and areas of abnormal growth were sub-cultured to fresh indicator plates (see text) for further study. +, Plates with a small number of plaques. ?, Plates with areas of abnormal growth, e.g. weak, or irregular. 0, Plates showing normal even matt of cell growth with no evidence of phage.

5.2 BACTERIOPHAGE HOST RANGE

The bacteriophage host range (Table 5.5) illustrates the specificity of phage host interactions. Phages JS 004 - JS 012 were all isolated using starch negative strains (Taxonomic cluster (1)) and Table 5.5 shows no evidence of infection to strains outside of this group. Similarly, phages JS 017 - JS 026 infect B. caldotenax and B. caldovelox at a similar frequency and to a lesser extent B. caldolyticus. B. stearothermophilus RS 93 (Cluster (1b)) also appears to be infected at a slightly lower level. The three phages isolated on B. stearothermophilus NCA 1503 did not infect any other strains examined except strain NW 10. None of the phages isolated infected strains of B. coagulans, B. subtilis or B. amyloliquefaciens.

5.3 HOST RESTRICTION AND MODIFICATION OF BACTERIOPHAGES

The examination of host range data must take into account the presence of host restriction and modification of the bacteriophages. No deliberate studies of restriction and modification systems were made, but evidence collected whilst carrying out phage isolations, host range studies and titring of phage preparations indicated its occurrence in thermophilic species of Bacillus (Table 5.6).

Examination of the efficiency of plating (e.o.p.) of phage JS 001 on B. caldotenax and B. stearothermophilus ATCC 12016 indicates restriction by B. caldotenax of phage harvested from ATCC 12016. The latter strain, however, did not appear to restrict (e.o.p. 0.75) phages previously harvested from B. caldotenax. Phage harvested from B. caldotenax appears to be modified, permitting a high level of infection when plated back to B. caldotenax.

Plating of phage JS 001 from B. caldotenax with B. caldovelox had an e.o.p. of 0.8 indicating the latter did not restrict phage from B. caldotenax. (Dilution and plating variations must be taken into account when examining values of e.o.p. An e.o.p. of 0.8 was not considered significantly different to an e.o.p. of 1, to clarify further repeat tests would be required.) The data from strains RS 15, RS 93, RS 108, RS 125, and EP 262 illustrates the variation in e.o.p. This data alone, while suggesting restriction, may indicate some inherent resistance in the population to phage infection possibly due to a lack of phage receptor sites. Clarification of the presence of a restriction and

modification system, requires the re-infection of these strains (and infection of B. caldotenax) with their own phage lysates.

Data using phage TP 1C harvested from B. caldotenax, indicates no restriction by B. caldovelox. The e.o.p. on B. stearothermophilus ATCC 12016 was 0.72, a similar value to that found using JS 001 (0.75) probably indicating a low level of restriction.

Phages JS 019, JS 024 and JS 025 indicated no evidence of restriction of phages harvested from B. caldotenax when plated on B. caldovelox. Conversely phages harvested from B. caldovelox when plated with B. caldotenax appear restricted with e.o.p. values of 0.28, 0.12, 0.125 respectively. Phages harvested from B. caldotenax had a low e.o.p. on B. caldolyticus and B. stearothermophilus RS 93.

Plating of phage JS 017 from B. caldotenax to B. caldovelox again showed no evidence of restriction, however plating on B. caldolyticus, B. thermocatenulatus and B. stearothermophilus ATCC 8005 all indicated the presence of a restriction system. Phage harvested from B. caldovelox also appeared restricted by B. thermocatenulatus and ATCC 8005. Phage isolated from B. thermocatenulatus was restricted by ATCC 8005 but unusually gave an e.o.p. of 20 when plated on B. caldotenax. This plating was not repeated but it suggests B. thermocatenulatus does not produce modification enzymes to inhibit the effect of its restriction system.

The evidence suggests that B. caldotenax has a restriction system which is able to reduce the e.o.p. of phages harvested from some strains of B. stearothermophilus by up to 10^{-4}. Restriction of phages harvested from B. caldovelox which is taxonomically very similar to B. caldotenax (Section 3.2.3) is evident but at a much lower level. A number of other strains appear to restrict phage infection including B. caldolyticus, RS 15, RS 93, RS 108 and RS 125.

Table 5.5

Thermophilic bacteriophage host range

Host strains	Bacteriophages						
	TP 1C	TP 84	12016B	ϕ1	ϕ29	JS 001	JS 002
B. caldotenax	++++	++++	++++	-	-	++++	++++
B. caldovelox	++++	++++	++++	-	-	++++	++++
B. caldolyticus	+++	++	++	-	-	++	-
B. stearothermophilus NCA 1503	+	++	-	-	-	*	+
B. stearothermophilus ATCC 12016	++++	++++	++++	-	-	++++	++
B. stearothermophilus strain NW 10	+	++	-	-	-	*	*
B. stearothermophilus strain 4S	++++	+++	-	-	-	*	*
B. stearothermophilus EP 136	-	-	-	-	-	*	*
B. stearothermophilus RS 93	+	+	-	-	-	+	+
B. stearothermophilus EP 262	+	++	+	-	-	+	*
B. stearothermophilus EP 240	-	+++	++++	-	-	-	*
B. stearothermophilus ATCC 8005	*	*	*	*	*	*	*
B. thermocatenulatus DSM 730	*	*	*	*	*	*	*
Thermophile RS 15	-	*	*	*	*	+++	*
Thermophile RS 38	-	*	*	*	*	-	-
Thermophile RS 108	*	*	*	*	*	+++	*
Thermophile RS 125	-	*	*	*	*	++	-
Thermophile RS 239	*	*	*	*	*	*	*
Thermophile RS 240	*	*	*	*	*	*	*
Thermophile RS 241	*	*	*	*	*	*	*
Thermophile RS 242	*	*	*	*	*	*	*
Thermophile TB 118	*	*	*	*	*	*	*
Thermophile TB 124	*	*	*	*	*	*	*
Thermophile TB 128	*	*	*	*	*	*	*
Thermophile TB 144	*	*	*	*	*	*	*
Thermophile TB 150	*	*	*	*	*	*	*
Thermophile TB 152	*	*	*	*	*	*	*
B. coagulans ATCC 8038	-	-	-	-	-	-	-
B. coagulans ATCC 12245	-	-	-	-	-	-	-
B. subtilis 168	-	-	-	++++	++++	-	-
B. amyloliquefaciens ϕ1	-	-	-	++++	++++	-	-

Table 5.5 (Cont'd)

Host strains	Bacteriophages					
	JS 003	JS 004	JS 005	JS 006	JS 007	JS 008
B. caldotenax	++++	-	-	-	-	-
B. caldovelox	++++	-	-	-	-	-
B. caldolyticus	-	-	-	-	-	-
B. stearothermophilus NCA 1503	+	-	-	-	-	-
B. stearothermophilus ATCC 12016	++	*	*	*	*	-
B. stearothermophilus strain NW 10	*	-	-	+	-	-
B. stearothermophilus strain 4S	*	*	*	*	*	*
B. stearothermophilus EP 136	*	-	-	*	*	-
B. stearothermophilus RS 93	+	+++	-	+++	++++	-
B. stearothermophilus EP 262	*	-	-	+	*	-
B. stearothermophilus EP 240	*	-	-	*	*	-
B. stearothermophilus ATCC 8005	*	*	*	*	*	*
B. thermocatenulatus DSM 730	*	*	*	*	*	*
Thermophile RS 15	*	*	*	*	*	*
Thermophile RS 38	-	*	*	*	*	*
Thermophile RS 108	*	*	*	*	*	*
Thermophile RS 125	-	*	*	*	*	*
Thermophile RS 239	*	++++	++++	-	++++	++++
Thermophile RS 240	*	-	-	-	++	-
Thermophile RS 241	*	++	++	-	++++	++++
Thermophile RS 242	*	+	-	++	+	-
Thermophile TB 118	*	-	*	-	-	*
Thermophile TB 124	*	-	*	-	-	*
Thermophile TB 128	*	++++	*	-	++++	*
Thermophile TB 144	*	-	*	-	-	*
Thermophile TB 150	*	-	*	-	-	*
Thermophile TB 152	*	-	*	*	-	*
B. coagulans ATCC 8038	-	-	-	*	*	-
B. coagulans ATCC 12245	-	-	-	*	*	-
B. subtilis 168	-	*	*	*	-	-
B. amyloliquefaciens ϕ1	-	*	*	*	-	-

Table 5.5 (Cont'd)

Host strains	Bacteriophages					
	JS 009	JS 010	JS 011	JS 012	JS 013	JS 014
B. caldotenax	-	-	-	-	-	-
B. caldovelox	-	-	-	-	-	-
B. caldolyticus	-	-	-	-	-	-
B. stearothermophilus NCA 1503	-	-	-	-	+++	+++
B. stearothermophilus ATCC 12016	*	*	*	*	-	-
B. stearothermophilus strain NW 10	-	-	*	*	++	++
B. stearothermophilus strain 4S	*	*	*	*	-	-
B. stearothermophilus EP 136	*	*	*	*	-	-
B. stearothermophilus RS 93	++++	+	*	*	+	-
B. stearothermophilus EP 262	*	*	*	*	*	*
B. stearothermophilus EP 240	*	*	*	*	*	*
B. stearothermophilus ATCC 8005	*	*	*	*	*	*
B. thermocatenulatus DSM 730	*	*	*	*	*	*
Thermophile RS 15	*	*	*	*	*	*
Thermophile RS 38	*	*	*	*	*	*
Thermophile RS 108	*	*	*	*	*	*
Thermophile RS 125	*	*	*	*	*	*
Thermophile RS 239	*	-	*	*	*	*
Thermophile RS 240	*	-	*	*	*	*
Thermophile RS 241	++++	+	*	*	*	*
Thermophile RS 242	++	++++	*	*	*	*
Thermophile TB 118	-	-	*	*	*	*
Thermophile TB 124	-	-	*	*	*	*
Thermophile TB 128	++++	+	+	+	*	*
Thermophile TB 144	-	-	*	*	*	*
Thermophile TB 150	-	-	*	*	*	*
Thermophile TB 152	-	-	*	*	*	*
B. coagulans ATCC 8038	*	*	*	*	-	-
B. coagulans ATCC 12245	*	*	*	*	-	-
B. subtilis 168	-	-	-	-	-	-
B. amyloliquefaciens ϕ1	-	-	-	-	-	-

Table 5.5 (Cont'd)

Host strains	Bacteriophages					
	JS 015	JS 016	JS 017	JS 018	JS 019	JS 020
B. caldotenax	-	*	++++	++++	++++	++++
B. caldovelox	-	*	++++	++++	++++	++++
B. caldolyticus	-	*	+	++++	+	++++
B. stearothermophilus NCA 1503	+++	*	-	-	-	-
B. stearothermophilus ATCC 12016	-	*	+++	-	-	-
B. stearothermophilus strain NW 10	++	*	++	-	-	-
B. stearothermophilus strain 4S	-	*	*	*	*	*
B. stearothermophilus EP 136	-	*	-	-	-	+
B. stearothermophilus RS 93	-	*	+++	+++	+++	+++
B. stearothermophilus EP 262	*	*	++	-	-	-
B. stearothermophilus EP 240	*	*	+	+	+	+
B. stearothermophilus ATCC 8005	*	*	++	*	*	*
B. thermocatenulatus DSM 730	*	*	++	*	*	*
Thermophile RS 15	*	*	*	*	*	*
Thermophile RS 38	*	*	*	*	*	*
Thermophile RS 108	*	*	*	*	*	*
Thermophile RS 125	*	*	*	*	*	*
Thermophile RS 239	*	*	*	*	*	*
Thermophile RS 240	*	*	*	*	*	*
Thermophile RS 241	*	*	*	*	*	*
Thermophile RS 242	*	*	*	*	*	*
Thermophile TB 118	*	*	*	*	*	*
Thermophile TB 124	*	*	*	*	*	*
Thermophile TB 128	*	*	*	*	*	*
Thermophile TB 144	*	*	*	*	*	*
Thermophile TB 150	*	*	*	*	*	*
Thermophile TB 152	*	*	*	*	*	*
B. coagulans ATCC 8038	-	*	-	-	-	-
B. coagulans ATCC 12245	-	*	-	-	-	-
B. subtilis 168	-	*	-	-	-	-
B. amyloliquefaciens ϕ1	-	*	-	-	-	-

Table 5.5 (Cont'd)

Host strains	Bacteriophages					
	JS 021	JS 022	JS 023	JS 024	JS 025	JS 026
B. caldotenax	++++	++++	++++	++++	++++	++++
B. caldovelox	++++	++++	++++	++++	++++	++++
B. caldolyticus	+++	+	-	+++	+++	+++
B. stearothermophilus NCA 1503	-	-	-	-	-	-
B. stearothermophilus ATCC 12016	+	-	-	-	-	++
B. stearothermophilus strain NW 10	-	-	-	-	-	-
B. stearothermophilus strain 4S	*	*	*	*	*	*
B. stearothermophilus EP 136	-	-	+	+	-	-
B. stearothermophilus RS 93	+++	++++	+++	+++	+++	+++
B. stearothermophilus EP 262	-	-	-	-	-	-
B. stearothermophilus EP 240	+	+	-	+	+	-
B. stearothermophilus ATCC 8005	*	*	*	*	*	*
B. thermocatenulatus DSM 730	*	*	*	*	*	*
Thermophile RS 15	*	*	*	*	*	*
Thermophile RS 38	*	*	*	*	*	*
Thermophile RS 108	*	*	*	*	*	*
Thermophile RS 125	*	*	*	*	*	*
Thermophile RS 239	*	*	*	*	*	*
Thermophile RS 240	*	*	*	*	*	*
Thermophile RS 241	*	*	*	*	*	*
Thermophile RS 242	*	*	*	*	*	*
Thermophile TB 118	*	*	*	*	*	*
Thermophile TB 124	*	*	*	*	*	*
Thermophile TB 128	*	*	*	*	*	*
Thermophile TB 144	*	*	*	*	*	*
Thermophile TB 150	*	*	*	*	*	*
Thermophile TB 152	*	*	*	*	*	*
B. coagulans ATCC 8038	-	-	-	-	-	-
B. coagulans ATCC 12245	-	-	-	-	-	-
B. subtilis 168	-	-	-	-	-	-
B. amyloliquefaciens ϕ1	-	-	-	-	-	-

Table 5.5 (Cont'd)

Thermophilic bacteriophage host range

Host strains	Bacteriophages isolated from thermophilic Bacillus						
	RS 50	RS 51	RS 52	RS 53	RS 55	RS 56	RS 58
B. caldotenax	++++	-	+++	++++	++++	-	+++
B. caldovelox	++++	-	+++	++++	++++	-	+++
B. caldolyticus	-	-	-	-	-	-	-
B. stearothermophilus	*	*	*	*	*	*	*
NCA 1503	+	-	+	+	+++	-	-
ATCC 12016	++	-	+++	++++	++	+++	+++
strain NW 10	*	*	*	*	*	*	*
strain NW 4S	+	*	+	+	+	*	*
strain EP 136	*	*	*	*	*	*	*
RS 93	-	-	-	+	-	+	-
strain EP 262	-	-	-	-	-	++	-
strain EP 240	-	-	-	-	-	-	-
RS 15	-	++	-	-	+	+	-
RS 23	-	-	-	-	-	-	-
RS 108	++	+++	++++	+++	++++	+++	-
RS 125	-	-	-	-	-	-	-
RS 239	-	*	-	-	-	*	*
RS 240	-	-	-	-	*	*	*
RS 241	-	-	-	-	*	*	*
RS 242	-	-	-	-	*	*	*
TB 128	*	*	*	*	*	*	*
B. coagulans ATCC 8038	-	-	-	-	*	*	*
B. coagulans ATCC 12245	-	*	-	-	-	*	*
B. subtilis 168	-	*	-	-	-	*	*
B. amyloliquefaciens ϕ1	*	*	*	*	*	*	*

Table 5.5 (Cont'd)

Host strains	Bacteriophages isolated from thermophilic Bacillus					
	RS 59	RS 62	RS 65	RS 67	RS 68	RS 69
B. caldotenax	-	-	+	++++	+++	++++
B. caldovelox	-	-	+	++++	+++	++++
B. caldolyticus	-	-	+++	+	-	++++
B. stearothermophilus	*	*	*	*	*	*
NCA 1503	-	-	-	-	-	-
ATCC 12016	+++	-	+	+	-	-
strain NW 10	*	*	*	*	*	*
strain NW 4S	*	*	*	+	*	*
strain EP 136	*	*	*	*	*	*
RS 93	-	+	*	-	-	-
strain EP 262	-	-	*	+	-	+
strain EP 240	-	-	*	-	-	-
RS 15	-	-	+	*	-	++
RS 23	-	+++	*	-	++	-
RS 108	-	-	*	++	-	+
RS 125	+++	-	+	-	-	-
RS 239	*	*	*	-	*	-
RS 240	*	*	*	-	*	-
RS 241	*	*	*	-	*	-
RS 242	*	*	*	-	*	-
TB 128	*	*	*	*	*	*
B. coagulans ATCC 8038	*	*	*	-	*	-
B. coagulans ATCC 12245	*	*	*	-	*	-
B. subtilis 168	*	*	*	-	*	-
B. amyloliquefaciens ϕ	*	*	*	*	*	*

Legend

Bacteriophage suspensions (10^6 p.f.u. ml^{-1}) were prepared as B. caldotenax or the most suitable phage host available. Dilutions were then plated with the test strains and observed for plaque formation following O/N incubation at 55 °C. Results were recorded as ++++ for e.o.p. of 1; +++ e.o.p. 0.1 - 0.01; ++ e.o.p. 0.01 - 0.001; + e.o.p. <0.001; - no evidence of phage infection; * not tested.

Table 5.6

Restriction and modification of phage by thermophilic hosts based on the efficiency of plating (e.o.p.)

Phage	Host used for preparation of phage	Hosts used for titre of phage	Plaque count	e.o.p.
JS 001	B. caldotenax	B. caldotenax	8×10^8	1
		B. stearo. (ATCC 12016)	6×10^8	0.75
JS 001	B. stearo. (ATCC 12016)	B. stearo. (ATCC 12016)	1×10^6	1
		B. caldotenax	1×10^2	0.0001
JS 001	B. caldotenax	B. caldotenax	2.7×10^5	1
		B. caldovelox	2.2×10^5	0.8
		thermophile RS 15	5.0×10^4	0.19
		thermophile RS 108	1.25×10^4	0.046
		thermophile RS 125	4.0×10^3	0.015
		B. stearo. RS 93	1.3×10^2	0.0005
		B. stearo. EP 262	1.0×10^1	0.00004
TP 1C	B. caldotenax	B. caldotenax	1.8×10^3	1
		B. caldovelox	1.9×10^3	1
		B. stearo. (ATCC 12016)	1.3×10^3	0.72
JS 019	B. caldotenax	B. caldotenax	2×10^4	1
		B. caldovelox	2×10^4	1
		B. stearo. RS 93	1×10^3	0.05
JS 019	B. caldovelox	B. caldovelox	1×10^6	1
		B. caldotenax	2.8×10^5	0.28
JS 024	B. caldotenax	B. caldotenax	1×10^7	1
		B. caldovelox	7×10^6	0.7
		B. caldolyticus	1×10^5	0.01
		B. stearo. RS 93	1.5×10^5	0.015
JS 024	B. caldovelox	B. caldovelox	1.2×10^7	1
		B. caldotenax	1.4×10^6	0.12
JS 025	B. caldotenax	B. caldotenax	1×10^7	1
		B. caldovelox	1×10^7	1
		B. caldolyticus	4×10^5	0.04
		B. stearo. RS 93	6.6×10^4	0.07
JS 025	B. caldovelox	B. caldovelox	8×10^6	1
		B. caldotenax	1×10^6	0.125

Table 5.6 (Cont'd)

Phage	Host used for preparation of phage	Hosts used for titre of phage	Plaque count	e.o.p.
JS 017	B. caldotenax	B. caldotenax	5×10^7	1
		B. caldovelox	5×10^7	1
		B. caldolyticus	1.2×10^2	0.000002
		B. thermocatenulatus	1.4×10^5	0.003
		B. stearo. (ATCC 8005)	1.5×10^5	0.003
JS 017	B. caldovelox	B. caldovelox	3.2×10^5	1
		B. thermocatenulatus	4.0×10^2	0.001
		B. stearo. (ATCC 8005)	7.0×10^2	0.002
JS 017	B. thermocatenulatus	B. thermocatenulatus	3.0×10^4	1
		B. caldotenax	6.0×10^5	20
		B. stearo. (ATCC 8005)	2.0×10^3	0.07

Legend

Phages were titred using the soft agar plating technique, plates were incubated overnight at 60 ^{o}C.

5.4 PLAQUE MORPHOLOGY OF THERMOPHILIC BACTERIOPHAGES

Table 5.7 details the plaque morphology of thermophilic bacteriophages when plated in trypticase soft agar supplemented with 0.015 M $CaCl_2$ and incubated overnight at 55 ^{o}C.

Considerable variations in plaque morphology were observed using identical conditions for plating and incubation. The plaque morphology of phage JS 004, following its initial isolation and purification by single plaque transfer, was described variously as "small, hazy pin heads", "2 mm hazy plaques", "1 - 2 mm hazy plaques with some resistant colonies", "0.5 mm, hazy irregular", "1 mm or less and clear with resistant colonies". Following approximately 12 consecutive single plaque transfers the morphology stabilised to that described in Table 5.7. Similar initial variations in plaque morphology were observed with JS 006, JS 007 and to a lesser extent the other phages isolated using strains RS 239 - 242.

Studies with phage JS 004 (Table 5.8) indicate a variation in plaque size and morphology with variation in temperature and calcium chloride levels in the medium.

5.5 THE EFFECT OF METAL IONS, TEMPERATURE AND u.v. RADIATION ON THE p.f.u. OF PHAGE JS 017

The effect of $CaCl_2$, $MgCl_2$ and $MnCl_2$ on the titre of phage preparations is shown in Table 5.9. All three were shown to stimulate p.f.u. at varying concentrations. A comparison with the data presented for phage JS 004 (Table 5.8) indicates the latter to have a slightly lower requirement for calcium ions for optimum p.f.u. $CaCl_2$ at 0.015 M was used for all phage assays, although this may not be the optimum for all phages examined it does generally appear to enhance the p.f.u.

Decreasing the incubation temperature has a significant effect on p.f.u. particularly below 60 ^{o}C (Fig. 5.5). This trend would not continue however since B. caldotenax has a minimum growth temperature in the region of 42 ^{o}C. Phage JS 004 (Table 5.8) indicated a similar rise in p.f.u. as incubation temperature decreased.

Exposure of phage JS 017 to u.v. radiation resulted in a rapid decrease in p.f.u. and exposure to 270 J m^{-2} reduced the p.f.u. by 99% (Fig. 5.3). Attempts to enhance the transduction frequency of JS 017 by exposure to u.v. radiation were unsuccessful (Section 8.1.2).

Table 5.7

Plaque morphology of thermophilic bacteriophage

Phage No.	Host	Plaque description
JS 001	B. caldotenax	1 - 3 mm diameter, circular, clear.
JS 002	B. caldotenax	1 - 2 mm diameter, circular, clear.
JS 003	B. caldotenax	1 - 2 mm diameter, circular, clear centre, hazy outer halo 2 - 3 mm.
JS 004	Strain RS 239	0.5 - 1 mm, diameter, circular, hazy.
JS 005	Strain RS 239	0.5 - 1 mm diameter, circular, slightly hazy.
JS 006	Strain RS 239	0.5 - 0.75 mm diameter, circular, clear.
JS 007	Strain RS 240	0.5 - 2 mm diameter, circular, hazy.
JS 008	Strain RS 241	1 - 3.5 mm diameter, circular, hazy.
JS 009	Strain RS 242	0.5 mm diameter, circular, hazy centre.
JS 010	Strain RS 242	3 mm diameter, irregular, hazy with clear areas at edge.
JS 011	Strain RS 239	0.7 mm diameter, circular, slightly hazy.
JS 012	Strain RS 239	0.5 - 2 mm diameter, irregular, clear.
JS 013	B. stearo. NCA 1503	0.75 - 1 mm diameter, circular, hazy.
JS 014	B. stearo. NCA 1503	0.5 - 1 mm diameter, circular, slightly hazy.
JS 015	B. stearo. NCA 1503	0.5 - 2 mm diameter, circular, slightly hazy.
JS 017	B. caldotenax	1 - 1.5 mm diameter, circular, turbid/hazy, centre.
JS 018	B. caldotenax	1 mm diameter, circular, clear centre.
JS 019	B. caldotenax	1 - 1.5 mm diameter, circular, clear centre, hazy at the edges.
JS 020	B. caldotenax	1 - 2 mm diameter, circular, clear centre, hazy outer halo 4 - 8 mm.
JS 021	B. caldotenax	2 - 3 mm diameter, clear centre, hazy outer halo.
JS 022	B. caldotenax	
JS 023	B. caldotenax	0.5 - 1.5 mm circular, clear centre, hazy edge.
JS 024	B. caldotenax	0.5 - 2 mm circular, clear centre, hazy edge with less hazy outer edge.
JS 025	B. caldotenax	1 - 1.5 mm circular, hazy.
JS 026	B. caldotenax	1 - 1.5 mm circular, hazy.
JS 027	RS 243	0.75 mm circular, clear.

Legend

Phages were plated in trypticase soft agar supplemented with 0.015 M $CaCl_2$ and overlayered on TSBA plates. Plates were incubated overnight at 55 ^{o}C.

Table 5.8

The effect of $CaCl_2$ and temperature on the plaque morphology and p.f.u. of phage JS 004

Temperature		$CaCl_2$ 0.01 M	$CaCl_2$ 0.015 M	$CaCl_2$ 0.025 M
55 °C	Phage titre	3.9×10^8	2.0×10^8	3.0×10^8
	Plaque diameter	1 - 3 mm	0.5 - 2 mm	0.5 - 4 mm
	Plaque morphology	clear with hazy outer area	clear, some with colonies at centre	hazy, some with colonies at centre
60 °C	Phage titre	2.2×10^8	1.9×10^8	1.0×10^8
	Plaque diameter	1 - 3 mm	0.5 - 2 mm	0.5 - 2.5 mm
	Plaque morphology	-	clear, some with colonies at centre	hazy
65 °C	Phage titre	3.0×10^6	9.8×10^6	2.5×10^6
	Phage diameter	<1 - 1 mm	<1 - 1 mm	<1 - 1 mm
	Plaque morphology	clear	clear	clear

Legend

Phage dilutions in PMBA were plated using the soft agar double layer technique with strain RS 239. $CaCl_2$ levels in the trypticase soft agar were modified and replicate sets of plates incubated overnight at 50 °C, 60 °C and 70 °C.

Table 5.9

The examination of $CaCl_2$, $MgCl_2$ and $MnCl_2$ on the p.f.u. of phage JS 017

Molarity	$CaCl_2$ p.f.u. ml^{-1}	*$MgCl_2$ p.f.u. ml^{-1}	*$MnCl_2$ p.f.u. ml^{-1}
0	7.4×10^7	1.4×10^8	1.4×10^8
0.001	8.6×10^7		
0.0015		6.8×10^7	2.54×10^8
0.05	1.2×10^8		
0.01	1.16×10^8		
0.015	1.45×10^8	2.29×10^8	1.6×10^8
0.05	1.3×10^8		
0.1	1.3×10^8	2.1×10^7	?
0.5	1.2×10^8	6.0×10^6	?

Legend

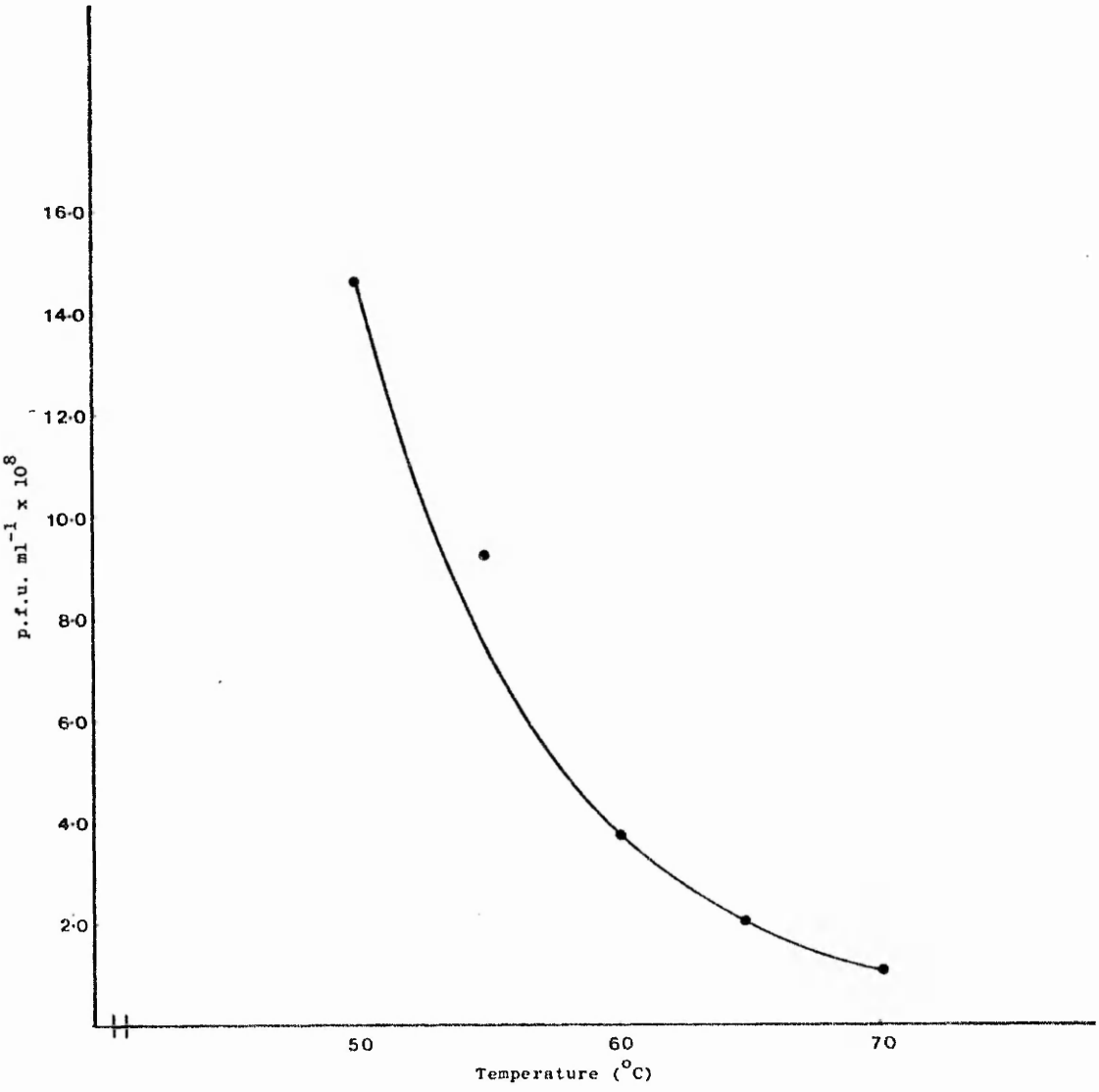
Phage populations were titred using the double layer soft agar technique. The trypticase soft agar was supplemented with various levels of $CaCl_2$, $MgCl_2$ and $MnCl_2$.

?, at this concentration $MnCl_2$ precipitates with the trypticase soft agar.

*The effect of $MgCl_2$ and $MnCl_2$ were examined using the same phage preparations but different to that used for examination of the effect of $CaCl_2$.

Figure 5.5

The effect of temperature on the p.f.u. of phage JS 017

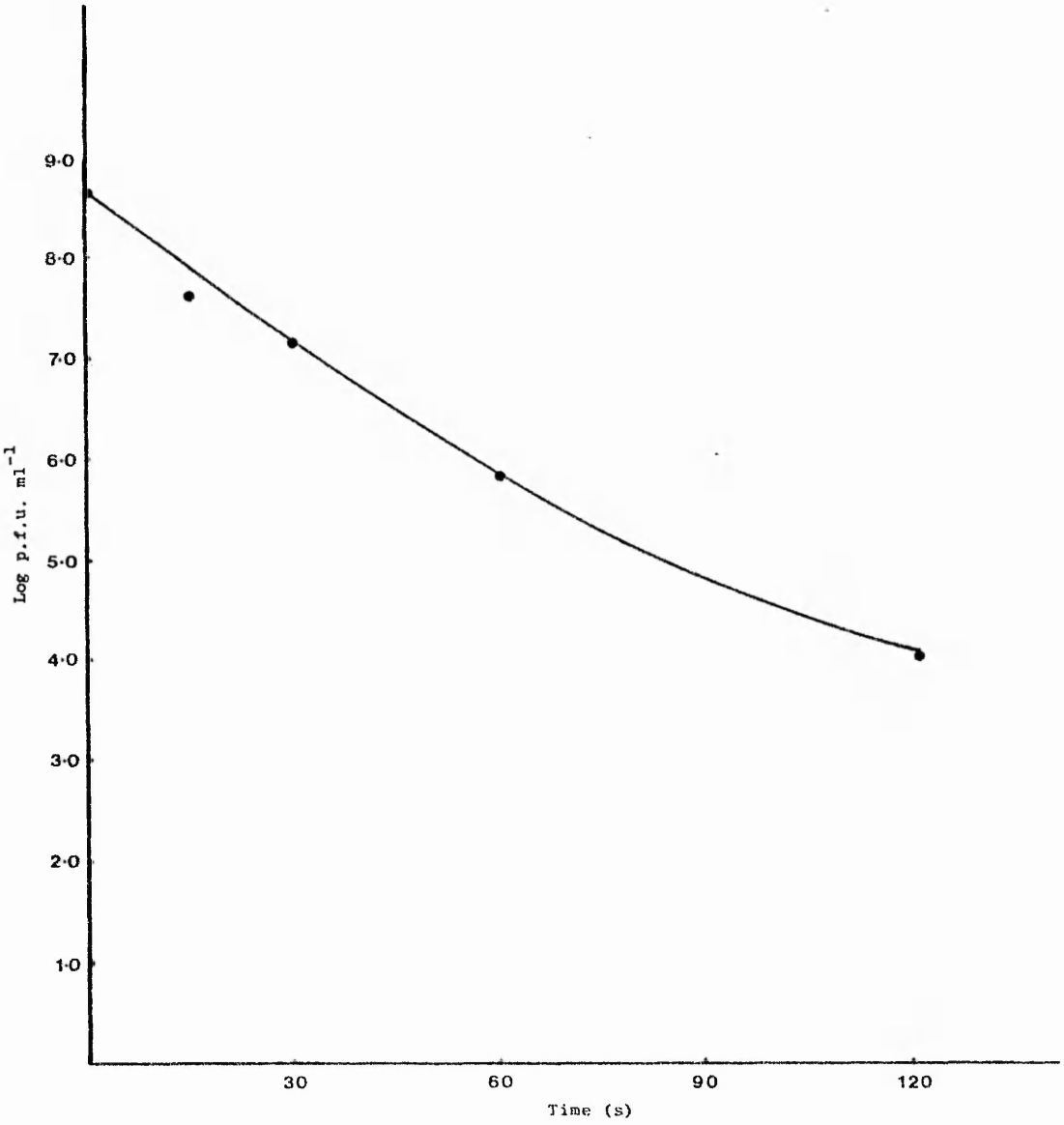


Legend

Bacteriophage dilutions were plated using the soft agar overlayer technique and the plates incubated at a range of temperatures.

Figure 5.6

Sensitivity of phage JS 017 to u.v. irradiation



Legend

A suspension (6×10^8 p.f.u. ml^{-1}) of phage JS 017 was exposed to 6.8 J m^{-2} s^{-1} of u.v. radiation for 2 min. At intervals, samples were assayed for p.f.u.

5.6 THERMAL INACTIVATION OF THERMOPHILIC BACTERIOPHAGES

Thermal inactivation was a useful factor for characterisation of the phages, but it was also necessary to know the thermal stability of phage suspensions when used to investigate possible transduction systems.

The temperature sensitivity of thermophilic bacteriophages held for 4 - 5 h at 50 °C, 60 °C and 70 °C is demonstrated in Fig. 5.11 - 5.16.

After 1 h at 70 °C all the phages examined showed a decrease of 10^3 - 10^6 p.f.u. which then began to plateau for the next 2 - 3 h showing only a small subsequent decrease.

At 60 °C a smaller but significant decrease in p.f.u. was observed within 1 - 2 h which was followed by plateauing of the p.f.u. Phage JS 014 differed, showing little distinction between the rate of inactivation at 60 °C or 50 °C.

Following 4 h at 50 °C most phages showed little decrease in p.f.u. (less than 1 log). Exceptions were JS 024 where the p.f.u. decreased by 99% and JS 025 which decreased by 99.9%.

5.7 INFECTION KINETICS OF BACTERIOPHAGES

Bacteriophages JS 001, JS 002 and JS 003, isolated by lysogenic induction, had similar phage plaque morphology and host range to phage TP 1C. Their infection kinetics were examined and compared with TP 1C (Fig. 5.14 - 5.17).

Adsorption of TP 1C to B. caldotenax appeared to occur between 15 min and 30 min after the addition of phage to the culture. The eclipse period (the time before viable phage progeny were identified within the cell) was between 30 - 40 min and the latent period (the time before viable phage progeny were released into the culture following cell lysis) between 75 - 90 min (Fig. 5.14).

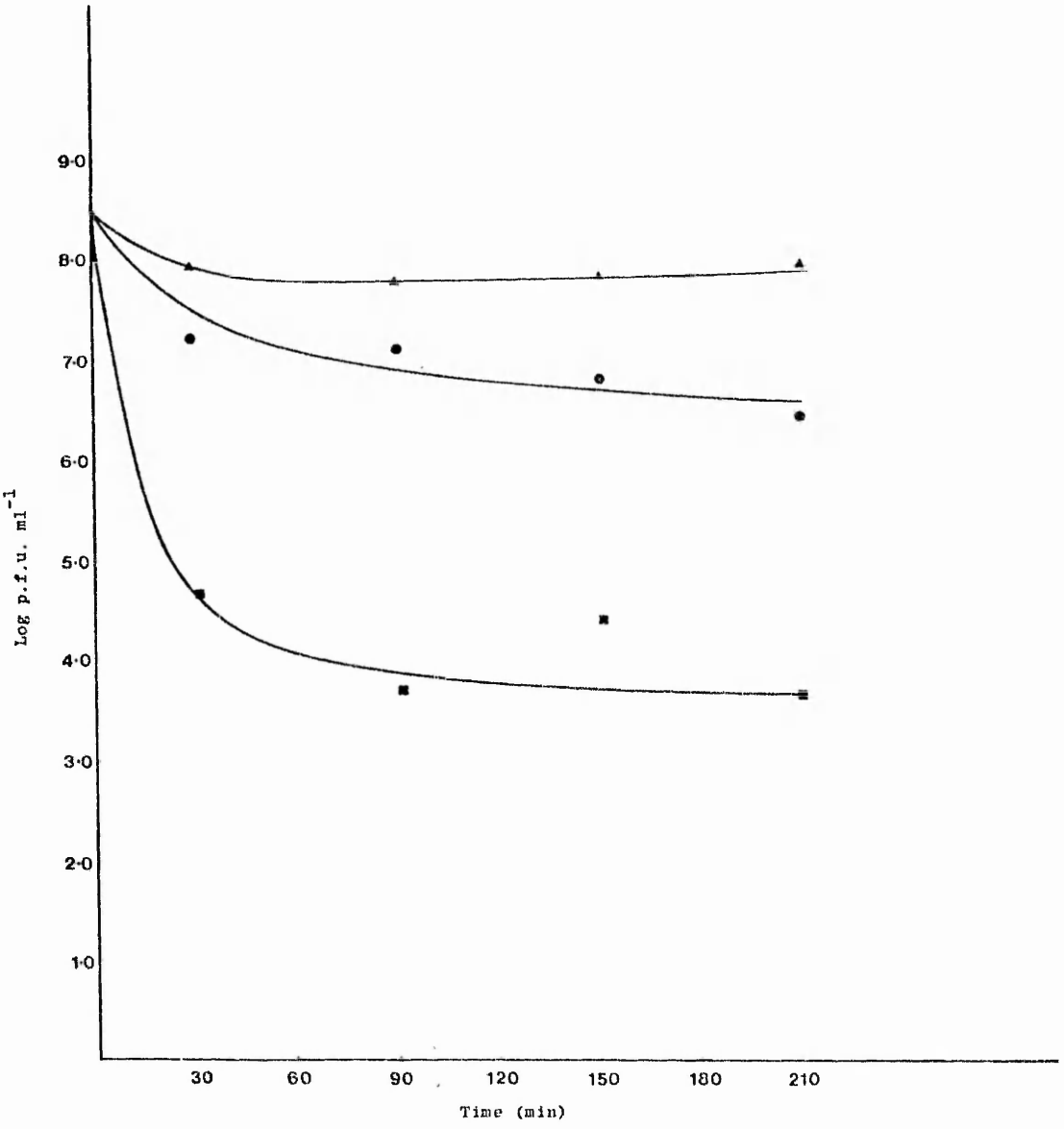
Phages JS 001, JS 002 and JS 003, unlike TP 1C did not show the initial rapid fall in free phage following infection of the culture. Phage JS 001 had an eclipse period between 15 - 30 min with a latent period between 60 - 75 min.

Phage JS 002 had an eclipse period between 15 - 30 min and a latent period between 45 - 60 min. The phage appeared to go through three cycles of infection.

Phage JS 003 had an eclipse period between 30 - 45 min and a latent period of between 60 - 75 min.

Figure 5.7

Thermal inactivation of phage TP 1C

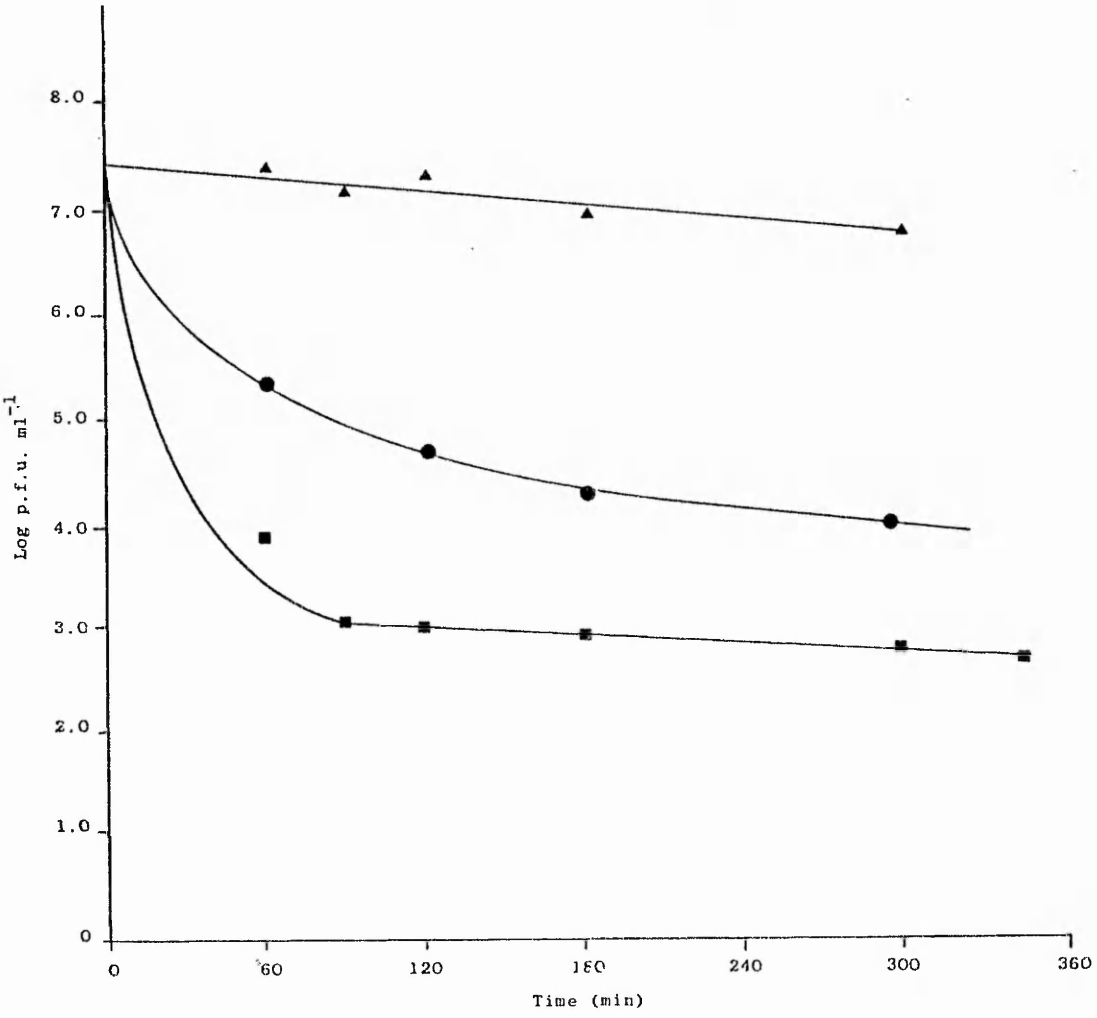


Legend

Phage suspensions in PMN buffer were adjusted to 10^7 - 10^8 p.f.u. ml^{-1}. Aliquots (3 ml) were held at 50 °C, 60 °C and 70 °C and titred at intervals up to 4.5 h. ▲, 50 °C; ●, 60 °C; ■, 70 °C.

Figure 5.8

Thermal inactivation of phage TP 84

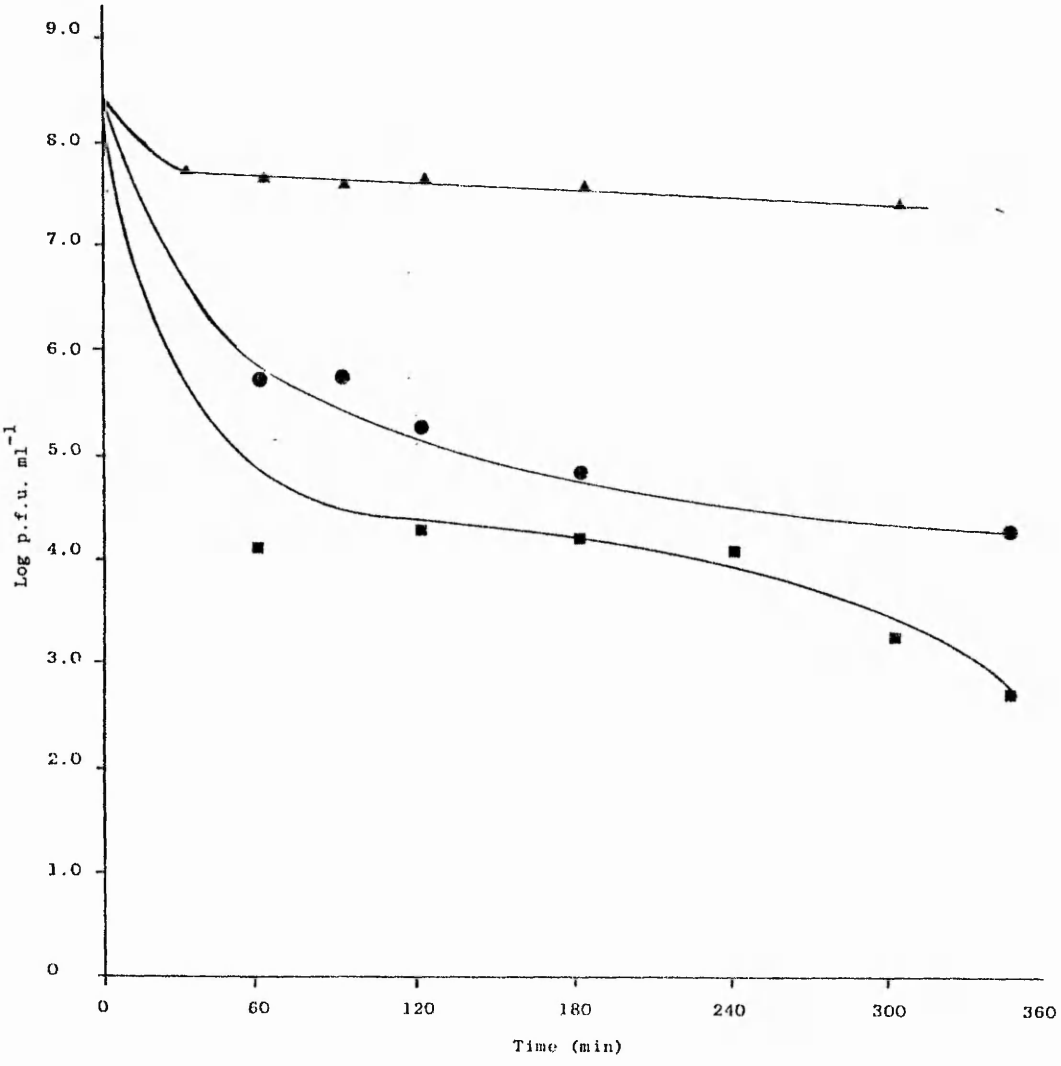


Legend

Phage suspensions in PMN buffer were adjusted to 10^7 - 10^8 p.f.u. ml^{-1}. Aliquots (3 ml) were held at 50 °C, 60 °C and 70 °C and titred at intervals up to 4.5 h. ▲, 50 °C; ●, 60 °C; ■, 70 °C.

Figure 5.9

Thermal inactivation of phage JS 002

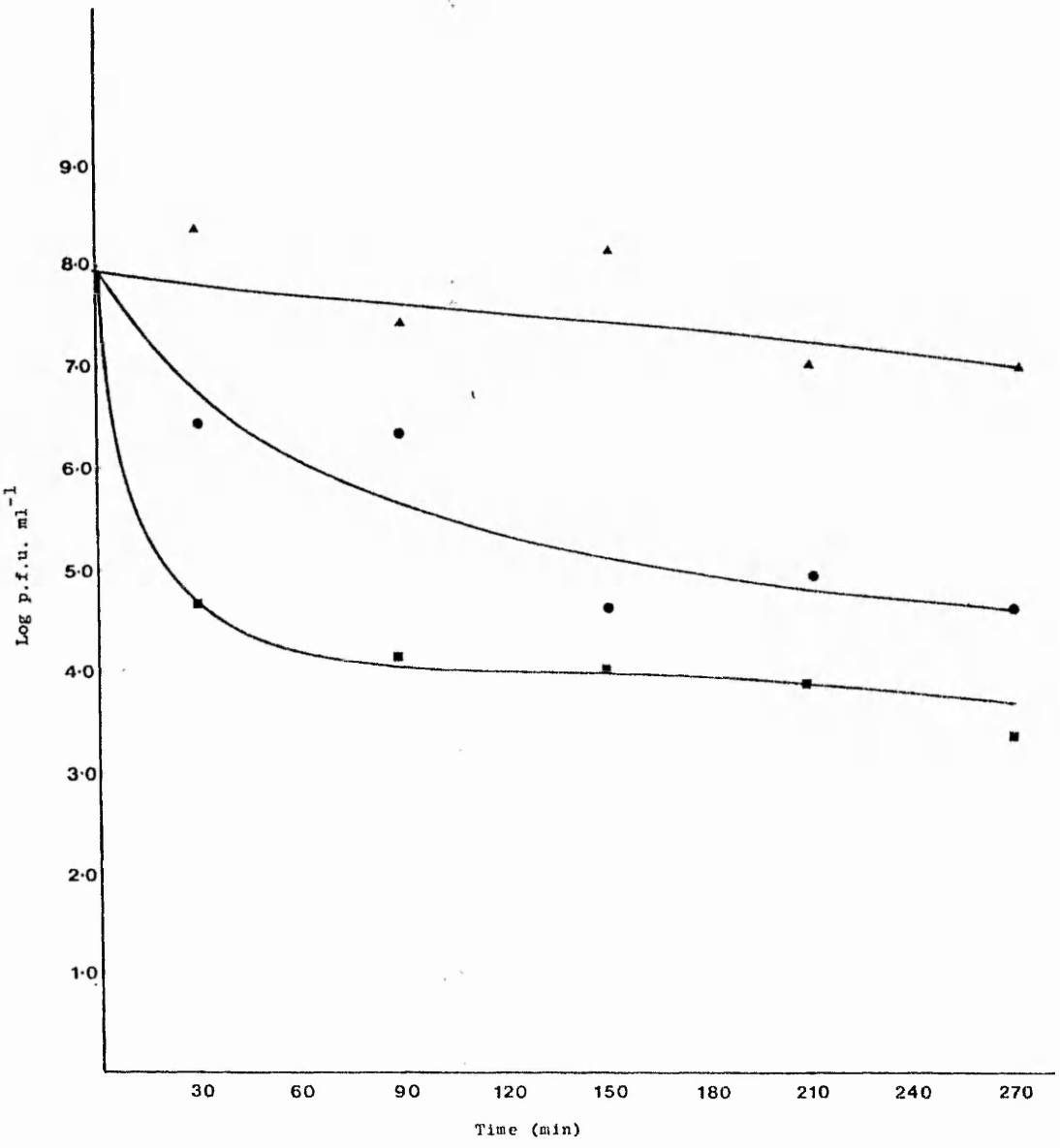


Legend

Phage suspensions in PMN buffer were adjusted to 10^7 - 10^8 p.f.u. ml^{-1}. Aliquots (3 ml) were held at 50 °C, 60 °C and 70 °C and titred at intervals up to 4.5 h. ▲, 50 °C; ●, 60 °C; ■, 70 °C.

Figure 5.10

Thermal inactivation of phage JS 005

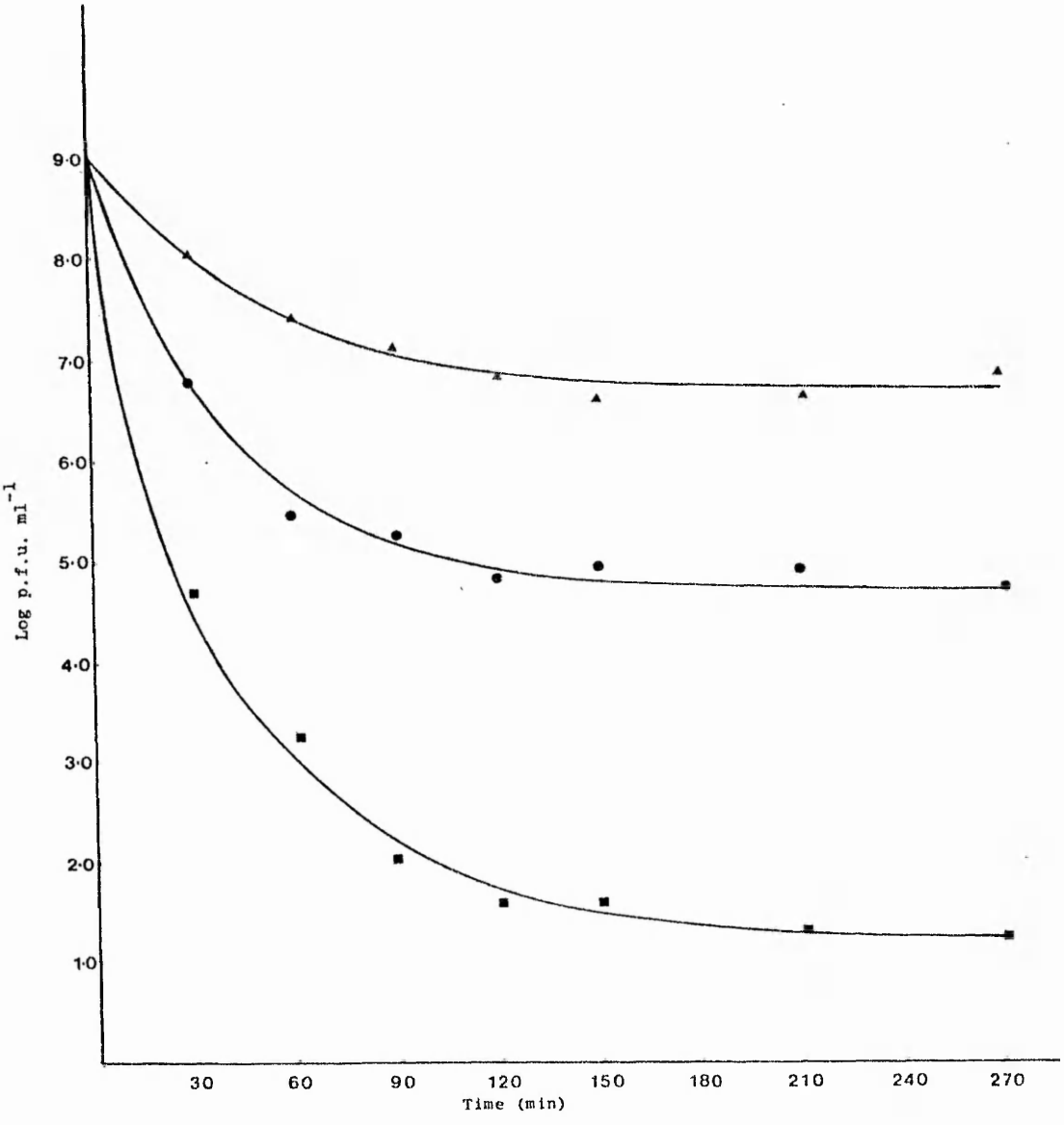


Legend

Phage suspensions in PMN buffer were adjusted to 10^7 - 10^8 p.f.u. ml^{-1}. Aliquots (3 ml) were held at 50 °C, 60 °C and 70 °C and titred at intervals up to 4.5 h. ▲, 50 °C; ●, 60 °C; ■, 70 °C.

Figure 5.11

Thermal inactivation of phage JS 007

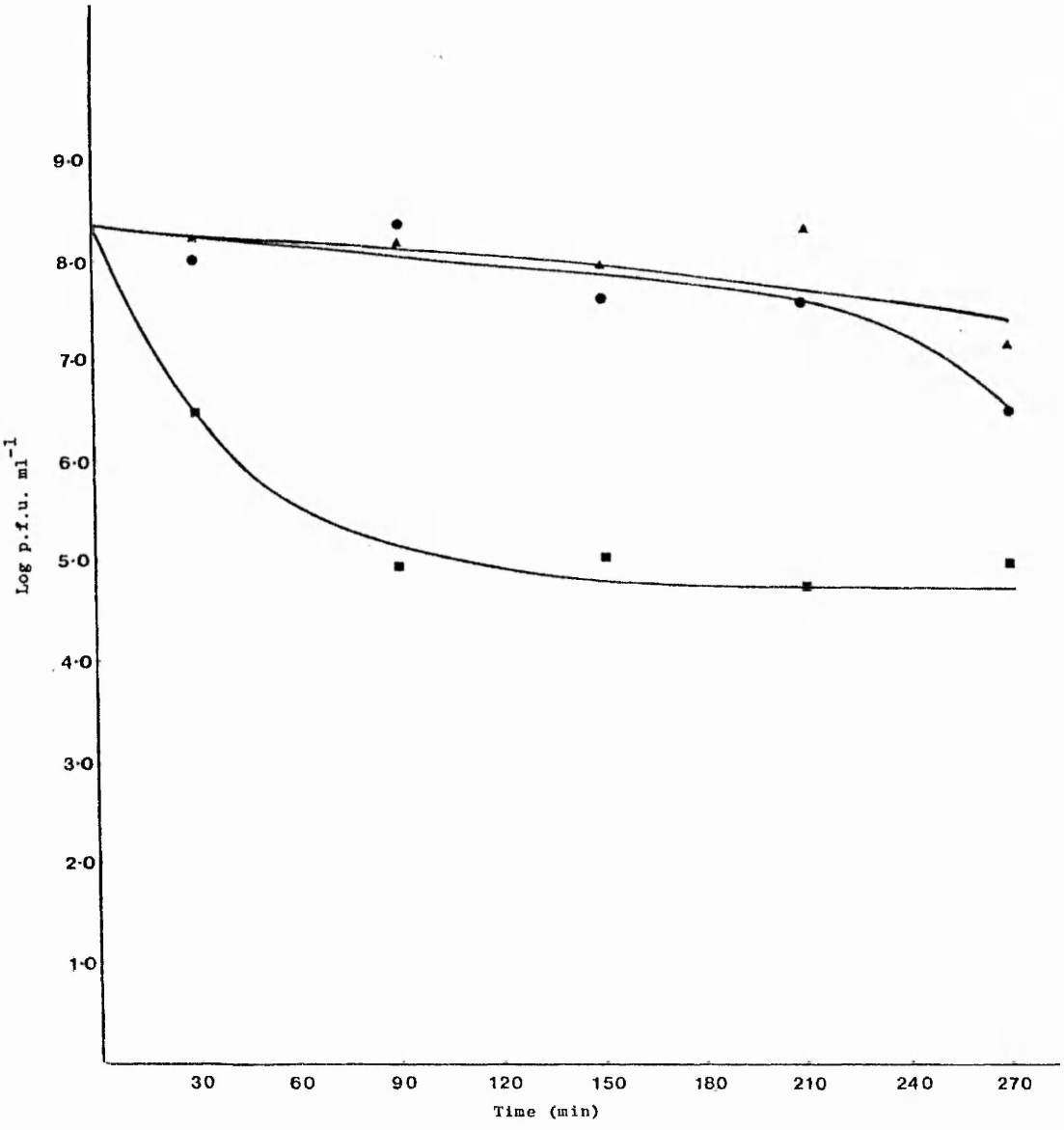


Legend

Phage suspensions in PMN buffer were adjusted to 10^7 - 10^8 p.f.u. ml^{-1}. Aliquots (3 ml) were held at 50 °C, 60 °C and 70 °C and titred at intervals up to 4.5 h. ▲, 50 °C; ●, 60 °C; ■, 70 °C.

Figure 5.12

Thermal inactivation of phage JS 014

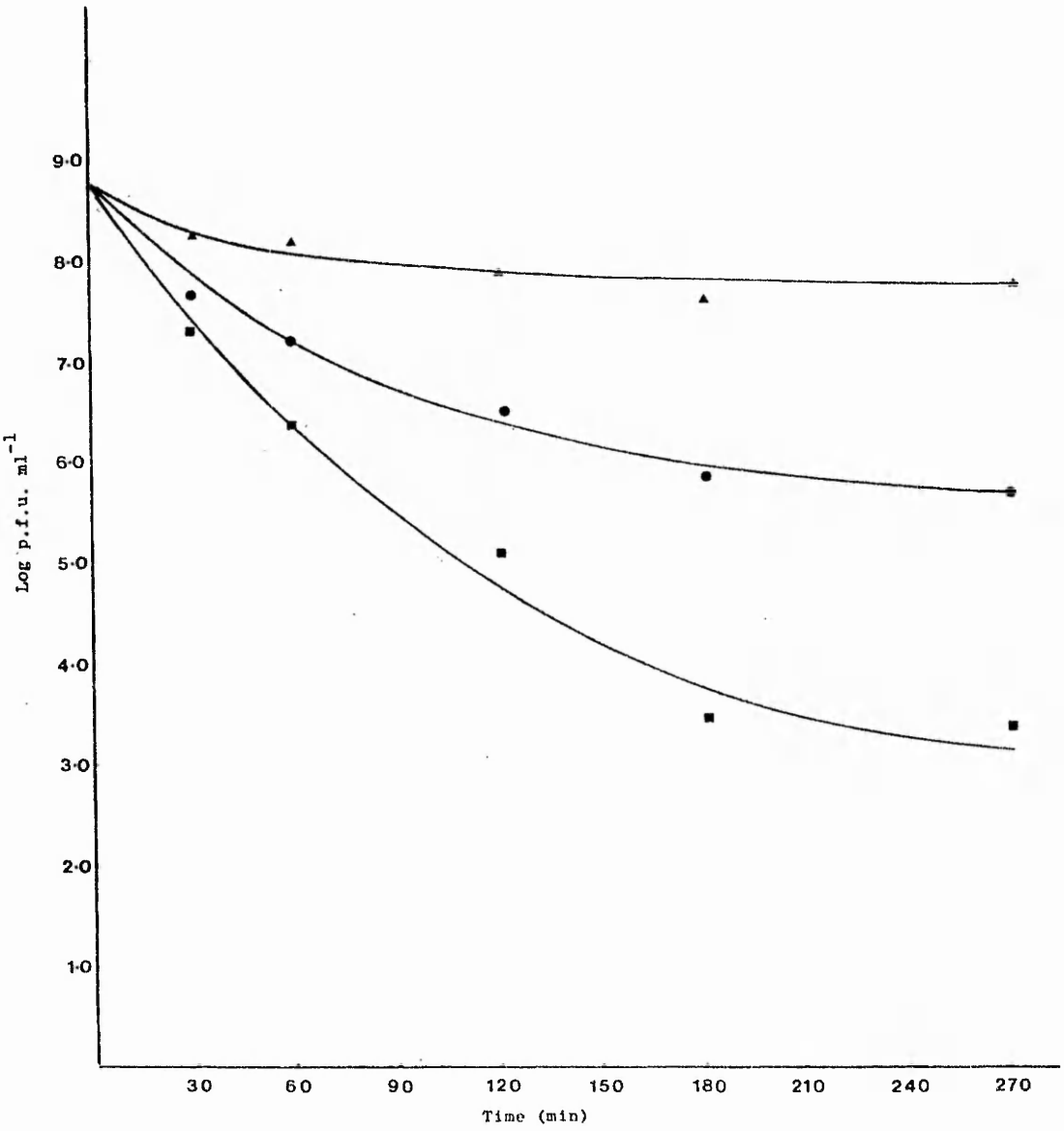


Legend

Phage suspensions in PMN buffer were adjusted to 10^7 - 10^8 p.f.u. ml^{-1}. Aliquots (3 ml) were held at 50 °C, 60 °C and 70 °C and titred at intervals up to 4.5 h. ▲, 50 °C; ●, 60 °C; ■, 70 °C.

Figure 5.13

Thermal inactivation of phage JS 017

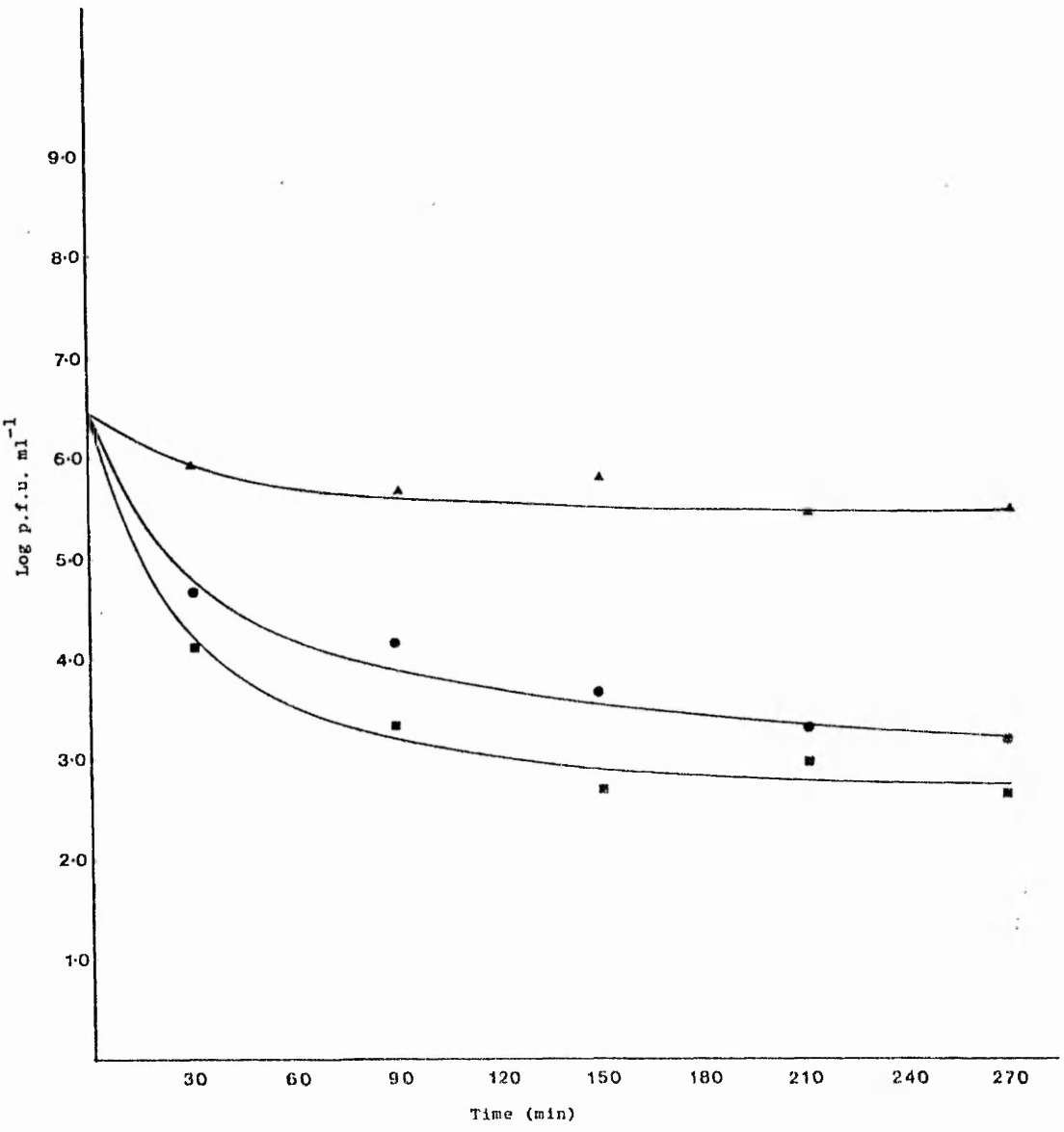


Legend

Phage suspensions in PMN buffer were adjusted to 10^7 - 10^8 p.f.u. ml^{-1}. Aliquots (3 ml) were held at 50 °C, 60 °C and 70 °C and titred at intervals up to 4.5 h. ▲, 50 °C; ●, 60 °C; ■, 70 °C.

Figure 5.14

Thermal inactivation of phage JS 019

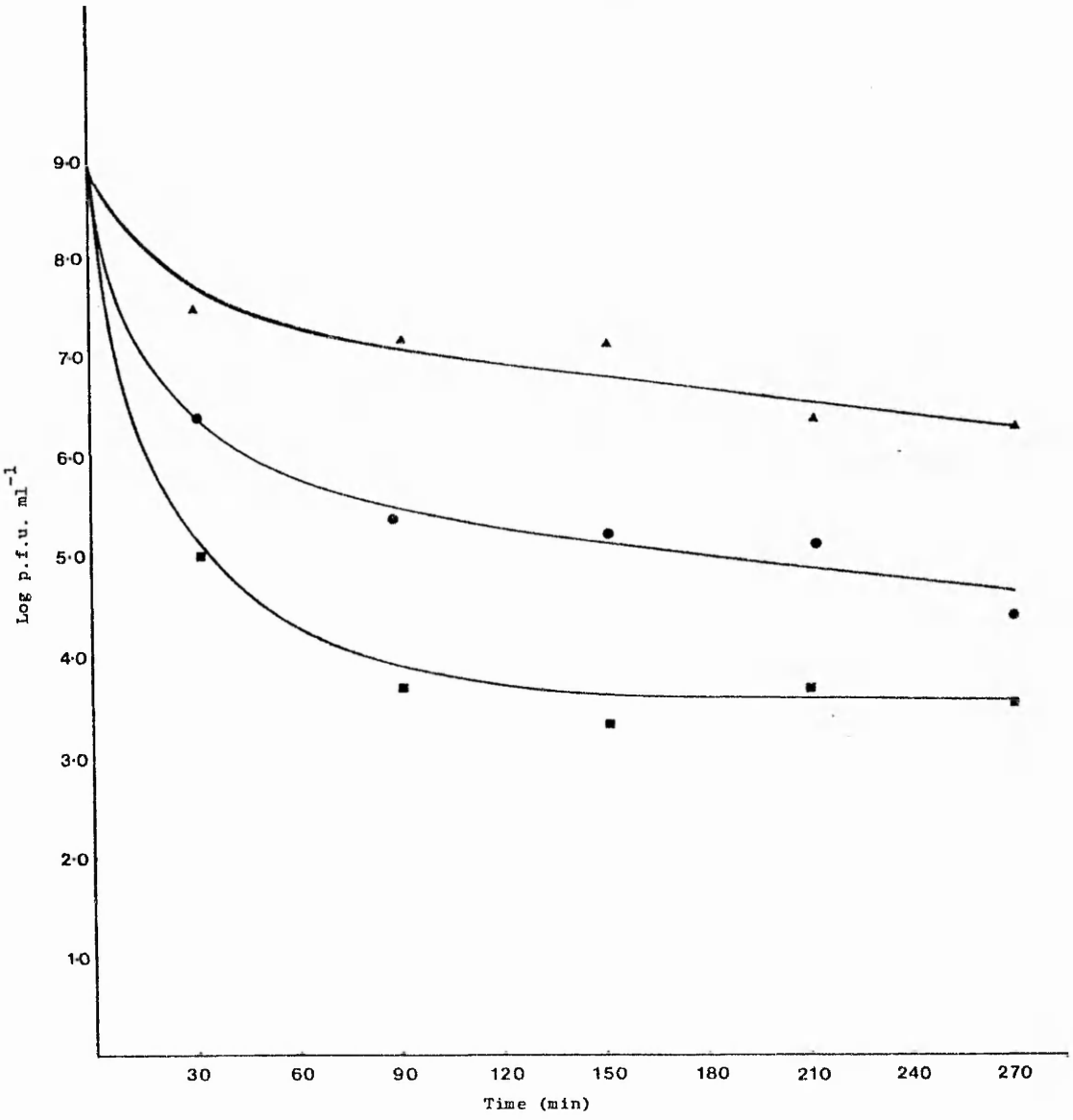


Legend

Phage suspensions in PMN buffer were adjusted to 10^7 - 10^8 p.f.u. ml^{-1}. Aliquots (3 ml) were held at 50 °C, 60 °C and 70 °C and titred at intervals up to 4.5 h. ▲, 50 °C; ●, 60 °C; ■, 70 °C.

Figure 5.15

Thermal inactivation of phage JS 024

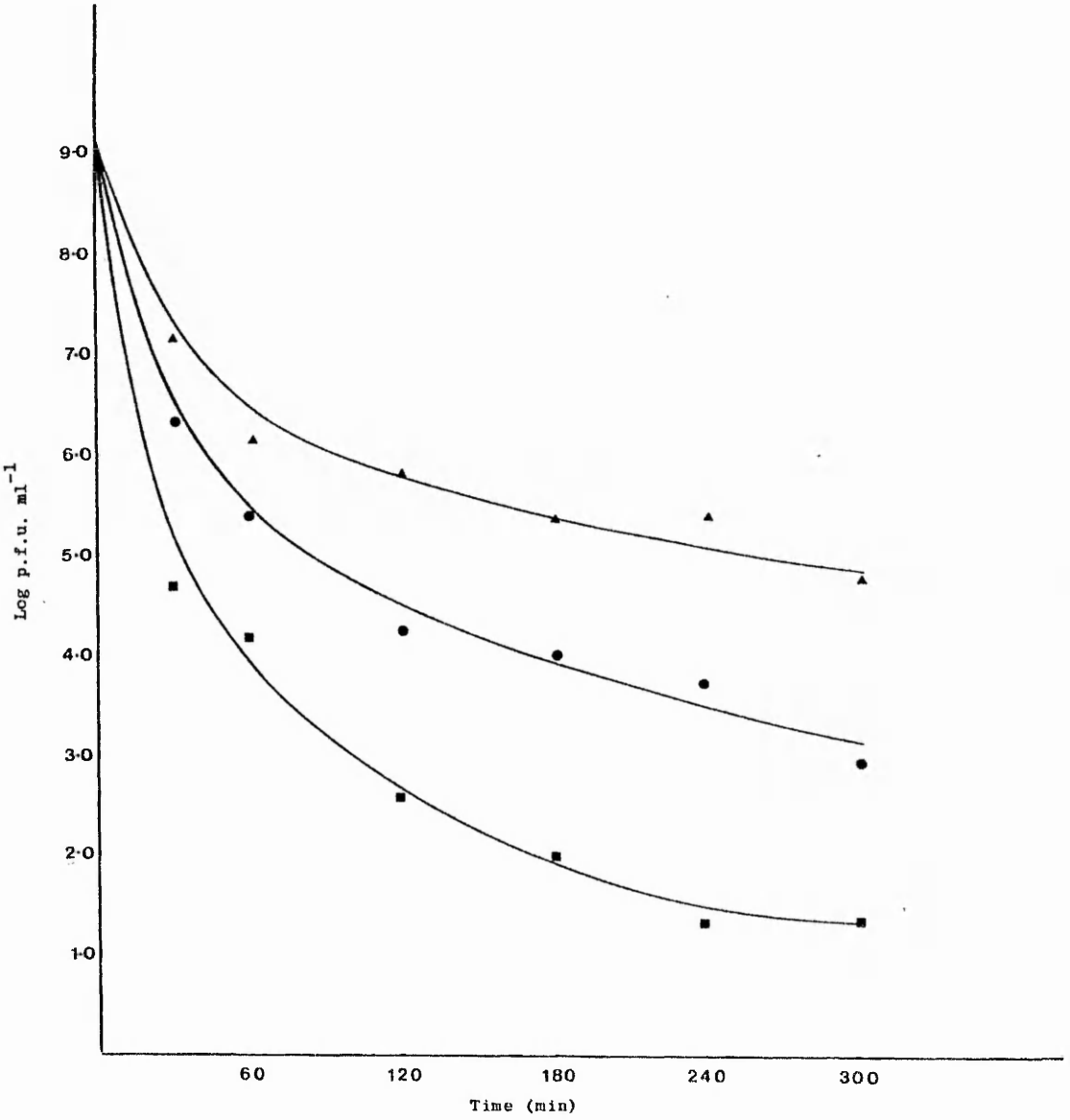


Legend

Phage suspensions in PMN buffer were adjusted to 10^7 - 10^8 p.f.u. ml^{-1}. Aliquots (3 ml) were held at 50 °C, 60 °C and 70 °C and titred at intervals up to 4.5 h. ▲, 50 °C; ●, 60 °C; ■, 70 °C.

Figure 5.16

Thermal inactivation of phage JS 025

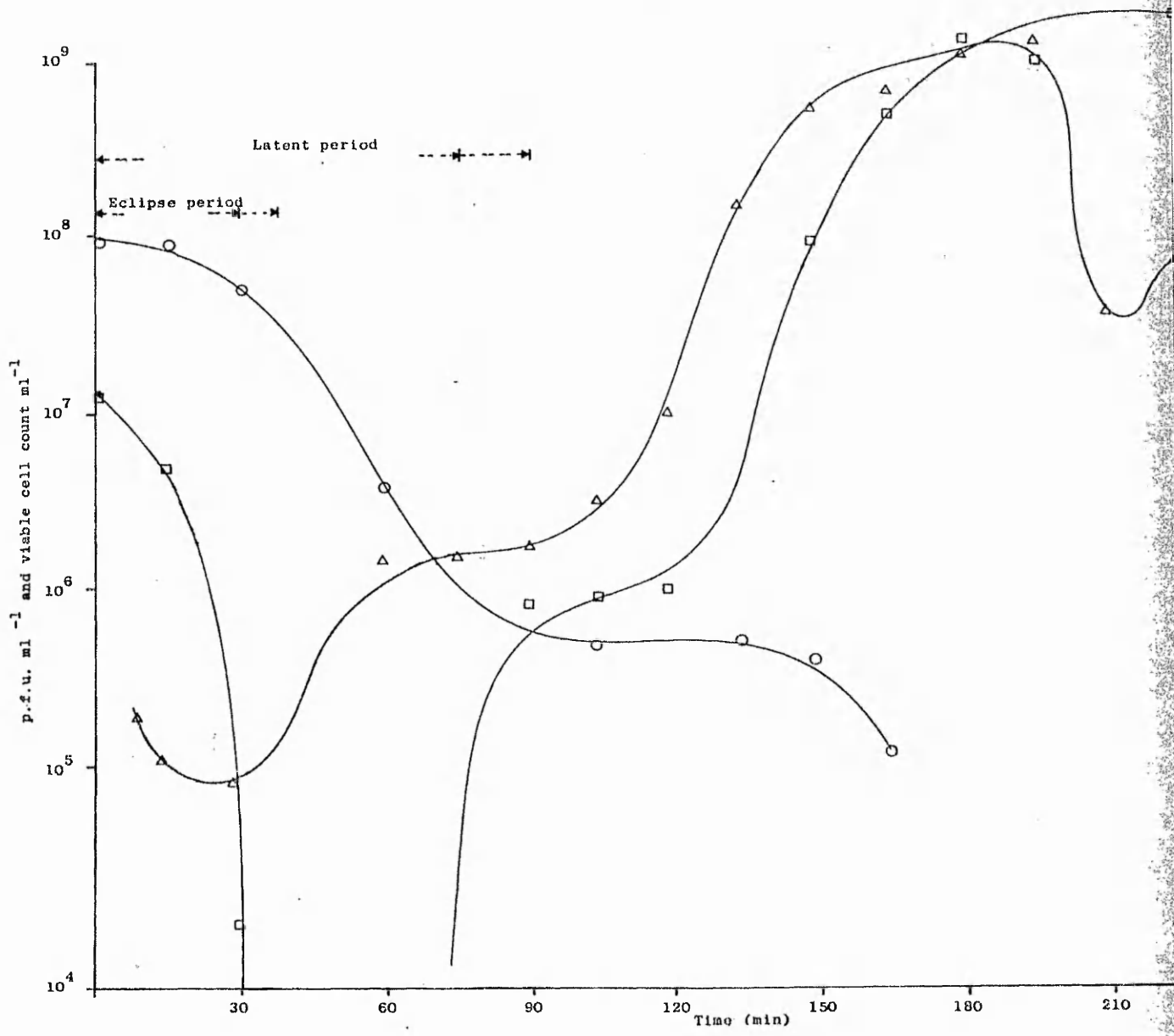


Legend

Phage suspensions in PMN buffer were adjusted to 10^7 - 10^8 p.f.u. ml^{-1}. Aliquots (3 ml) were held at 50 °C, 60 °C and 70 °C and titred at intervals up to 4.5 h. ▲, 50 °C; ●, 60 °C; ■, 70 °C.

Figure 5.17

The kinetics of infection of B. caldotenax with bacteriophage TP 1C

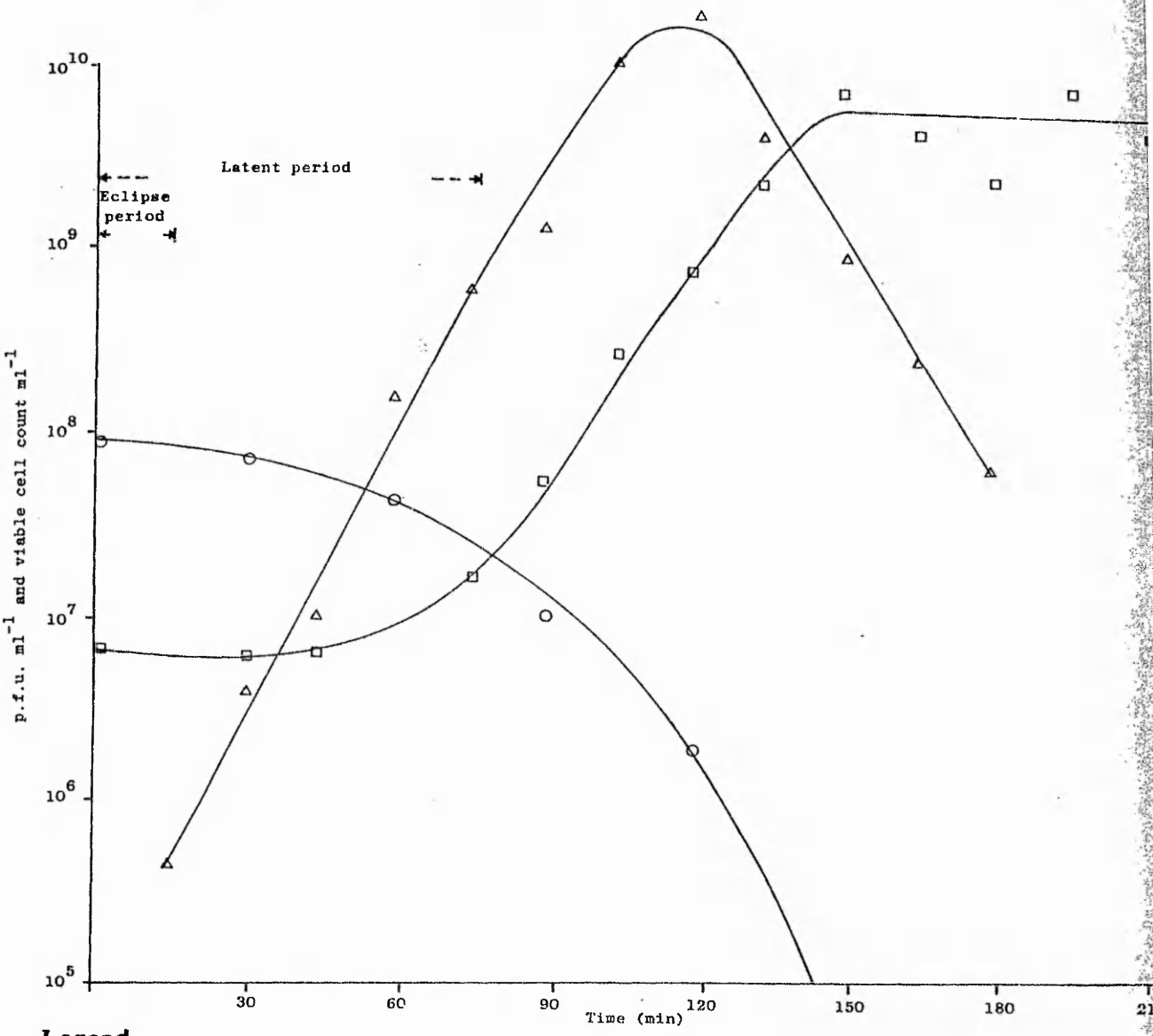


Legend

A culture of B. caldotenax grown at 55 °C in TSB medium was infected with bacteriophage at an m.o.i. of 0.1. The eclipse and latent periods were recorded. ○, viable cells; □, free phage in supernatant; △, viable phage particles released by premature lysis with sonication were monitored.

Figure 5.18

The kinetics of infection of B. caldotenax with bacteriophage JS 001

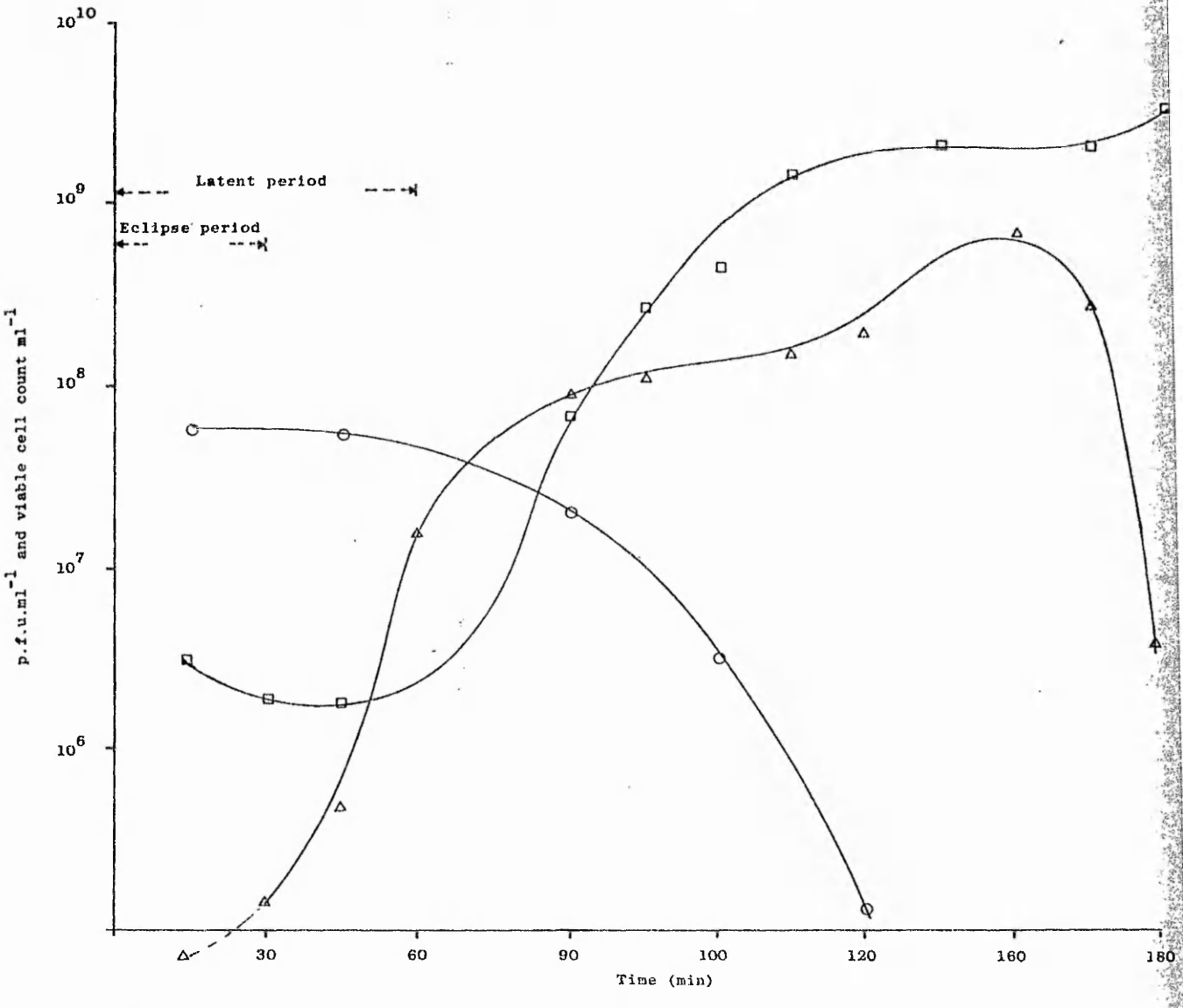


Legend

A culture of B. caldotenax grown at 55 ^{o}C in TSB medium was infected with bacteriophage at an m.o.i. of 0.1. The eclipse and latent periods were recorded. ○, viable cells; □, free phage in supernatant; △, viable phage particles released by premature lysis with sonication were monitored.

Figure 5.19

The kinetics of infection of B. caldotenax with bacteriophage JS 002

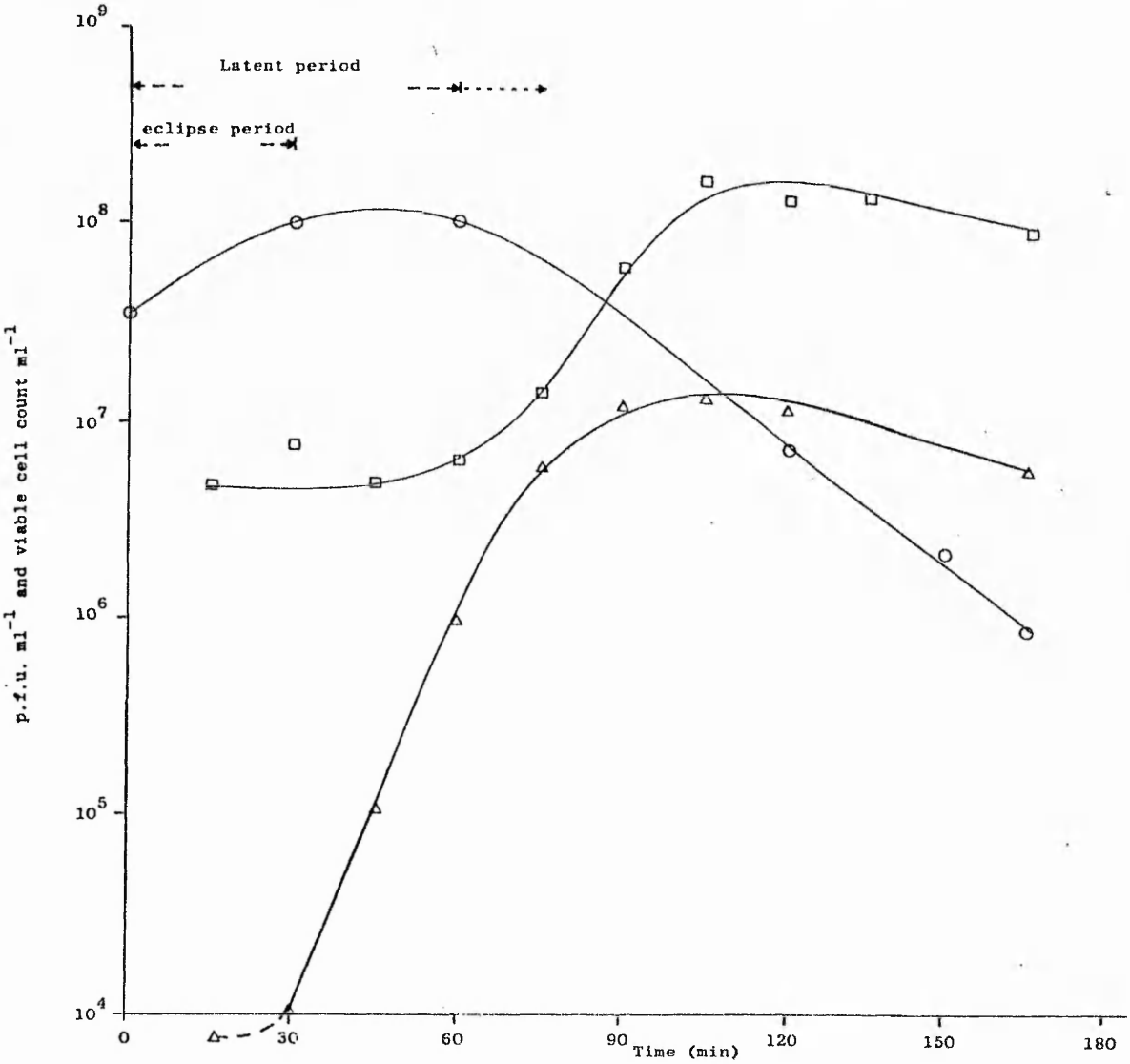


Legend

A culture of B. caldotenax grown at 55 °C in TSB medium was infected with bacteriophage at an m.o.i. of 0.1. The eclipse and latent periods were recorded. ○, viable cells; □, free phage in supernatant; △, viable phage particles released by premature lysis with sonication were monitored.

Figure 5.20

The kinetics of infection of B. caldotenax with bacteriophage JS 003



Legend

A culture of B. caldotenax grown at 55 °C in TSB medium was infected with bacteriophage at an m.o.i. of 0.1. The eclipse and latent periods were recorded. ○ , viable cells; □ , free phage in supernatant; △ , viable phage particles released by premature lysis with sonication were monitored.

Table 5.10

Determination of buoyant density of bacteriophages using calibrated percoll gradients

Bacteriophages	Buoyant density of high p.f.u. band	Buoyant density of low p.f.u. band where present
λ	1.04 - 1.055	-
T 7	1.025 - 1.05	-
TP 1C	1.1	1.035 - 1.045
TP 84	1.075	-
JS 002	1.08	1.05
JS 005	1.04	-
JS 017	1.0, 1.02, 1.025, 1.035	-
JS 019	1.045	-
JS 022	1.065	1.04
JS 025	1.02	1.045

Legend

Percoll gradients were calibrated using density marker beads (Pharmacia (UK) Ltd.). Where more than one band was apparent in the gradient they were each examined for the presence of high numbers of viable phage particles. Where the band was wide or consisted of several closely spaced bands with high p.f.u. the buoyant density range was recorded.

5.8 BACTERIOPHAGE BUOYANT DENSITY ESTIMATION

Buoyant density estimations using calibrated percoll gradients indicated several of the phages formed two or more bands in the gradients (Table 5.10). The bands were collected using a syringe and examined for the presence of viable bacteriophages. The buoyant density values correspond to either single phage bands or bands with a high p.f.u. The bands with a low p.f.u. were considered to contain phage debris such as heads or tails, or ghost particles. Although none of these bands were examined by electron microscopy, electron micrographs of phage preparations have shown the presence of many ghost phage particles and large numbers of free tails in the preparations.

5.9 SIZE AND MORPHOLOGY OF THERMOPHILIC BACTERIOPHAGES

Data derived from electron micrographs (Plate 5.1 - 5.15) of 13 thermophilic bacteriophages are presented in Table 5.11. The phages exhibited four distinct morphological types.

Phage JS 004, JS 005 and JS 027 were characterised by a comparatively large head, a short stubby tail and no evidence of a tail plate. Phages JS 002, JS 019, JS 022, JS 024 and JS 025 JS 028 all possessed polyhedral phage heads of approximately 60 nm diameter with tails between 130 nm and 250 nm long. Four phages in this group showed evidence of a tail plate.

Phages JS 007 and JS 015 had polyhedral heads, 90 - 100 nm in diameter and long flexible tails between 400 nm and 600 nm long with helical symmetry.

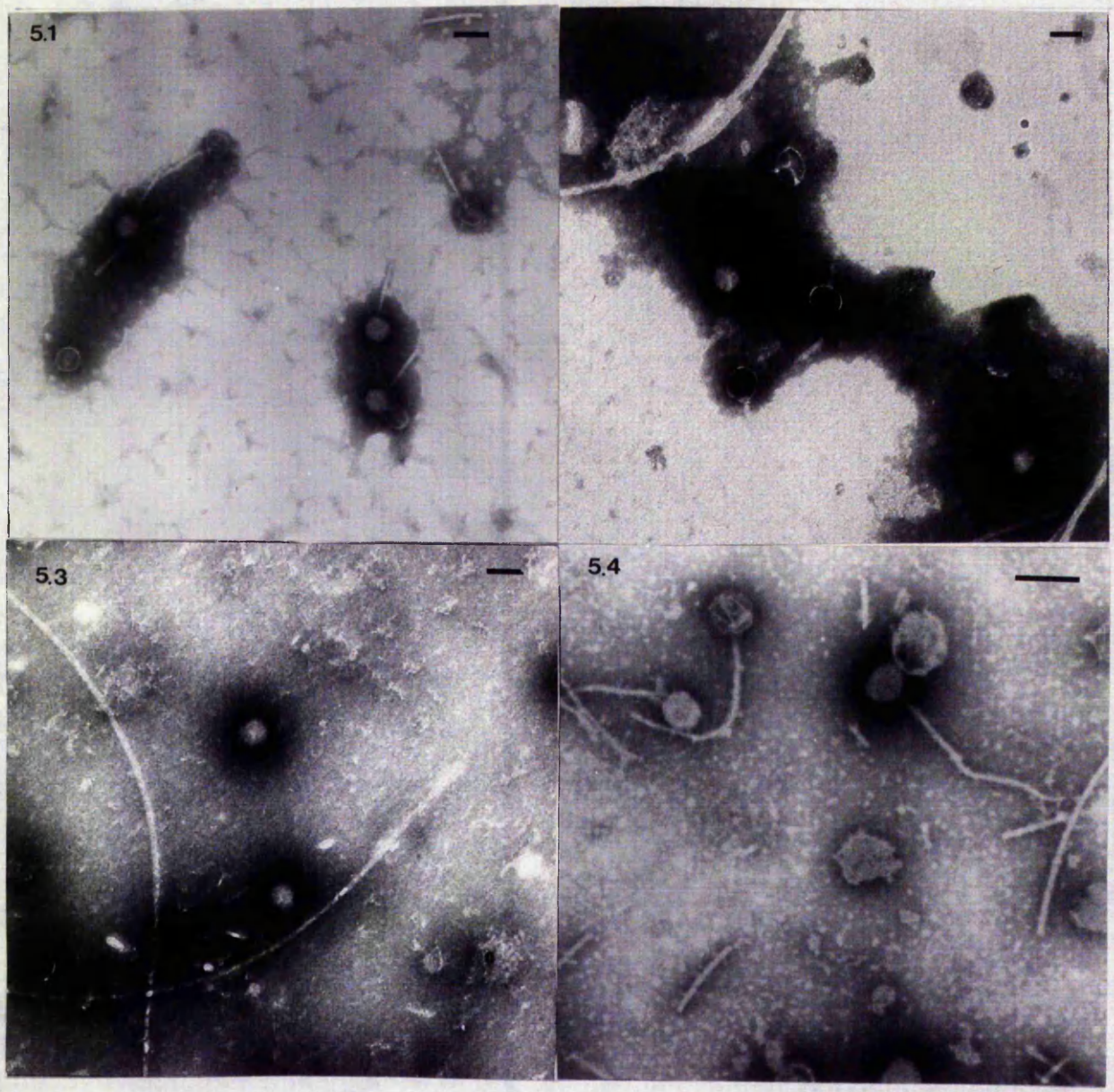
Phages JS 017 and JS 026 were cylindrical with rounded ends with tails varying between 120 nm and 160 nm. Between 1% and 5% of the JS 017 population had phage heads of twice the normal length (Plate 5.13). The occurrence of phage JS 017 with longer tails (160 nm) was also observed (Plate 5.14). The phage head was slightly narrower (40 nm) than the normal head and the tail showed clear evidence of striations or segmentation. Phage hosts of JS 026 and JS 017 showed evidence of being waisted at the centre (Plate 5.15) and some of the larger phage heads of JS 017 appeared to have striations around the head. Both phages appeared to have a V shaped tail plate.

Table 5.11

Size and morphology of thermophilic phages

Phage	Shape of head	Size of head	Size of tail	Evidence of tail plate	Comments
JS 002	Polyhedral	65 - 70 nm diameter	130 - 140 nm	-	-
JS 004	Polyhedral	80 nm diameter	50 - 60 nm	-	Tail appears curved on some
JS 005	Polyhedral	80 nm diameter	50 - 60 nm	-	-
JS 007	Polyhedral	60 - 65 nm diameter	180 nm	-	Flexible tail with helical symmetry
JS 015	Polyhedral	90 nm diameter	400 - 500 nm	-	Flexible tail with helical symmetry
JS 017	Cylindrical	80 - 90 nm x 40 - 50 nm	120 nm	+	Mutant bodies and tails central symmetry
JS 019	Polyhedral	55 - 65 nm diameter	150 nm	+	-
JS 022	Polyhedral	60 nm diameter	160 - 180 nm	+	-
JS 024	Polyhedral	60 nm diameter	160 - 180 nm	+	-
JS 025	Polyhedral	60 nm diameter	140 - 160 nm	+	-
JS 026	Cylindrical	80 - 90 nm x 50 nm	120 nm	+	No evidence of morphological variation
JS 027	Polyhedral	80 nm diameter	15 nm	-	Signs of a very short stubby tail on a few particles
JS 028	Polyhedral	60 - 65 nm diameter	180 nm	-	Straight or curved tail with helical symmetry

Plate 5.1 - 5.4

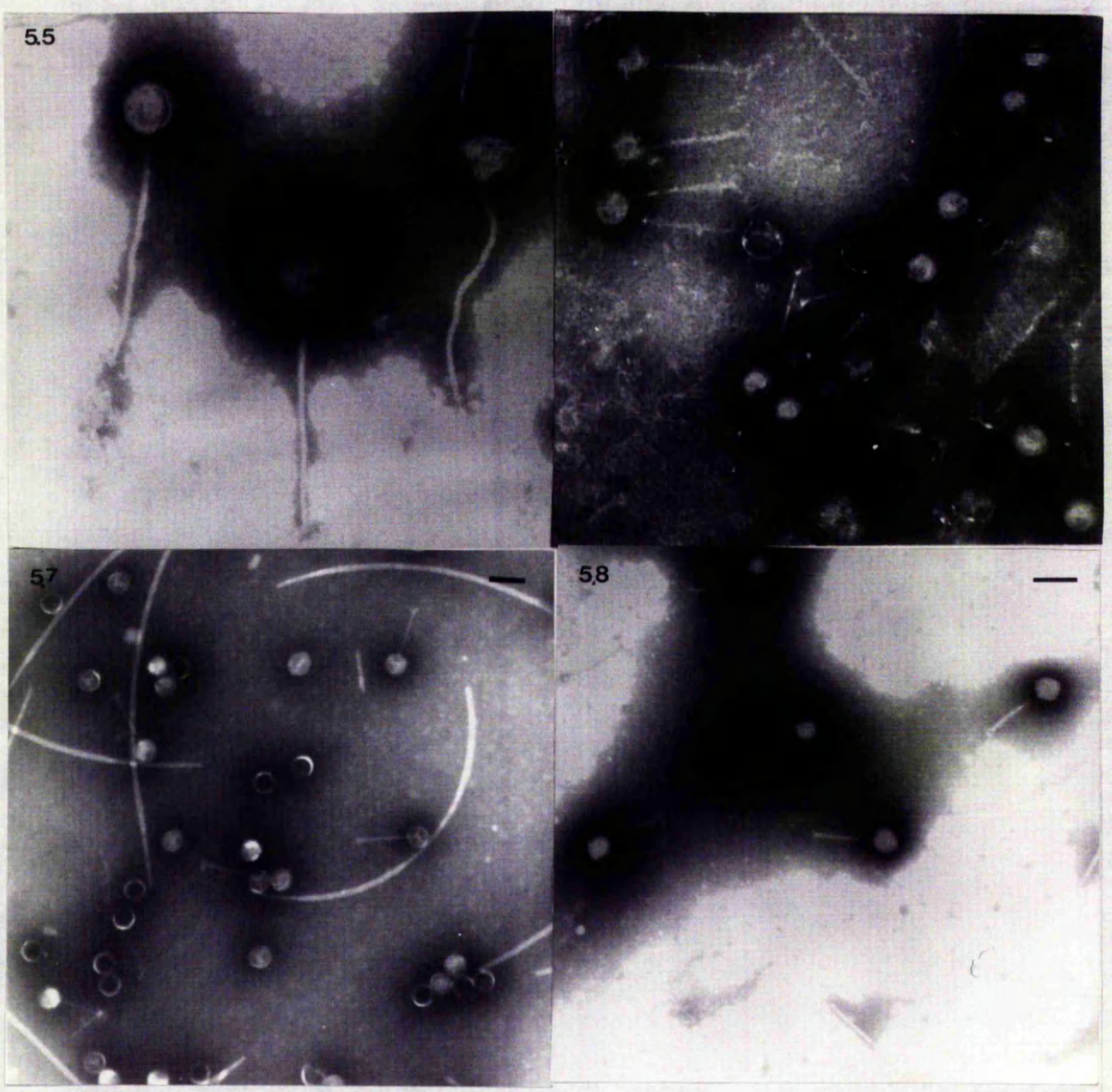


Legend

Phage preparations were examined in a Philips EM 300 or a Philips EM 400T Electron Microscope. Micrographs were recorded on Ilford EM technical film at magnifications x 28,000 to x 46,000 and printed as x 2 or x 3 enlargements. Plate 5.1, Bacteriophage JS 002 x 56,000; Plate 5.2, Bacteriophage JS 004 x 73,000; Plate 5.3, Bacteriophage JS 005 x 73,000; Plate 5.4, Bacteriophage JS 007 x 72,000.

Calibration markers are 100 nm.

Plate 5.5 - 5.8

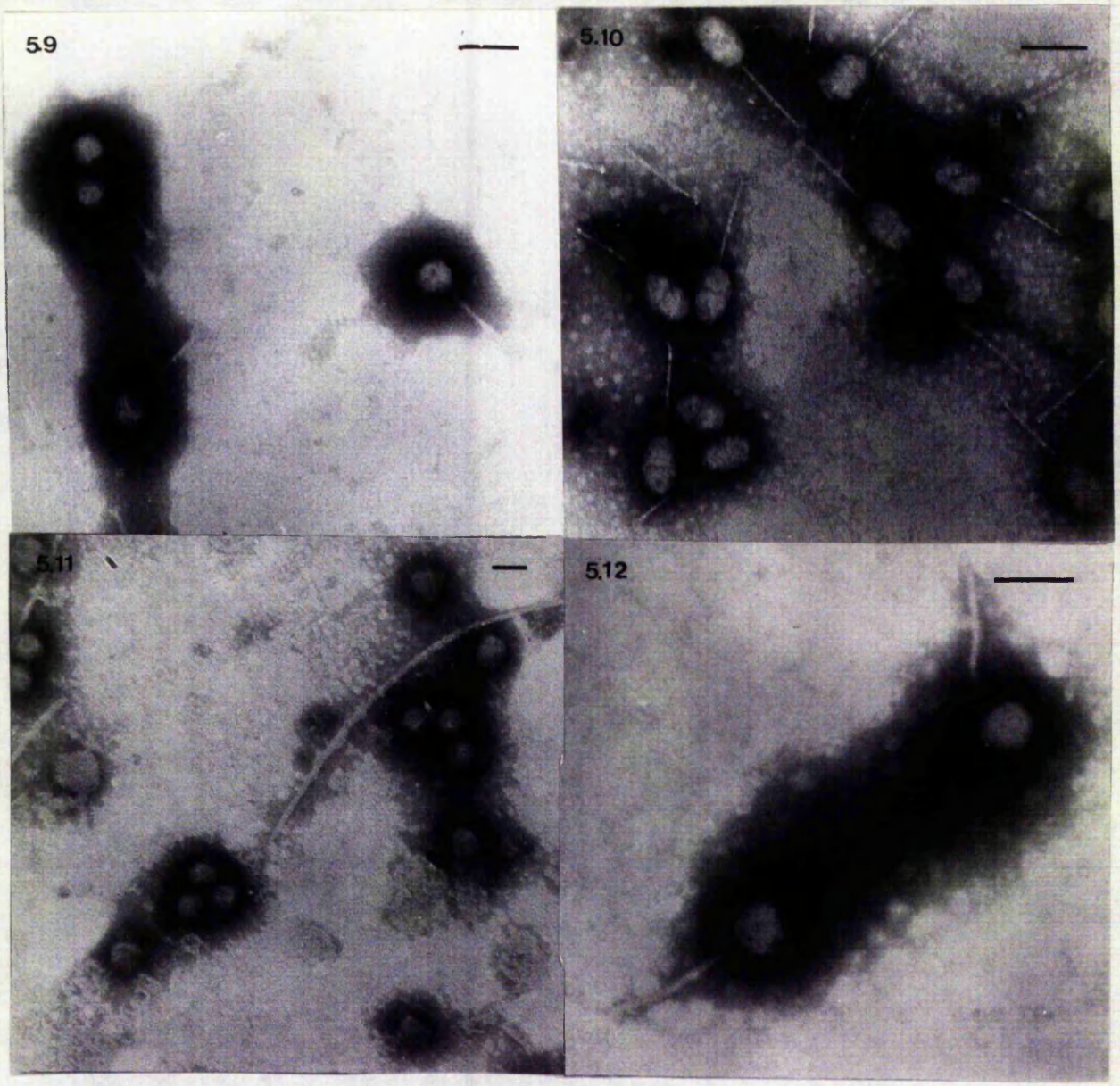


Legend

Phage preparations were examined in a Philips EM 300 or a Philips EM 400T Electron Microscope. Micrographs were recorded on Ilford EM technical film at magnifications x 28,000 to x 46,000 and printed as x 2 or x 3 enlargements. Plate 5.5, Bacteriophage JS 015 x 92,000; Plate 5.6, Bacteriophage JS 019 x 120,000; Plate 5.7, Bacteriophage JS 022 x 56,000; Plate 5.8, Bacteriophage JS 024 x 72,000.

Calibration markers are 100 nm.

Plate 5.9 - 5.12

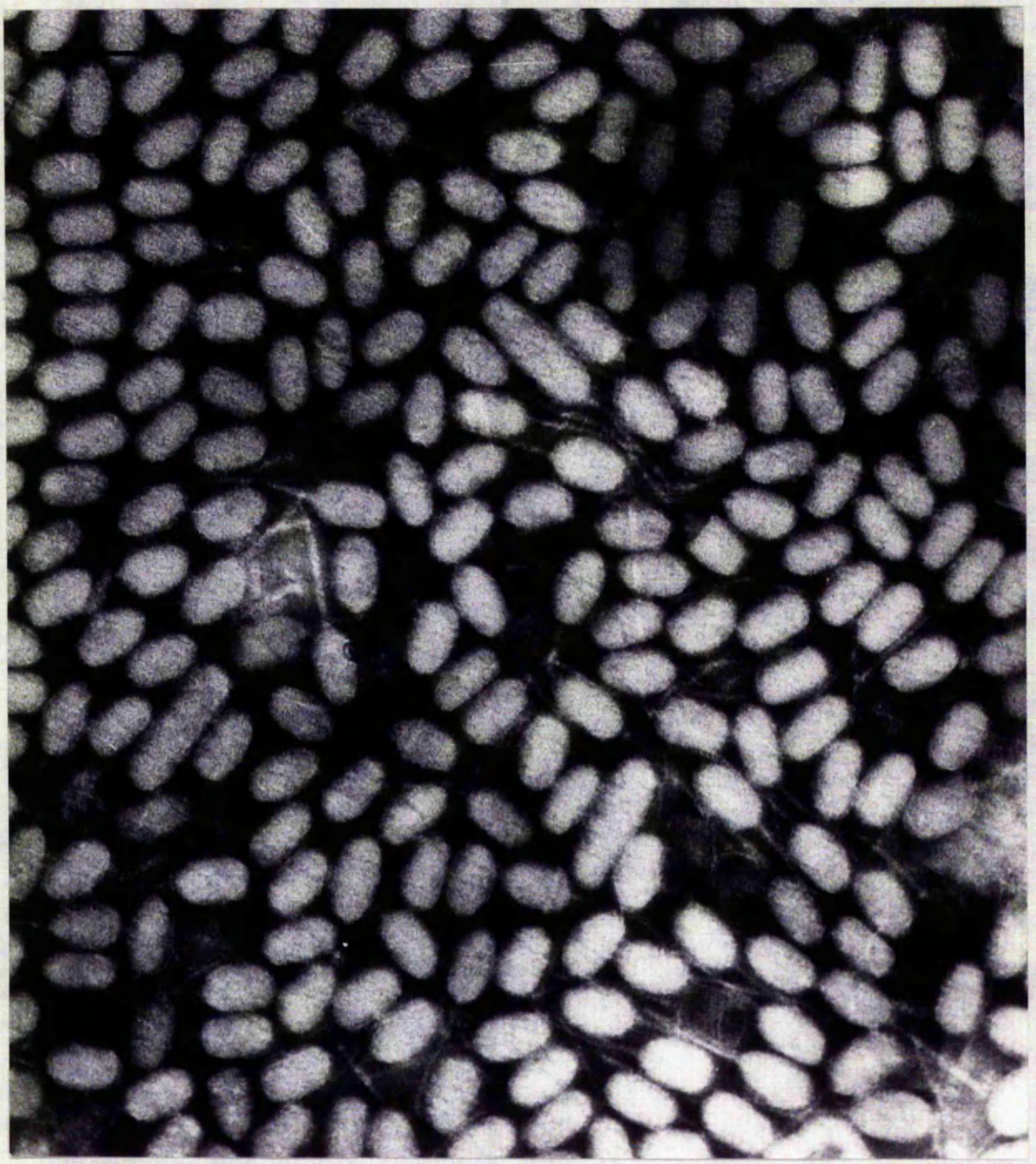


Legend

Phage preparations were examined in a Philips EM 300 or a Philips EM 400T Electron Microscope. Micrographs were recorded on Ilford EM technical film at magnifications x 28,000 to x 46,000 and printed as x 2 or x 3 enlargements. Plate 5.9, Bacteriophage JS 025 x 120,000; Plate 5.10, Bacteriophage JS 026 x 126,000; Plate 5.11, Bacteriophage JS 027 x 73,000; Plate 5.12, Bacteriophage JS 028 x 115,000.

Calibration markers are 100 nm.

Plate 5.13

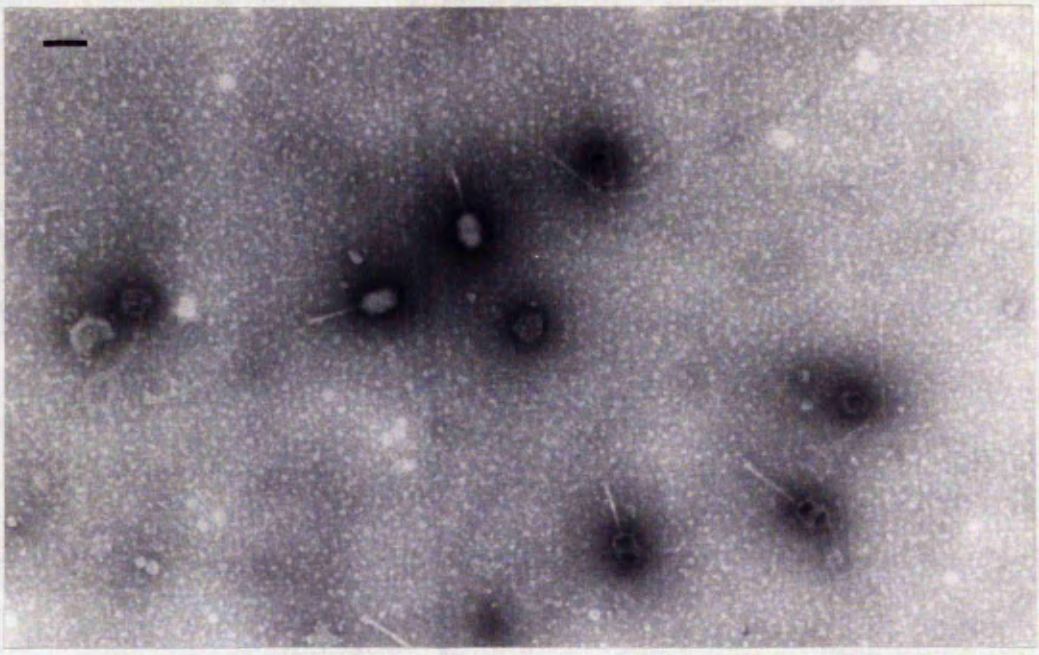


Legend

Bacteriophage JS 017 x 160,000 showing variation in the length of phage heads from 80 - 150 nm.

Calibration markers are 100 nm

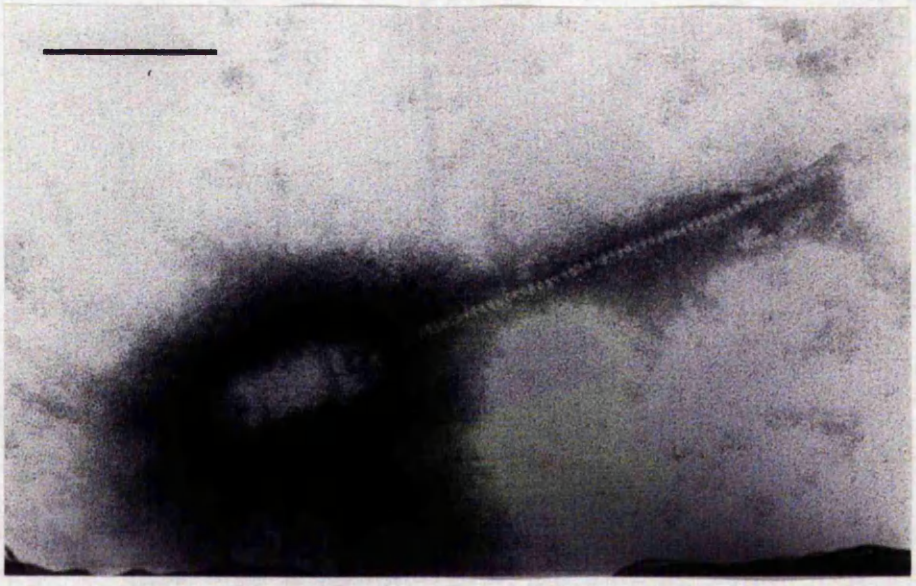
Plate 5.14



Legend

Bacteriophage JS 017 x 56,000 with phage ghosts showing evidence of central symmetry of the phage head. Calibration markers are 100 nm.

Plate 5.15



Legend

Bacteriophage JS 017 x240,000 showing a variation in the length of the phage head and tail. The head is 100 nm in length and the tail between 250 nm and 300 nm in length. Calibration markers are nm.

5.10 EXAMINATION OF PHAGE GENOME WITH RESTRICTION ENDONUCLEASES

DNA from five thermophilic bacteriophages was examined following digestion with restriction endonucleases and agarose gel electrophoresis (Plate 5.16 - 5.20; Table 5.12). This technique enabled the differentiation of phages which otherwise appeared morphologically identical. Phage JS 017, which was morphologically distinct to the other three phages had a distinctive restriction endonuclease cleavage pattern. Ten BamI sites and 18 BclI sites were evident while none of the remaining four phages showed evidence of DNA cleavage with these enzymes. A number of enzymes appeared to cause general digestion of the phage JS 017 DNA resulting in smearing of the gel with nucleotide fragments. Phages JS 022, JS 024 and JS 025 which were all morphologically similar showed several differences in their endonuclease cleavage patterns. JS 024 had between 10 and 12 BalI sites compared with eight and six for JS 022 and JS 025, it had approximately 30 BglI sites compared with three and four for JS 022 and JS 025. It also appeared to have more SmaI sites and had no XbaI site compared with three and four for JS 022 and JS 025. The differences between phages JS 022 and JS 025 were small and mainly involved slight differences in the number of restriction fragments produced by the different enzymes. Phage JS 028, which was the only phage able to infect B. thermodenitrificans (DSM 465), also had a distinctive restriction enzyme cleavage pattern. Unlike the other four phages examined, it had no HphI or SmaI site.

Following the extraction of DNA from phage JS 024 using proteinase K and formamide (Section 2.13.3) the DNA was further purified by banding on an ethidium bromide/CsCl gradient. Visualisation of the gradient under u.v. light indicated the presence of three distinct bands of DNA. These bands were collected and examined independently with restriction endonucleases, all showed identical cleavage patterns. This suggests that the three DNA samples were of similar composition and the three bands probably comprised linear, open circular and supercoiled DNA.

Table 5.12

Restriction endonuclease cleavage fragments in thermophilic bacteriophages

	λ	JS 017	JS 022	JS 024	JS 025	JS 028
BamHI	5	10	0	0	0	0
BalI	15	*	8	10 - 12	6	3
BclI	7	18	0	0	0	0
BglI	22	*	4	28 - 30	0/3	6
EcoRI	5	0	0	0	0	0
HhaI	>50	11	12	11 - 14	6/*	>16
HindIII	6	>12	0	0	0	0
HphI	>50	*	15 - 21	16	17	0
KpnI	2	*	2 - 3	0/*	0	0
PstI	18	*	0	0	0	0
PvuI	3	3	0	0	0	4
SalI	2	0	0	0	0	2
SmaI	3	7	11	17 - 22	13	0
SstI	2	*	0	0	0	2
SstII	4	*	0	0	0	5
XbaI	1	0	3 - 4	0	4/0	0

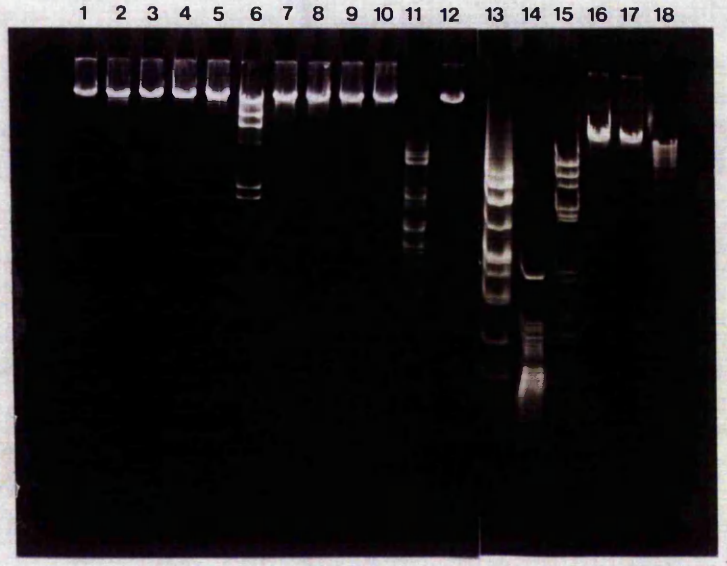
Legend

All endonuclease digestions were carried out at 37 °C (BclI at 60 °C) for 3 h. Enzymes were inactivated at 70 °C for 5 min and loaded to a 0.8% agarose gel and run at 30 mA for 3 h (Section 2.16.9).

*Smearing of gel showing general digestion of DNA.

Plate 5.16

Agarose gel of bacteriophage JS 024 DNA restriction enzyme digest



Legend

All endonuclease digestions were carried out at 37 °C (BclI at 60 °C) for 3 h. Enzymes were inactivated at 70 °C for 5 min and loaded to a 0.8% agarose gel and run at 30 mA for 3 h (Section 2.16.9).

1, HindIII; 2, BamHI; 3, EcoRI; 4, BclI; 5, PstI; 6, λ DNA digest with EcoRI; 7, SstI; 8, SstII; 9, SalI; 10, PvuII; 11, HphI; 12, Undigested control; 13, BalI; 14, HhaI; 15, SmaI; 16, XbaI; 17, KpnI; 18, BglI.

CHAPTER 6

THERMOCIN ISOLATION AND CHARACTERISATION

6. THERMOCIN ISOLATION AND CHARACTERISATION

6.1 THE DETECTION OF THERMOCIN PRODUCTION BY BACILLUS THERMOPHILES

The results of screening thermophilic strains of Bacillus for the production of thermocin are presented in Table 6.1. The majority (67) of the strains examined show evidence for the production of some growth inhibitory compound which inhibited growth of one or more test strains. It was not determined if these growth inhibitory substances were thermocins or toxic by-products of metabolism. B. stearothermophilus strain RS 93, which is a strong thermocin producer (Sharp et al., 1979), was isolated as a contaminant of a culture of B. stearothermophilus NCA 1503. The bacteriocinogenic property of the contaminant was observed on plates used to check culture purity. Similarly, thermocin production by a number of strains (e.g. RS 249) was observed during the initial strain isolation. Observations of a number of different colonies in close proximity occasionally revealed one, or a number of colonies, inhibiting growth of others in the vicinity.

6.2 THE RANGE OF ACTIVITY OF THERMOCINS

The range of activity of a number of thermocins isolated in this study together with ten thermocin producing strains supplied by Dr. B.D. Barridge is presented in Table 3.2 in Section 3.6. The host range of the bacteriocins was examined in Section 3.6 as a means of typing thermophilic strains of Bacillus. Thermocin 93 was more extensively studied than the other thermocins isolated and a number of mesophilic species were examined for their sensitivity (Table 6.2).

Table 6.1

Production of bacteriocin (thermocin) by strains of thermophilic Bacillus

INDICATOR STRAIN	RS 1	RS 2	RS 3	RS 5	RS 6	RS 7	RS 9	RS 10	RS 11	RS 13	RS 15	RS 16	RS 17	RS 18	RS 19	RS 20	RS 21	RS 22	RS 23	RS 25	RS 27	RS 28	RS 29	RS 30	RS 31	RS 32
RS 15	0	0	0	2	0	6	0	0	0	0	0	0	0	0	0	0	0	2	0	0	1H	4H	2H	2H	0	2H
RS 48	0	2	0	2	0	13	0	0	0	0	4	0	0	2	0	0	0	2	0	0	0	2H	0	0	0	2H
RS 56	0	0	0	2	2	4H	0	0	0	0	4	4H	4H	0	0	0	0	0	0	0	0	2H	0	0	0	0
RS 58	3H	0	3H	0	0	3	0	3	0	0	0	0	0	0	0	2	0	0	0	0	0	2H	0	2H	2H	2H
RS 62	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2H	0	25H	0	14H
RS 74	0	0	0	0	1	2H	0	0	0	0	3	2H	2H	0	0	0	0	0	0	0	0	5H	0	0	0	0
RS 89	4	2	4	4	5H	2	3	5	0	0	1H	2H	1H	0	0	0	2	0	0	0	0	3	0	0	0	0
RS 95	1	4	2	2	3	2	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	8H	8H	8H	6H	6H
RS 108	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0
RS 116	0	0	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RS 129	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	1	0	0
RS 144	0	0	0	3H	0	3H	0	2H	0	0	0	0	0	1	0	0	0	2	0	0	0	0	0	0	0	0
RS 161	1	1	1	3	1	4	2	1	0	0	5	1H	0	3	0	0	0	3	0	0	1	3	1	0	0	0
RS 166	0	0	0	3	0	2	1	1	0	0	3	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0
RS 173	0	0	0	2	1	2	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	1H	2H	2

Table 6.1 (Cont'd)

INDICATOR STRAIN	RS 33	RS 34	RS 35	RS 36	RS 38	RS 39	RS 40	RS 42	RS 43	RS 46	RS 48	RS 50	RS 51	RS 52	RS 53	RS 54	RS 55	RS 56	RS 58	RS 59	RS 62	RS 63	RS 65	RS 67	RS 68	RS 69
RS 15	0	3H	0	2H	1	0	0	0	0	0	0	0	0	0	0	3H	2	0	0	0	0	2H	2H	1	0	0
RS 48	0	1	0	0	0	0	1H	1H	2H	1	0	2	2H	1H	1H	1H	0	0	1H	0	0	0	0	0	2H	0
RS 56	0	3	0	0	0	0	0	0	0	2	0	0	0	1	3H	2	4H	0	0	0	0	3H	5H	0	0	0
RS 58	2H	0	2H	2H	2	1	1	2H	2H	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4H
RS 62	0	0	0	1H	0	9H	0	1H	2H	11H	8H	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RS 74	2	0	0	0	0	0	0	0	0	0	0	0	0	3	3	0	4	0	0	0	0	0	0	0	0	3
RS 89	2	1	1	1	0	0	0	3	2	4	0	0	0	1	0	1	2H	0	2H	0	1	1H	0	0	2	6
RS 95	5H	4H	9H	6H	5H	5H	4H	4H	4H	5H	7H	0	4H	0	0	0	0	0	0	0	0	0	0	0	0	0
RS 108	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2H	0	0	0	0	0	0	0
RS 116	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RS 129	0	1H	0	0	0	2	1	0	1H	0	0	0	0	0	3H	0	0	0	0	0	0	0	0	0	0	0
RS 144	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RS 161	0	0	3	0	0	0	0	0	0	4	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	2H
RS 166	0	0	0	0	0	0	0	0	0	2	0	0	0	1	0	1	3	0	0	0	0	0	0	0	0	0
RS 173	0	0	1	0	1H	1	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	2	2	0	0

Table 6.1 (Cont'd)

INDICATOR STRAIN	RS 70	RS 72	RS 73	RS 74	RS 75	RS 76	RS 77	RS 78	RS 79	RS 80	RS 81	RS 82	RS 83	RS 85	RS 86	RS 87	RS 88	RS 90	RS 91	RS 92	RS 94	RS 95	RS 96	RS 97	RS 98	RS 99
RS 15	2H	0	0	0	0	0	0	0	0	5	0	3H	3	3	4	0	6	0	0	0	0	0	0	0	3	3
RS 48	0	0	2H	0	0	0	3	0	0	4	0	0	3	3	4	0	6	0	0	0	0	2H	0	0	4	4
RS 56	5H	0	0	0	0	0	4	0	0	4	0	4H	3	2	0	0	6	0	0	0	0	2H	0	0	2	3
RS 58	0	0	0	0	0	0	1H	0	0	4	0	0	2	2	3H	0	6	0	0	0	0	1H	1H	0	2	1
RS 62	1	0	1H	0	0	0	3	0	0	2	0	5	3	4	3	0	7	0	0	0	0	4	0	0	4	4
RS 74	2H	0	0	0	0	0	3	0	0	4	0	4	5	0	0	0	6	0	0	0	0	4H	0	0	4H	4H
RS 89	4H	0	0	0	0	0	3	0	0	2	0	4	4	4	3	0	4	0	0	0	0	3H	0	0	5H	5H
RS 95	0	1H	0	0	0	0	0	0	0	0	0	1H	3H	0	0	0	0	0	0	0	0	0	0	0	2H	2H
RS 108	0	0	0	0	0	0	0	0	0	0	0	0	1	1H	2H	0	5	0	0	0	0	6H	5H	0	2	2
RS 116	3	2H	0	0	0	0	0	0	0	0	0	3H	2H	0	0	0	3H	0	0	0	0	0	0	0	3	2
RS 129	2	0	2H	0	0	2H	0	0	0	0	0	1H	1H	1H	2H	0	0	2H	0	2H	2H	1H	2H	0	2	2
RS 144	2H	2	0	0	0	0	0	0	0	0	0	5H	3	6H	5	0	6	0	0	0	0	4	0	0	3	2
RS 161	3	0	0	0	4H	4	0	0	0	2	0	5H	5	2	2	0	5	0	0	0	0	0	0	0	5	3
RS 166	2	0	0	0	3	3	0	0	0	2	0	3	5	2	2	0	7	0	0	0	0	3	0	0	4	4
RS 173	4H	0	0	0	0	2	2H	0	0	1H	0	3H	2H	2	3	0	6	3H	0	0	0	2H	0	0	2H	1H

Legend

Culture supernatant was examined for bacteriocin activity using a well diffusion assay. Bio-assay plates with TSBA were seeded with 2 ml of a 6 h culture of the bacteriocin indicator strain. Fifteen indicator strains were used to detect evidence of bacteriocin production. After allowing 1 h for plates to dry, 20 wells were cut into each bio-assay plate. Supernatant samples (100 µl) into each well. The plates were incubated overnight at 55 ^{o}C and observed for evidence of growth inhibition. Zones of inhibition were measured and recorded in mm.

H, denotes hazy zone of inhibition.

Table 6.2

Activity of thermocin 93 on thermophiles and mesophiles

Organism	Temp. of incubation	Inhibitory zone radii (mm)
B. stearothermophilus RS 93	60 °C	0
B. stearothermophilus NCA 1503	60 °C	7.0
B. stearothermophilus ATCC 12016	60 °C	6.0
B. stearothermophilus EP 240	60 °C	5.0
B. stearothermophilus EP 262	60 °C	6.5
B. stearothermophilus NW 10	60 °C	8.0
B. stearothermophilus NW 4S	60 °C	7.5
B. stearothermophilus RS 44	60 °C	4.0
B. caldolyticus	60 °C	7.0
B. caldovelox	60 °C	7.0
B. caldotenax	60 °C	7.0
B. amyloliquefaciens RUB 500	37 °C	0
B. subtilis 168	37 °C	0
B. subtilis ATCC 10783	37 °C	1.0
B. cereus 569H/9	37 °C	0
B. megaterium KM	37 °C	0
B. globigii NCIB 8056	37 °C	0
B. coagulans ATCC 8038	37 °C	0
B. coagulans ATCC 12245	37 °C	0
E. coli K 12 W3110	37 °C	0
Proteus vulgaris ATCC 13315	37 °C	0
Pseudomonas aeruginosa NCTC 6750	37 °C	0
Staphylococcus aureus NCTC 8530	37 °C	0
Salmonella typhimurium ATCC 23564	37 °C	0
Rhodotorula glutinis ATCC 4054	30 °C	0

Legend

Cultures used were grown for 6 h, prior to seeding of a TSBA plate with 1 ml of the culture. 8 mm diameter wells were cut into the plates and inoculated with 0.1 ml of B. stearothermophilus RS 93 culture supernatant. Zones of growth inhibition were recorded.

6.3 CHARACTERISATION OF THERMOCINS

6.3.1 The nature of thermocins

Bacteriocins are reported to exist in two very distinct basic types (Bradley, 1967):

i. As a small molecule which is generally thermostable and cannot be sedimented by ultracentrifugation or resolved by the electron microscope.

ii. As a much larger particle which is easily sedimented and appears as a phage like particle (or phage component, e.g. tails) under the electron microscope.

Preliminary work on ten thermocins (Table 6.3) showed that most of them could be dialysed for 48 h, without significant loss in their activity. Attempts to demonstrate the sedimentation of bacteriocin particles at 40,000 x g was successful for two thermocins RS 53 and RS 82 and to a lesser extent for RS 77 and RS 80. All of them however, still retained considerable thermocin activity in the supernatant fraction. This may indicate a mixture of particle sizes, or a sub-unit structure in which dissociated sub-units (which still retain activity) do not sediment.

Thermocin 93, which was shown to be dialysable but did not sediment following centrifugation at 40,000 x g for 3 h, was further examined to determine its structure and molecular weight.

6.3.2 Composition of thermocin 93

Studies of the digestion of thermocin 93 with nucleases and pronase are presented in Table 6.4. There was no apparent loss in activity of thermocin 93 following exposure to RNase and DNase. Digestion with pronase at 50 μg ml^{-1} resulted in up to 93% decrease in thermocin activity. (The degree of activity retained was estimated by reference to a standard curve of sensitivity zone diameter and concentration of bacteriocin (Fig. 6.1).)

Using gel filtration with a pre-calibrated Sephadex G-75 column the molecular weight of thermocin 93 was observed to be in the region of 13,500 Daltons.

6.3.3 Stability of thermocin 93

Thermocin 93 showed no loss in activity following rotary evaporation at 70 ^{o}C for 2 h. (Ten fold concentrates diluted 10^{-1} in distilled water showed the same original zone size on assay.) The half life for activity at 75 ^{o}C was about 80 min.

Table 6.3

Examination of residual bacteriocin activity following centrifugation at 40,000 x g and dialysis for 48 h

Thermocin producer	RS 6	RS 7	RS 53	RS 70	RS 77	RS 80	RS 82	RS 85	RS 86	RS 93
Original activity	1.0	0.5	3.0	1.0	2.5	3.0	3.0	0.5	5.0	5.0
Supernatant activity	0.5	1.0	4.0	0.5	2.0	3.0	2.0	0	4.0	5.0
Resuspended pellet	-	-	3.0	0	1.0	1.0	3.0	0	0	0
Dialysed supernatant	0.5	0.7	2.0	0	3.0	2.0	4.0	-	-	4.0

Legend

Crude thermocin samples were assayed and then centrifuged at 40,000 x g for 3 h. The supernatant and resuspended pellet were each examined for thermocin activity. A second sample was dialysed for 48 h and then reassayed for thermocin activity.

Table 6.4

The effect of pronase, RNase and DNase on the activity of thermocin 93

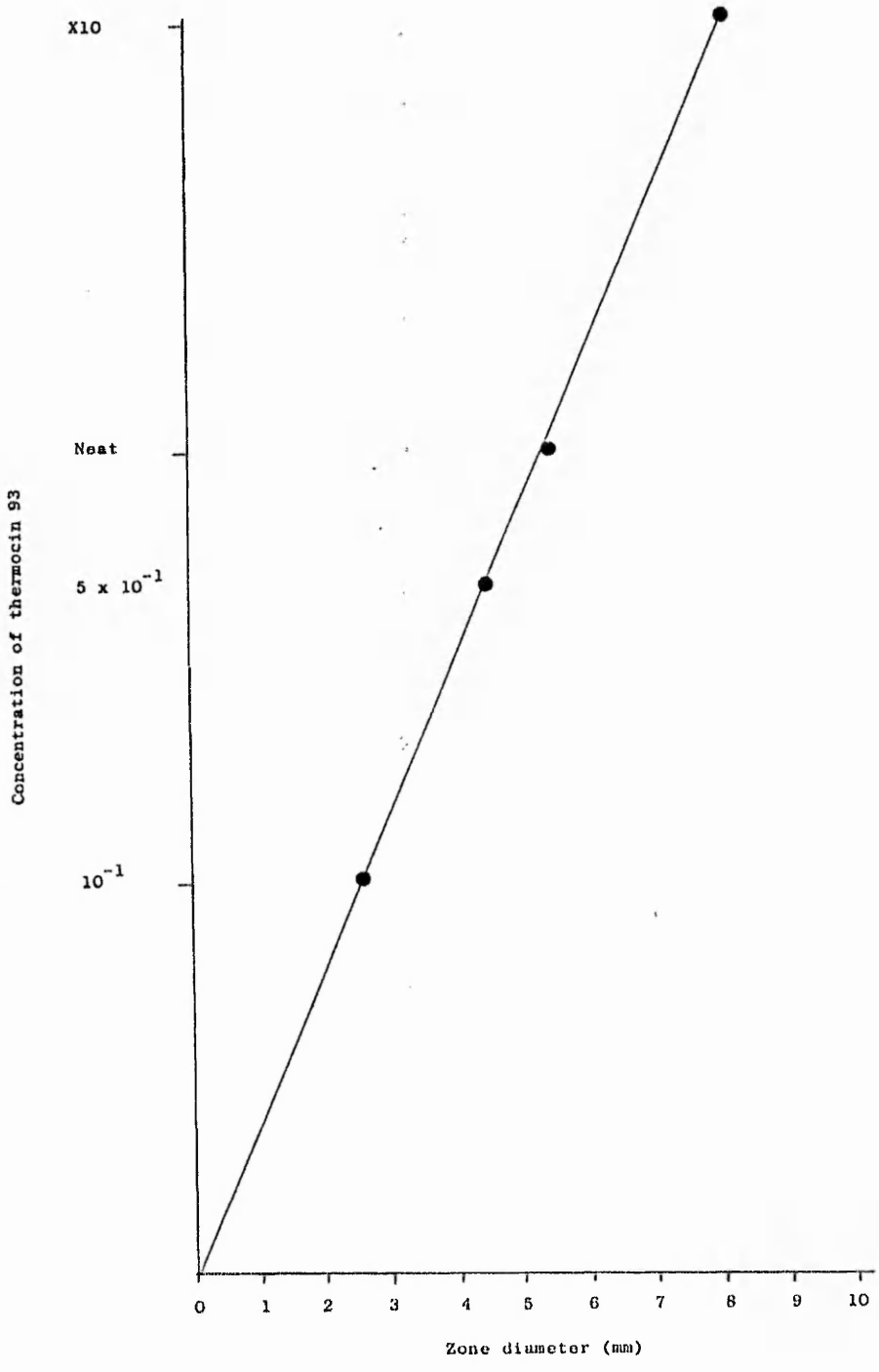
Sample treatment	Post incubation assay (mm)	Percentage activity retained
thermocin 93 + buffer at 4 ^{o}C	6	100
thermocin 93 + buffer	6	100
thermocin 93 + RNase 100 µg ml^{-1}	6	100
thermocin 93 + DNase 100 µg ml^{-1}	6	100
medium + RNase 100 µg ml^{-1}	0	-
medium + DNase 100 µg ml^{-1}	0	-
thermocin 93 + pronase 10 µg ml^{-1}	6	100
thermocin 93 + pronase 50 µg ml^{-1}	4	35
thermocin 93 + pronase 250 µg ml^{-1}	2	6.8
medium + pronase	0	0
PMFS + buffer	1	-

Legend

Thermocin 93 (0.2 ml) was digested at 37 ^{o}C for 4 h. Digests were assayed for activity on B. caldolyticus using the well diffusion assay. The percentage activity retained was observed by reference to a standard curve of sensitivity zone size and thermocin concentration.

Figure 6.1

The effect of thermocin 93 concentration on the sensitivity zone diameter



Legend

A crude preparation of thermocin 93 was concentrated ten fold using rotary film evaporation. The concentrate, the original sample and 10^{-1} and 5×10^{-1} dilutions were assayed using the well diffusion assay.

Optimum activity of thermocin 93 was observed at pH 7.0; adjustment of the pH to 4.1 (with HCl) or 9.4 (with NaOH) resulted in an apparent loss in activity of up to 70%. This effect was reversible since on return to pH 7.0 full activity was restored.

The majority of thermophilic strains of Bacillus examined produced growth inhibitory elements which were released into the culture medium. These were considered to be thermocins some of which could be sedimented by centrifugation at 40,000 x g. Others appeared to be smaller elements which could not be sedimented. A more extensive study of thermocin 93 produced by strain RS 93 indicated it to be a low molecular weight (13,500 Daltons) protein with considerable thermal stability.

The active centre of the molecule was protein, but it was not established if the thermocin was a glycoprotein or lipoprotein.

Thermocin 93 inhibited the growth of most thermophilic strains of Bacillus stearothermophilus, except those starch negative strains in Cluster (1) (Section 3.2). Mesophilic strains of Bacillus were also insensitive to thermocin 93.

CHAPTER 7

ISOLATION OF MUTANTS FROM THERMOPHILIC BACTERIA

7. ISOLATION OF MUTANTS FROM THERMOPHILIC BACTERIA

7.1 PENICILLIN ENRICHMENT OF AUXOTROPHIC MUTANTS OF B. CALDOTENAX

The sensitivity of B. caldotenax and other thermophiles to ampicillin using assay discs (10 µg) is shown in Table 3.8. Sensitivity zone diameter for B. caldotenax was recorded as 25 mm. Initial studies of penicillin enrichment of B. caldotenax mutants (following exposure to NTG or u.v. irradiation) using ampicillin (50 µg ml^{-1}), cycloserine (25 µg ml^{-1}) and benzyl penicillin (60 µg ml^{-1}) were carried out in BS media. A rapid decrease in cell density occurred within 1 h of the addition of the antibiotics (Fig. 7.1).

In Cal II minimal medium these three antibiotics had little effect on the growth of B. caldotenax (Fig. 7.2); cycloserine at 100 µg ml^{-1} did however, indicate a slight decrease in cell density. When combined, these three antibiotics still had little effect on cell growth; cycloserine and benzyl penicillin at 100 µg ml^{-1} indicated a decrease in OD after almost 2.5 h (Fig. 7.3). Reduction in the level of phosphate in the Cal II medium (KH_2PO_4 reduced from 0.5 g to 0.05 g) or the addition of lysine, alanine and glutamine to the minimal medium had no effect on the activity of the three antibiotics. The effect of three other β-lactam antibiotics (methicillin, cloxacillin and amoxicillin) and vancomycin and bacitracin (which also act at the site of cell wall synthesis) is presented in Fig. 7.4. The addition of bacitracin (50 µg ml^{-1}) resulted in a decrease in OD within 2 h of the addition of the antibiotic. In subsequent attempts to enrich mutant populations, bacitracin was used at a conc. of 100 µg ml^{-1}.

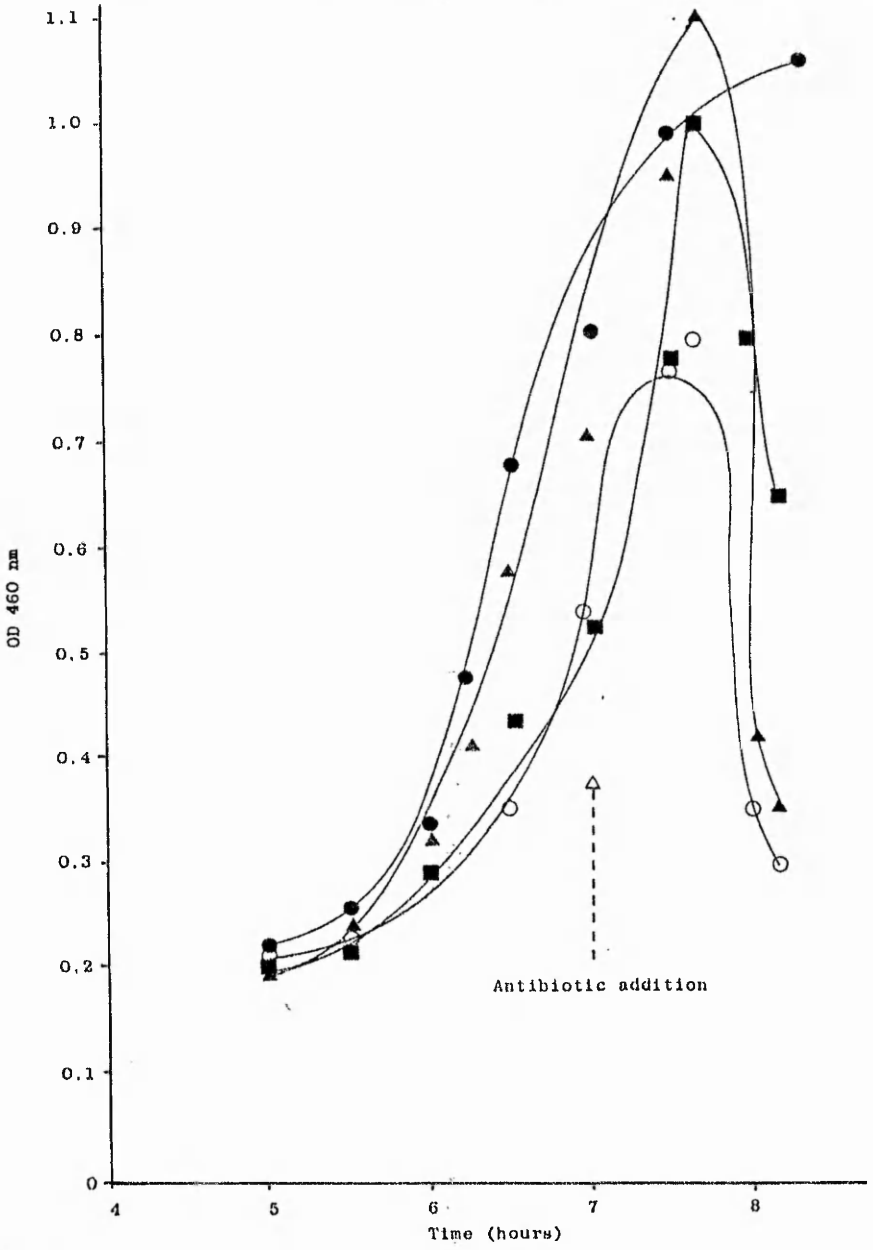
7.2 ISOLATION OF AUXOTROPHIC MUTANTS

7.2.1 Mutagenesis with NTG and EMS

Mutagenesis of actively growing cells of B. caldotenax with NTG followed by bacitracin enrichment of the mutant population yielded a number of amino acid auxotrophs. Preliminary examination indicated seven met$^-$, three his$^-$ and two glu$^-$, mutants with five double pro$^-$/arg$^-$, and several multiple mutants which were not typed. While analysing the growth requirements of these mutants it became evident that they were all "leaky" being able to grow weakly on minimal medium.

Figure 7.1

The effect of benzyl penicillin, ampicillin and cycloserine on the growth of B. caldotenax in BS complex medium

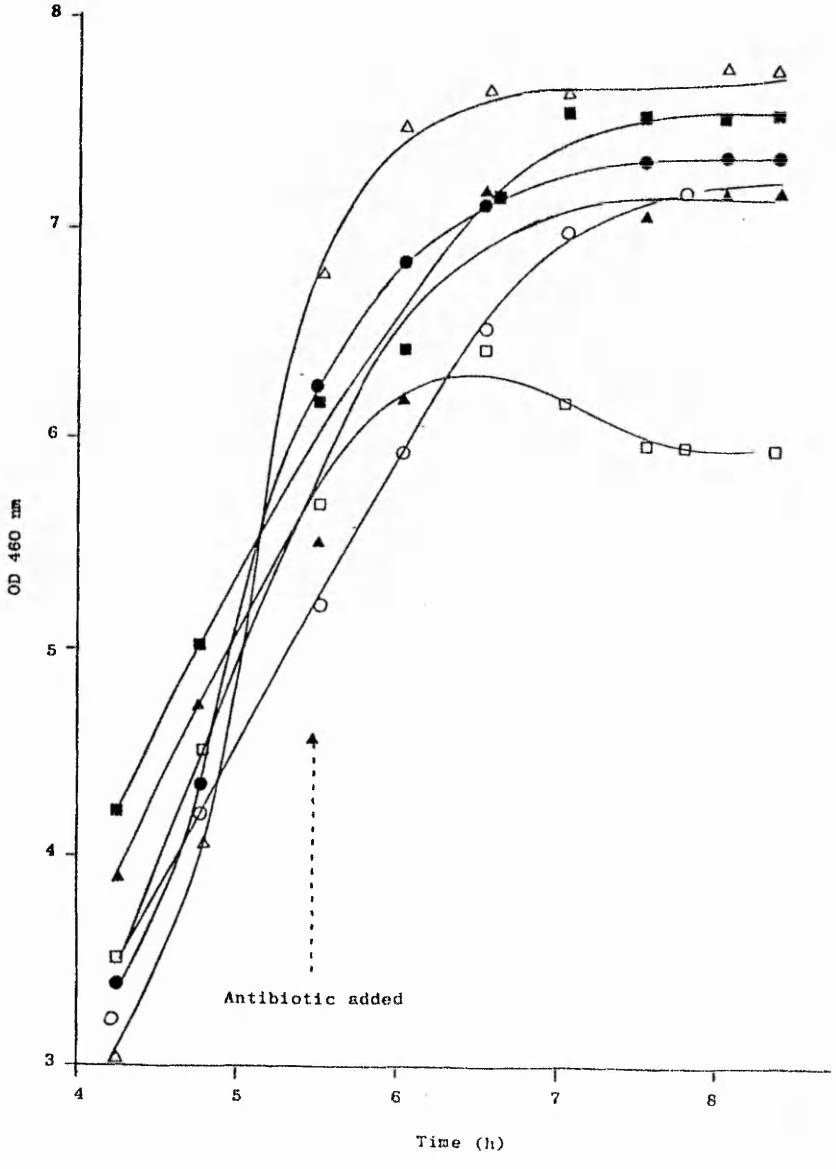


Legend

B. caldotenax was cultured in BS medium in T tubes at 55 °C using a Gallenkamp shaking water bath. Antibiotics were added to log phase cells and incubation continued. ● , control; ▲, crystopen (60 μg ml^{-1}); ■, cycloserine (25 mg ml^{-1}); ○, ampicillin (50 μg ml^{-1}).

Figure 7.2

The effect of benzyl penicillin, ampicillin and cycloserine on the growth of B. caldotenax in Cal II minimal medium

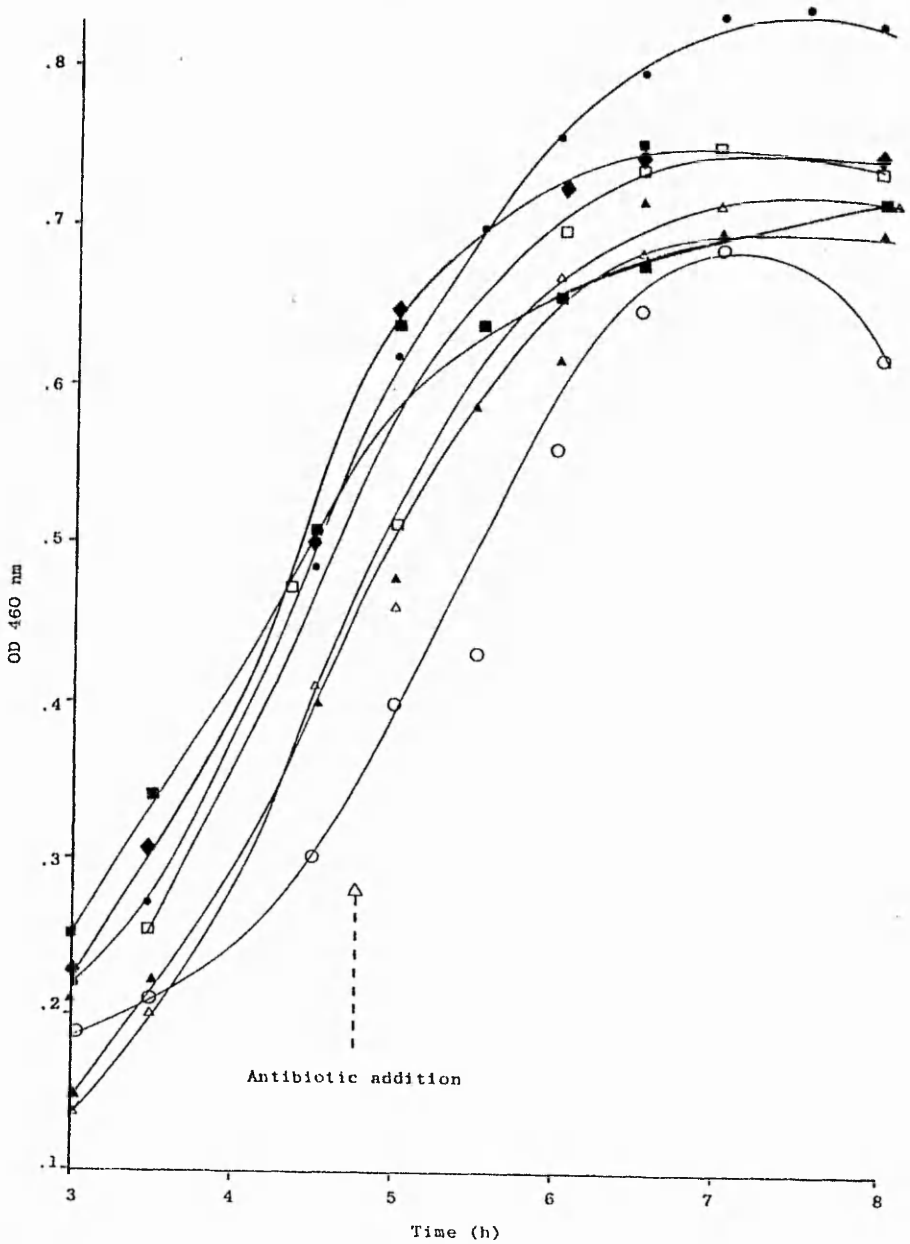


Legend

B. caldotenax was cultured in Cal II minimal medium in T tubes at 55 °C using a Gallenkamp shaking water bath. Antibiotics were added to log phase cells and incubation continued. ● , control; ▲, benzyl penicillin (60 μg ml^{-1}); ■ benzyl penicillin (90 μg ml^{-1}); △ , cycloserine (50 μg ml^{-1}); □ , cycloserine (100 μg ml^{-1}); ○ , ampicillin (50 μg ml^{-1}).

Figure 7.3

The effect of benzyl penicillin, ampicillin and cycloserine in combination on the growth of B. caldotenax in Cal II minimal medium

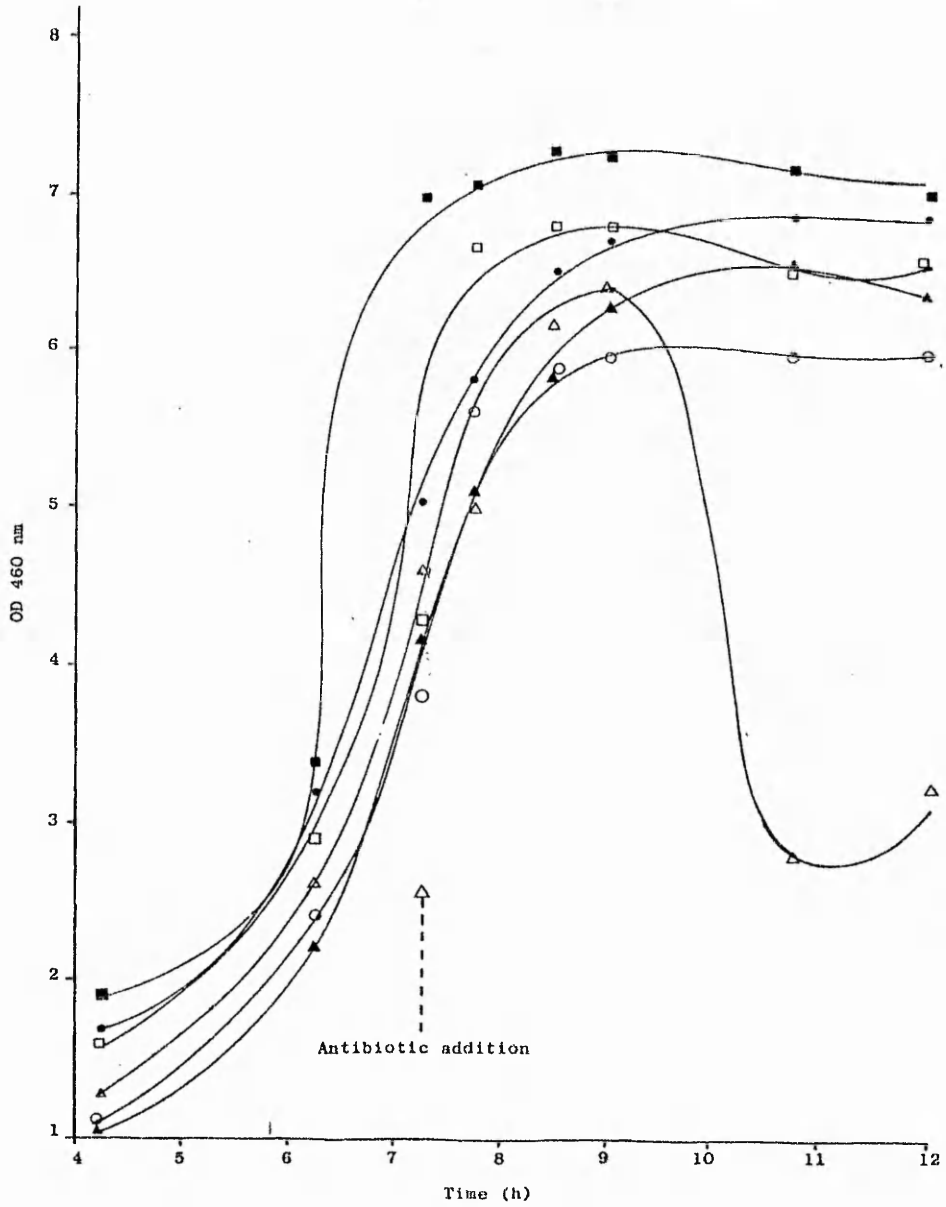


Legend

B. caldotenax was cultured in Cal II minimal medium in T tubes at 55 °C using a Gallenkamp shaking water bath. Combinations of antibiotics were added to log phase cells and incubation continued. ●, control; ■, cycloserine/benzyl penicillin (25/25 μg ml^{-1}); ▲, cycloserine/benzyl penicillin (50/50 μg ml^{-1}); ○, cycloserine/benzyl penicillin (100/100 μg ml^{-1}); △, cycloserine/benzyl penicillin/ampicillin (25/25/25 μg ml^{-1}); x, cycloserine/benzyl penicillin/ampicillin (50/50/50 μg ml^{-1}); □, cycloserine/ampicillin (50/50 μg ml^{-1}).

Figure 7.4

The effect of methicillin, cloxacillin, amoxicillin, bacitracin and vancomycin on the growth of B. caldotenax in Cal II minimal medium

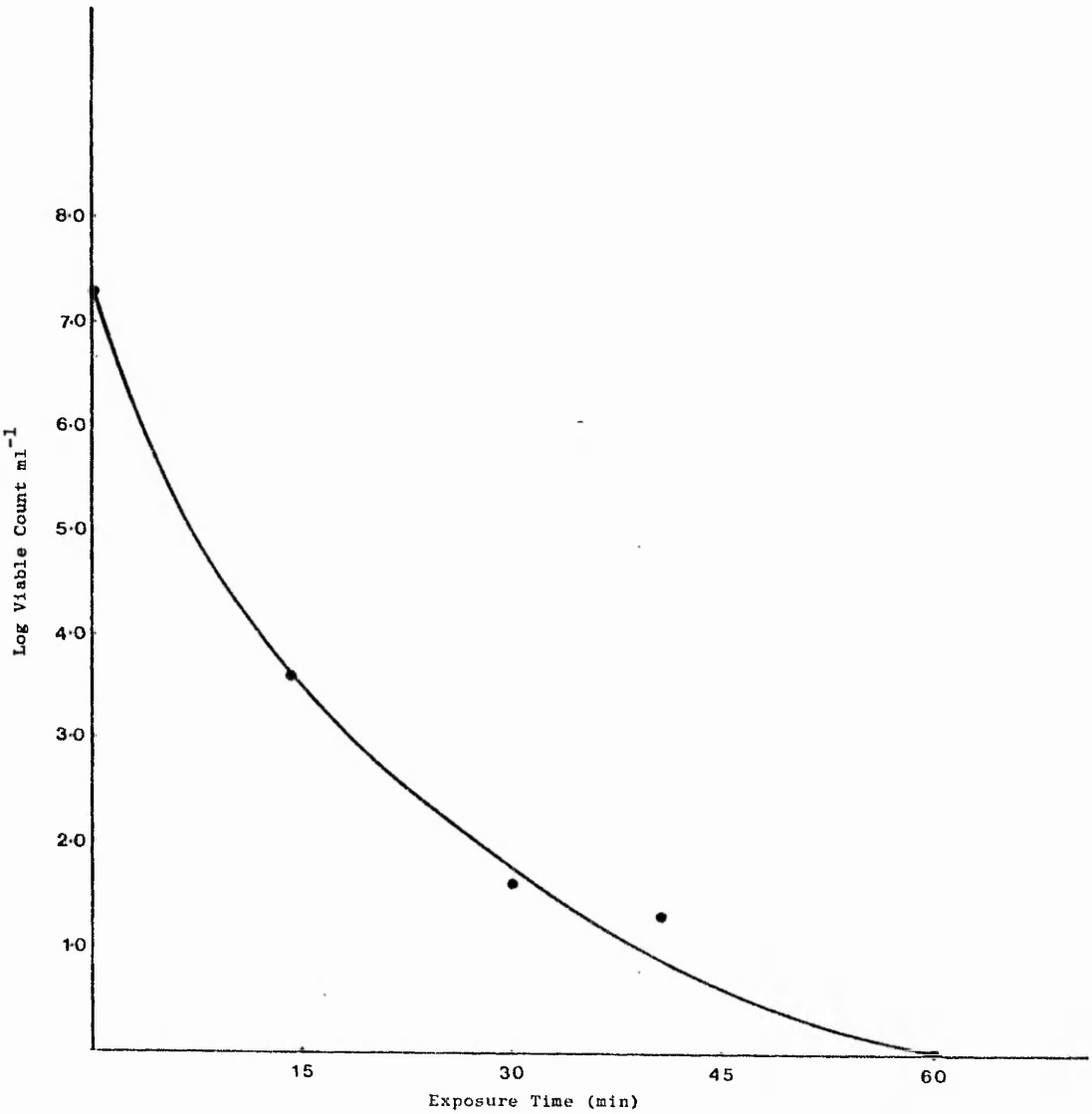


Legend

B. caldotenax was cultured in Cal II minimal medium in T tubes at 55 °C using a Gallenkamp shaking water bath. Antibiotics were added to log phase cells and incubation continued. ●, control; ○, methicillin (50 μg ml^{-1}); □, cloxacillin (50 μg ml^{-1}); ■, amoxicillin (50 μg ml^{-1}); △, bacitracin (50 μg ml^{-1}); ▲, vancomycin (50 μg ml^{-1}).

Figure 7.5

The effect of NTG on washed cells of B. caldotenax

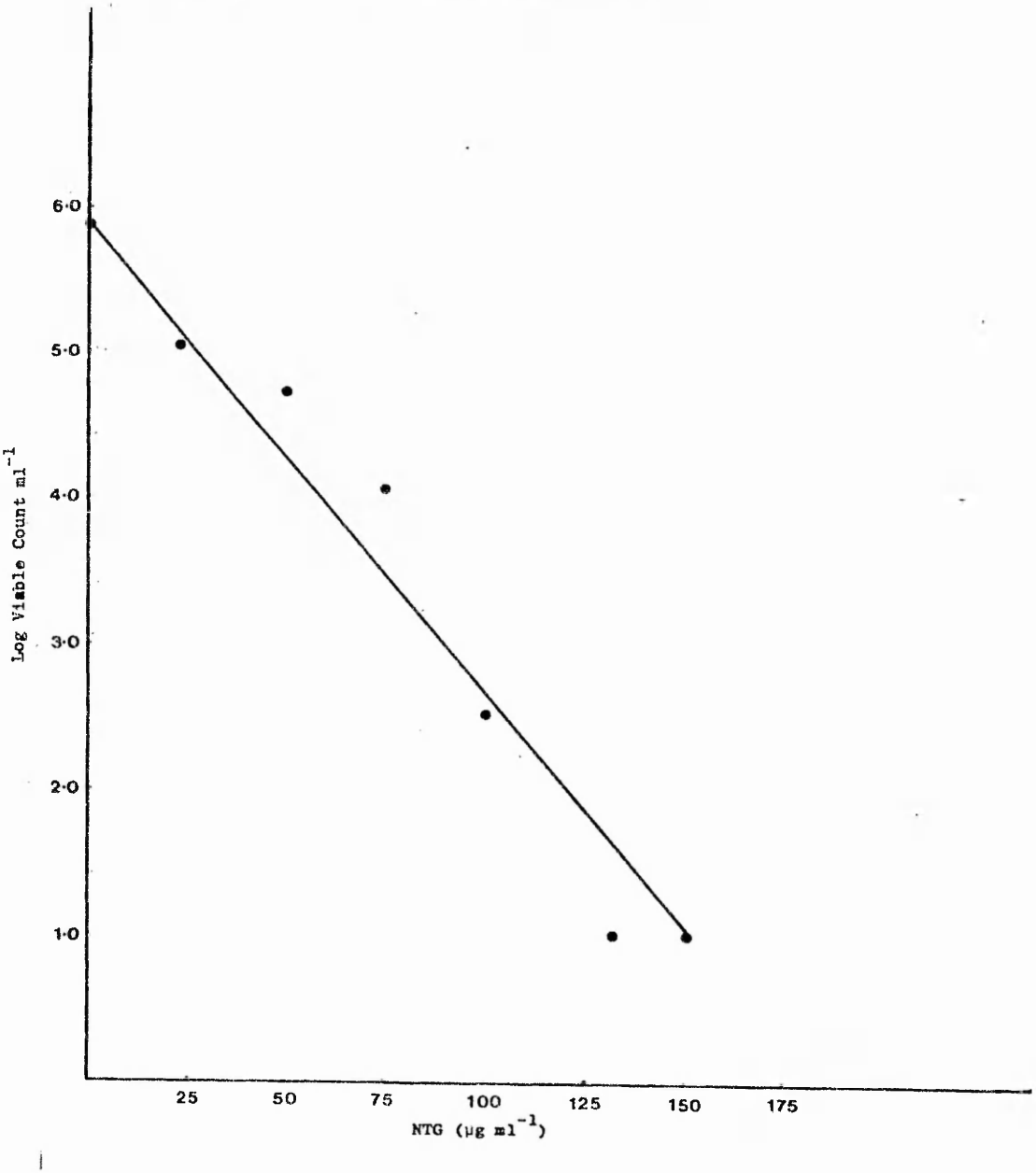


Legend

The cells from 20 ml of a B. caldotenax culture grown in BS broth for 4 h at 60 °C were washed and resuspended in Tris-maleate buffer. 100 µg ml^{-1} of NTG were added to 2 ml aliquots of resuspended cells and sampled at 15 min intervals for viable cell count.

Figure 7.6

The effect of NTG on growing cells of B. caldotenax

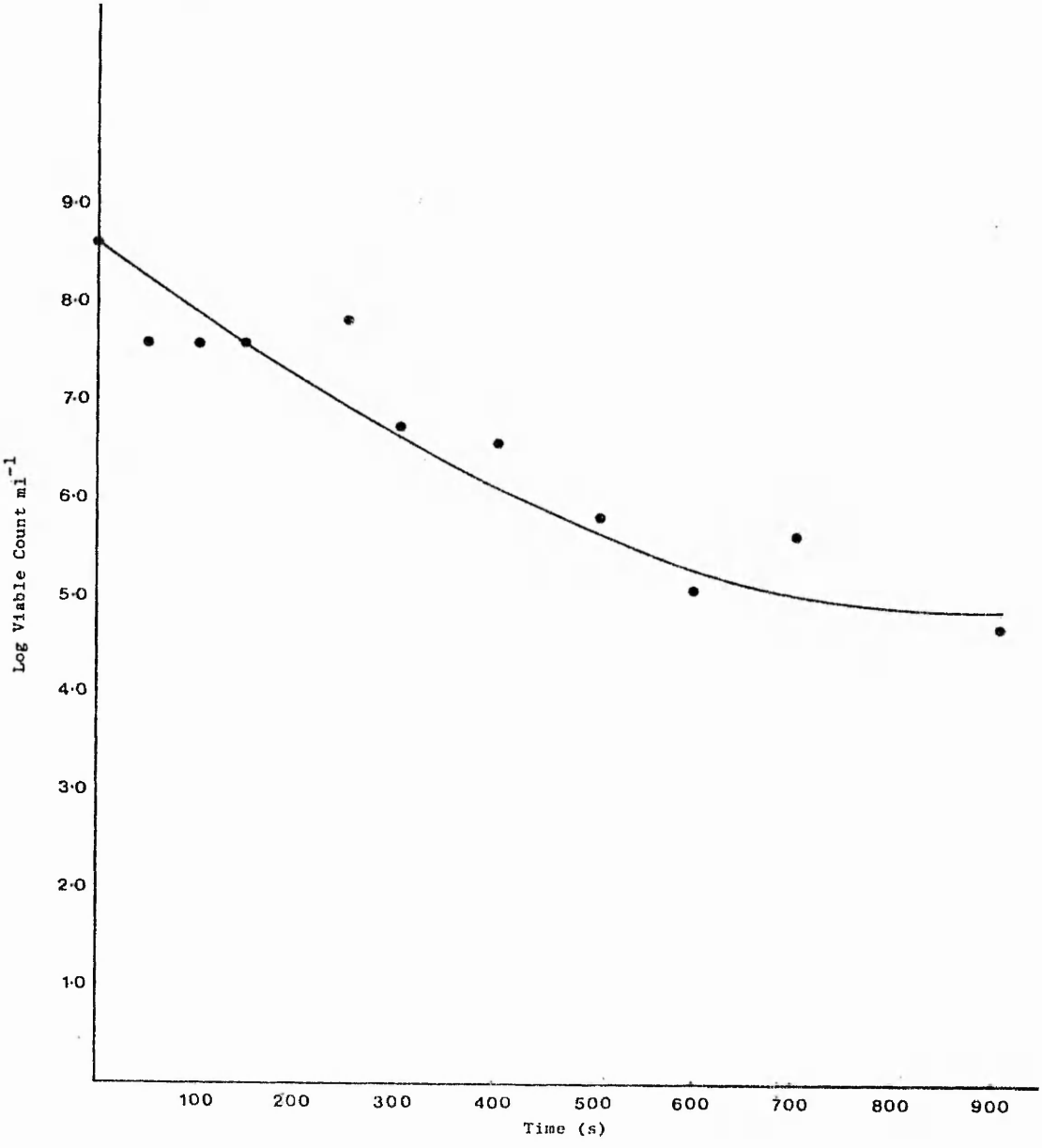


Legend

4 h cultures of B. caldotenax grown at 60 ^{o}C in BS media were inoculated with 25 - 100 µg ml^{-1} of NTG. Incubation was continued for a further 15 min, the cells were washed to remove NTG and examined for viable cell count.

Figure 7.7

The effect of u.v. irradiation on B. caldotenax



Legend

10 ml of a B. caldotenax culture grown in BS media to an OD of 1.3 (420 nm) was exposed to 3.1 J m^{-2} s^{-1} of u.v. irradiation for 900 s. The suspension was gently mixed during exposure and samples removed for viable count.

Further attempts to isolate mutants using "growing" and "washed" cells (Fig. 7.5) followed by bacitracin enrichment resulted in the further isolation of "leaky" or unstable mutants.

The direct plating of survivors from a growing culture of B. caldotenax, exposed to 75 μg ml^{-1} NTG (Fig. 7.6), to TSBA plates was more successful. Of 40 colonies replica plated to minimal medium, 28 indicated growth requirements for one or more amino acid. During further examination a number of these reverted or became "leaky", some were found difficult to type and probably required a number of growth factors. Four mutants were finally isolated with single amino acid requirements, these were met$^-$, his$^-$, arg$^-$ and leu$^-$. The arg$^-$ and leu$^-$ strains reverted to the wild type following storage in liquid nitrogen; the met$^-$ and his$^-$ strains however, stored in liquid nitrogen for two years showing no apparent loss in viability or reversion.

No mutants were isolated from growing cultures of B. stearothermophilus EP 136 following exposure to EMS (17.5 μl ml^{-1}). The survivors were examined for the presence of amino acid auxotrophs following direct plating to TSBA and replica plating on defined media.

7.2.2 Isolation of mutants following exposure to u.v. irradiation

Several attempts were made to isolate amino acid auxotrophs from B. caldotenax and B. stearothermophilus RS 93 following exposure to u.v. irradiation. The cells were exposed to give a 99% kill and the survivors plated directly onto TSBA, or subjected to bacitracin enrichment before plating to TSBA. Replica plating from TSBA to Cal II defined medium for the isolation of amino acid auxotrophs was unsuccessful. Replica plating of survivors to TSBA supplemented with 25 μg ml^{-1} and 50 μg ml^{-1} streptomycin, resulted in the isolation of a number of Str^r mutants for both the strains. The B. caldotenax mutants initially isolated were resistant to 100 μg ml^{-1} of streptomycin. Further selection from these resulted in the isolation of mutants resistant to 1,000 μg ml^{-1}. A u.v. survival curve of B. caldotenax is presented in Fig. 7.7. The cells exposed to u.v. showed no evidence of sporulation.

7.2.3 Isolation of thymine auxotrophic mutants

Thymine requiring mutants of B. caldotenax were isolated at a frequency of 2.1 x 10^{-5} following selection on Cal II agar supplemented with thymine (50 μg ml^{-1}) and trimethoprim (25 μg ml^{-1}). Thymine auxotrophic mutants of B. caldovelox, B. caldolyticus, B. stearothermophilus strains, ATCC 8005, EP 262, RS 93, NCA 1503 and

B. thermocatenulatus were all isolated at similar frequencies. B. stearothermophilus strains EP 136, EP 240 and ATCC 12016 did not yield any thymine requiring mutants using this procedure. Although the gene has not been further characterised or mapped, it was designated as thy since it appears analogous to the thy gene of E. coli or B. subtilis.

Thy$^-$ mutants of strain RS 93 were either high thymine requirers (20 μg ml^{-1}) or low thymine requirers (<2.5 μg ml^{-1}). Thy$^-$ mutants of B. caldolyticus appeared to grow well on 2 μg ml^{-1} thymine while B. caldovelox mutants required >5 μg ml^{-1} thymine.

The thymine requirements and resistance to trimethoprim of 68 thy$^-$ mutants of B. caldotenax were studied in greater detail (Table 7.1) and showed three levels of requirement for thymine. Three isolates were able to grow well on 0.5 μg ml^{-1} of thymine, a further 16 were able to grow well on 2 μg ml^{-1} and 49 required 5 μg ml^{-1} for normal growth. Resistance to trimethoprim (100 μg ml^{-1} and 200 μg ml^{-1}) was variable. Auxotrophic strains and Str^r strains of B. caldotenax were used to isolate thymine auxotrophs using trimethoprim selection, resulting in the production of doubly marked mutants.

Table 7.1

Characterisation of 68 thymine requiring mutants of B. caldotenax isolated following growth in the presence of trimethoprim

Thymine mutant	Number of mutants isolated	Growth on thymine				Trimethoprim resistant	
		0.5 μg ml^{-1}	2 μg ml^{-1}	5 μg ml^{-1}	20 μg ml^{-1}	100 μg ml^{-1}	200 μg ml^{-1}
1	3	+	+	+	+	W	W
2	4	W	W	+	+	W	W
3	4	-	+	+	+	+	+
4	9	-	+	+	+	+	W
5	2	-	+	+	+	+	VW
6	1	-	+	+	+	VW	VW
7	4	-	W	+	+	+	+
8	4	-	W	+	+	+	W
9	2	-	W	+	+	+	VW
10	5	-	W	+	+	W	W
11	4	-	W	+	+	W	VW
12	2	-	W	+	+	W	-
13	10	-	W	+	+	VW	VW
14	6	-	W	+	+	-	-
15	3	-	-	+	+	+	+
16	5	-	-	+	+	W	W

Legend

Colonies grown on Cal II agar supplemented with 50 μg ml^{-1} thymine were emulsified in 1 ml of saline. 50 μl were dropped to the surface of dried Cal II agar plates. Plates were incubated 24 h at 60 ^{o}C before scoring results. No strains grew on minimal media and all grew on 20 μg ml^{-1} thymidine. 50 μg ml^{-1} of thymine was included in the plates used to examine trimethoprim sensitivity.

7.3 EVIDENCE FOR THYMINELESS DEATH IN thy⁻ MUTANTS OF B. STEAROTHERMOPHILUS RS 93 AND B. CALDOTENAX

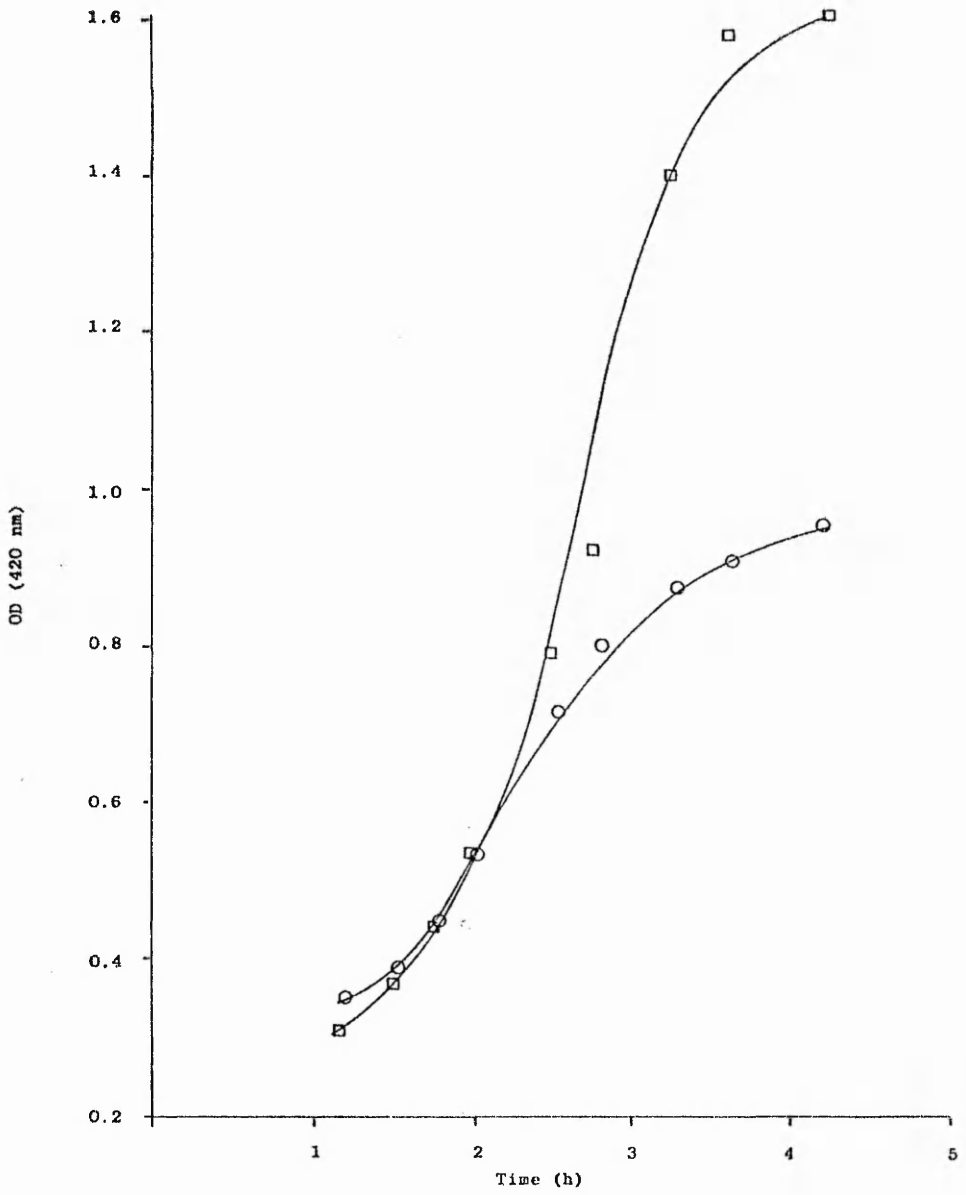
The growth of a high thymine requiring (20 μg ml^{-1}) mutant of B. stearothermophilus RS 93 was examined in L broth and L broth supplemented with 100 μg ml^{-1} thymine. Fig. 7.8 shows the OD of both cultures continuing to increase in parallel for 50 - 60 min before the growth of the starved culture began to decrease.

The cells incubated with thymine showed no evidence of change in cell morphology after 180 min growth. The cells incubated in the absence of thymine began to form short chains after 100 - 120 min and after 180 min the majority of the cells in the culture formed very long thin filaments.

The behaviour of a number of thy⁻ B. caldotenax strains was also examined. Two mutants from group 1 (Table 7.1) which required 0.5 μg ml^{-1} of thymine for growth showed signs of thymineless death. Cells grown in the presence of thymine appeared as normal single cells, while in the absence of thymine one mutant formed a mixture of normal cells and filaments and the other formed a mixture of chains and filaments with considerable twisting and curling. A thymine starved thy⁻ mutant from group 15 (Table 7.1) (which required 5 μg ml^{-1} of thymine and was resistant to 200 μg of trimethoprim) formed cells which were double the length of normal cells and a small number showed evidence of the formation of long filaments. A thymine starved mutant from group 16 (which required 5 μg ml^{-1} of thymine but was only weakly resistant to trimethoprim) showed no evidence of morphological change.

Figure 7.8

Thymineless death in B. stearothermophilus strain RS 93



Legend

A thymine auxotroph of B. stearothermophilus RS 93 was cultured in 200 ml of L broth supplemented with 100 µg ml^{-1} of thymine in a 1,000 ml Erlenmeyer flask at 150 r.p.m. and 60 °C. On reaching an OD of 1.0 (420 nm), 40 ml of culture was centrifuged to collect the cells which were then washed and transferred to two flasks, one of which was supplemented with 100 mg ml^{-1} of thymine. The growth was monitored by OD and samples examined by Gram staining at intervals.

□ , flask supplemented with 100 µg ml^{-1} of thymine; ○ , flask unsupplemented with thymine.

CHAPTER 8

TRANSDUCTION IN THERMOPHILIC SPECIES OF *BACILLUS*

8. TRANSDUCTION IN THERMOPHILIC SPECIES OF BACILLUS

8.1 ISOLATION OF THERMOPHILIC TRANSDUCING PHAGE

In the early part of this study the range of markers available for examination of transducing ability was restricted. Subsequently, other auxotrophic mutants were isolated and a more detailed analysis was possible.

The results (Table 8.1) indicated four B. caldotenax phages (JS 017, JS 019, JS 025 and JS 026) capable of transducing the thy$^+$ marker from a prototrophic strain of B. caldotenax to a thy$^-$ auxotroph. The transduction frequency for this marker was approximately 10^{-7}. After two successful transductions of a thy$^-$ auxotroph of B. caldotenax phages JS 019 and JS 025 appeared to lose their transducing ability. Attempts to re-isolate transducing phage from the original phage stocks were unsuccessful and work with these two phages was discontinued. Following the examination of the plaque morphology, host range, electron micrographs and transducing frequencies, phages JS 017 and JS 026 were considered identical. Further studies of transduction were restricted to phage JS 017.

8.2 OPTIMISATION OF thy$^+$ TRANSDUCTANTS USING PHAGE JS 017

8.2.1 The effect of growth media on transduction frequency

The transduction frequency obtained using the procedure described in 2.21.1 was approximately 5×10^{-7}. The examination of the transduction frequency of thy$^+$ following growth to an OD of 1.0 (420 nm) in double strength L broth and TSB, indicated a frequency of 5×10^{-6} - 10^{-7}. Comparable growth in BS broth, TYF broth and Cal II minimal medium indicated a slightly lower frequency of 10^{-7} - 5×10^{-7}.

Plating transductants on TSBA, NA, Cal II agar and Cal II agar supplemented with 0.1% (w/v) casein hydrolysate indicated no significant differences in the total number of transductant colonies. Growth of transductant colonies on TSBA and NA became apparent earlier, but the trace levels of thymine in the media resulted in a faint background of thymine starved cells. Growth on the supplemented Cal II agar was slightly faster than on Cal II minimal agar but since further work involved the use of amino acid auxotrophs Cal II minimal agar with glucose as carbon source became the general plating medium for subsequent studies.

Table 8.1

Results of preliminary screen for transducing ability of thermophilic phages with B. caldotenax, B. stearothermophilus NCA 1503 B. stearothermophilus RS 93 and B. stearothermophilus RS 239

Phages	Markers examined for transduction				
	Str^r	thermocin prod.	amylase	protease	thy$^+$
ATCC 12016B	NT	NT	NT	NT	-
TP 1C	NT	NT	NT	NT	-
TP 84	NT	NT	NT	NT	-
JS 001	NT	NT	NT	NT	-
JS 002	NT	NT	NT	NT	-
JS 003	NT	NT	NT	NT	-
JS 017	-	NT	NT	NT	+
JS 018	-	NT	NT	NT	-
JS 019	-	-	-	-	+/-
JS 020	-	-	-	-	-
JS 021	-	-	-	-	-
JS 022	-	-	-	-	-
JS 023	-	-	-	-	-
JS 024	-	-	-	-	-
JS 025	-	-	-	-	+/-
JS 026	-	NT	NT	NT	+
JS 004	-	-	NT*	NT*	-
JS 007	-	-	NT*	NT*	-
JS 009	-	-	NT*	NT*	-
JS 027	NT	-	NT*	NT*	NT
JS 013	-	NT*	NT*	NT*	-
JS 014	-	NT*	NT*	NT*	-
JS 015	-	NT*	NT*	NT*	-

Legend (Table 8.1)

+ - Evidence of transduction.

\- - No evidence of transduction.

NT - Not tested.

NT* - Phage not examined since suitable markers were not available in the known host range of the phage.

✧ - Phage suspensions prepared on wild type B. caldotenax and used to attempt the transduction of thy⁻ B. caldotenax (requiring 20 - 25 µg thymine for growth).

◓ - Phage suspensions prepared on Str^r B. caldotenax (resistant to 100 µg ml^{-1} streptomycin) used to attempt transduction of Str^s B. caldotenax (sensitive to 50 µg ml^{-1} streptomycin).

△ - Phage suspensions prepared on RS 93 and used to infect B. caldotenax.

● - Phage suspensions prepared on B. caldotenax and used to infect RS 93.

✦ - Phage suspensions prepared on RS 239 Str^r and used to infect Str^s RS 93.

□ - Phage suspensions prepared on RS 93 and used to infect RS 239.

■ - Phage suspensions prepared on wild type RS 93 and used to infect RS 93 thy⁻ strain (requiring 20 - 25 µg ml^{-1} thymine).

▲ - Phage suspensions prepared on wild type NCA 1503 and used to infect NCA 1503 thy⁻ strain (requiring 20 - 25 µg ml^{-1} thymine).

8.2.2 The effect of $CaCl_2$ on transduction frequency

Addition of $CaCl_2$ (0.001 M - 0.1 M) to the transducing mixture had no effect on the frequency of thy^+ transduction. $CaCl_2$, $MnCl_2$ and $MgCl_2$, however, all affected the p.f.u. of phage JS 017 in trypticase agar (Section 5.1).

8.2.3 The effect of incubation temperature on transduction frequency

No transductant colonies were isolated following incubation of the phage/bacteria mixture at 70 °C. However at 65 °C, 60 °C, 55 °C and 50 °C, 15, 18, 22 and 19 transductants respectively were isolated. The absence of transductants at 70 °C was expected since a ten fold reduction in p.f.u. was observed within 10 min at 70 °C (Fig. 5.13). Transduction was carried out at 55 °C in all subsequent studies.

8.2.4 The effect of cell density on transduction frequency

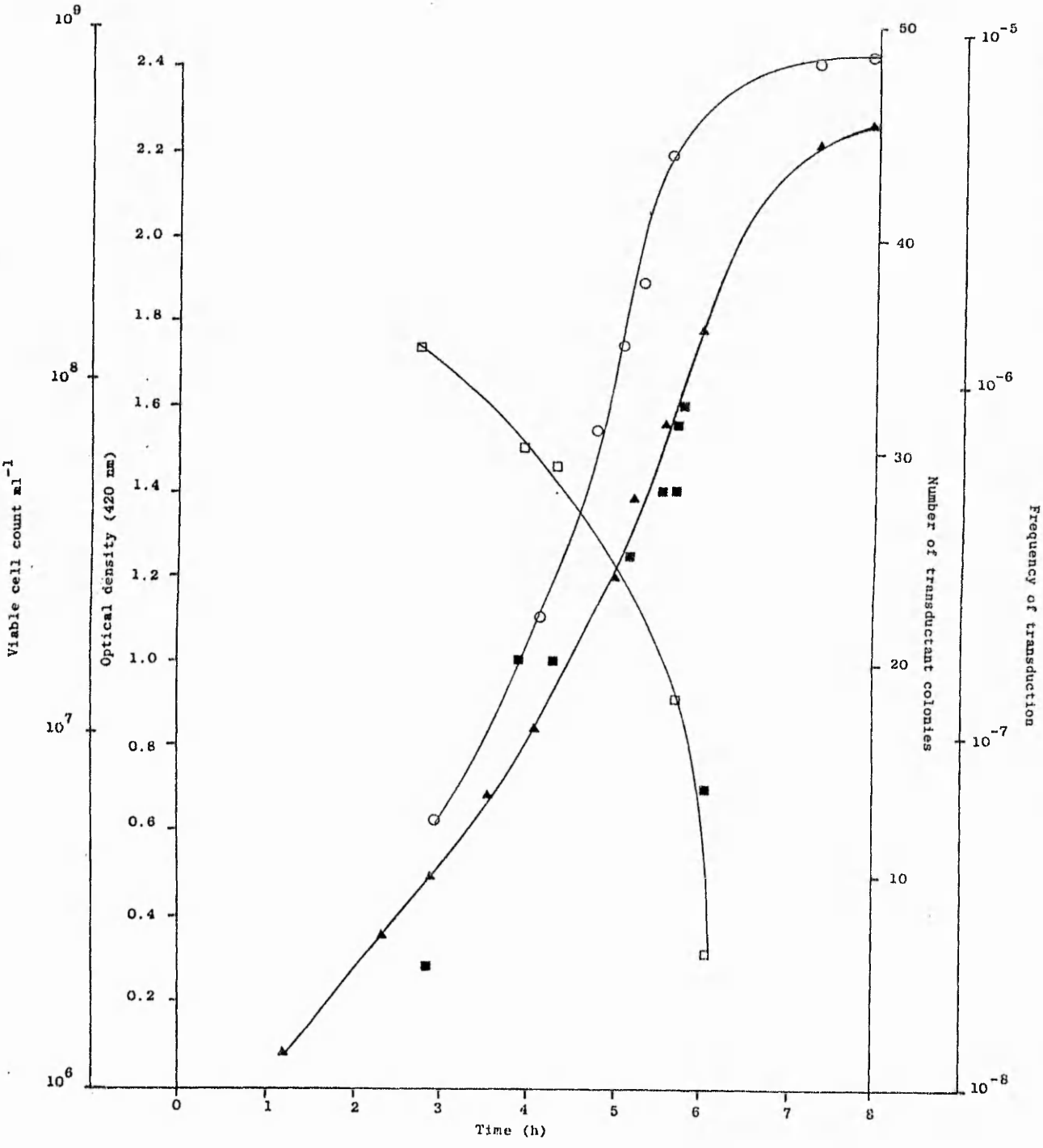
Transduction of thy^- B. caldotenax cells at various stages of the growth cycle indicated a higher frequency during mid log phase, (5×10^{-5}) than during late log phase and stationary phase (1.3×10^{-7}). The number of transductant colonies detected on the plates changed only slightly through a twenty fold increase in viable count (Fig. 8.1).

The results from two studies to determine the effect of cell concentration on the number of transductants are presented in Table 8.2. The results suggest that dilution of the cells in fresh medium enhances the number of transductant colonies and the transduction frequency. A 1/10 dilution of the cell population resulted in at least a ten fold increase in the transduction frequency.

The addition of fresh pre-warmed media may stimulate the cells and provide conditions similar to those encountered earlier in the growth cycle when the number of transductants was higher. Alternatively dilution may decrease the concentration of inhibitors of phage adsorption or transduction. Higher cell densities may inhibit phage attachment although this would seem unlikely. The two fold increase in the number of transductants (Table 8.2) when the volume of cells was doubled, indicated that the number of transducing particles was not limiting.

Figure 8.1

The effect of cell density on transduction frequency



Legend

Samples of a thymine auxotrophic strain of B. caldotenax grown in Cal II medium supplemented with 50 µg ml^{-1} of thymine was transduced at intervals throughout the growth cycle. The culture was monitored by OD and viable cell count. The transduction frequency was calculated from the number of viable cells and transductant colonies from each sample. ○, viable cell count; ▲, OD (420 nm); ■, transductant colonies observed; □, transducing frequency (viable cell count/transductants).

Table 8.2

The effect of dilution of the recipient thy$^-$culture on the frequency of thy$^+$transduction frequency

Culture OD: 1.32
Transducing phage titre: 1×10^9 p.f.u. ml^{-1}

Culture dilution	Viable count ml	No. of transductant colonies	Transduction frequency
Neat	1.5×10^8	9	1.7×10^{-7}
0.75	1.125×10^8	13	8.6×10^{-6}
0.5	7.5×10	10	7.5×10^{-6}
0.15	3.75×10	23	1.6×10^{-6}
0.1	1.5×10^7	23	6.5×10^{-5}

Culture OD: 1.12
Transducing phage titre: 9.7×10^8 p.f.u. ml^{-1}

Culture dilution	Viable count ml	No. of transductant colonies	Transduction frequency
*Neat	$2 \times 9.5 \times 10^7$	24	7.7×10^{-6}
Neat	9.5×10^7	10	9.5×10^{-6}
0.75	7.12×10^7	39	1.8×10^{-6}
0.5	4.75×10^7	19	2.5×10^{-6}
0.2	1.9×10^7	14	1.3×10^{-6}
0.1	9.5×10^6	8	1.2×10^{-6}

Legend

1 ml of phage suspension (JS 017) was added to 1 ml of the recipient cell population (neat or diluted with L broth as indicated). *2 ml of neat recipient population was added to 1 ml of phage suspension.

8.2.5 The effect of bacteriophage concentration on transduction frequency

The effect of phage concentration on the efficiency of transduction is presented in Fig. 8.2. An increase in p.f.u. from 10^8 - 10^9 resulted in a three to four fold increase in the number of *thy*$^+$ transductants. The higher number of transductants isolated using cells taken at a lower OD supports the evidence for higher transduction frequencies with early log phase cells. An m.o.i. of ten was found to be the most suitable ratio for obtaining an acceptable number of transductants.

8.2.6 The effect of u.v. irradiation on transducing frequency

Irradiation of phage for a period of 30 s, 60 s, 120 s and 180 s (with 6.8 J m^{-2} s^{-1}) significantly reduced the level of transductants and p.f.u. (Fig. 8.3). Exposure for 120 s reduced the numbers of transductants by 96% and p.f.u. by 99.99%.

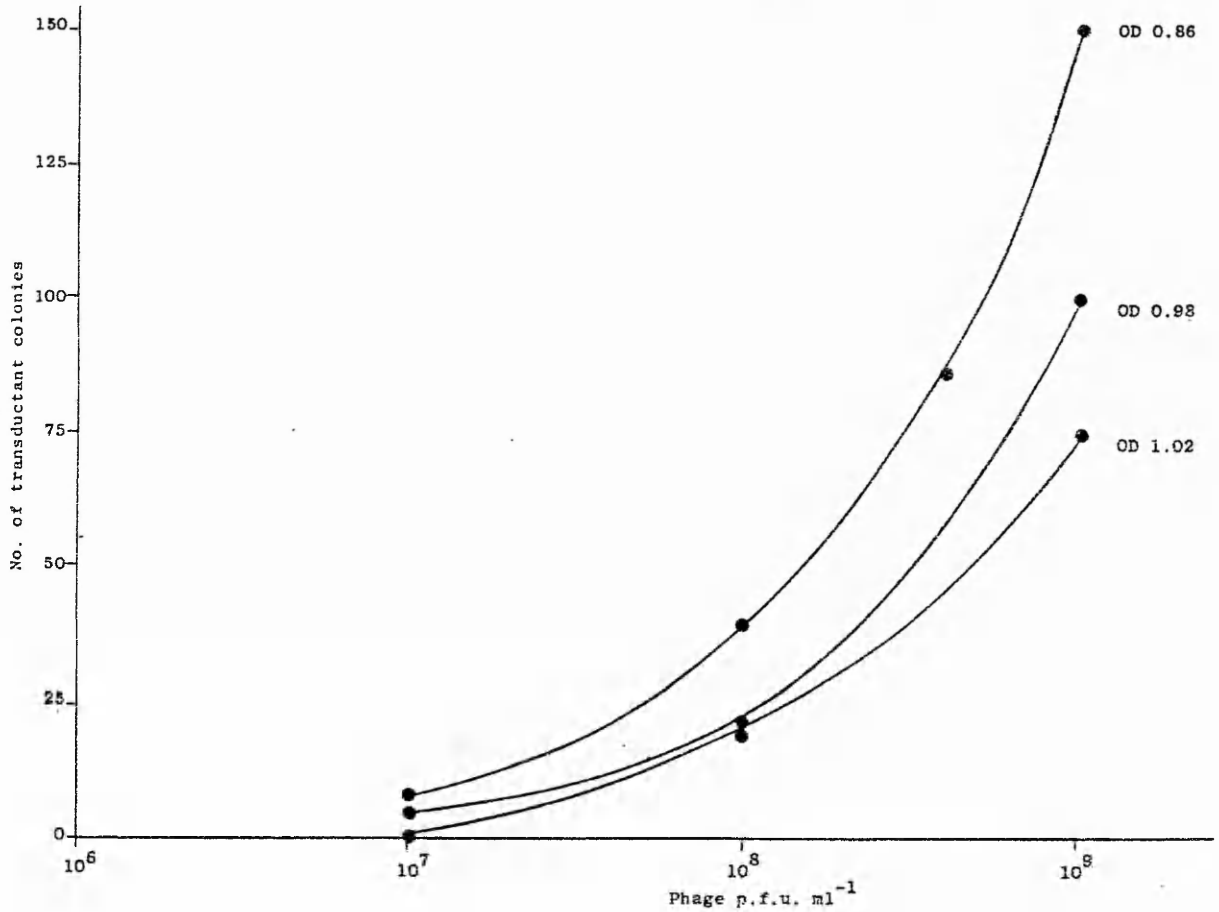
8.3 ANALYSIS OF THE *B. CALDOTENAX* *thy*$^-$ TRANSDUCING SYSTEM

8.3.1 Sub-culture of *thy*$^+$ transductant and revertant colonies

Transductant and revertant colonies of *B. caldotenax* from six independent transduction studies were replica plated (Table 8.2) onto selective media (Cal II minimal agar; Cal II minimal agar + thymine (50 µg ml^{-1}); Cal II minimal agar + thymine (50 µg ml^{-1}) + trimethoprim (10 µg ml^{-1}); and TSBA). The number of putative transductant colonies recorded from the test plates did not take into account the occurrence of *thy* revertant colonies. In the first two studies (Table 8.2) the number of revertants (estimated by the absence of colonies on the control plate) was negligible and the colonies appearing on the test plates were considered to be true transductants. Further analysis indicated that their growth on selective media was apparently identical to that of normal wild type cells, since they were able to grow in the presence or absence of thymine and were inhibited by trimethoprim. Examination of the putative revertant colonies from studies 6 and 7 (Table 8.3) indicated that these were not true revertants since they were unable to survive following sub-culture to fresh minimal media. Similarly, following the sub-culture of the putative transductants, 30% were found to still require thymine for normal growth. These were considered to be unstable revertants rather than unstable transductants.

Figure 8.2

The effect of phage concentration on transduction frequency

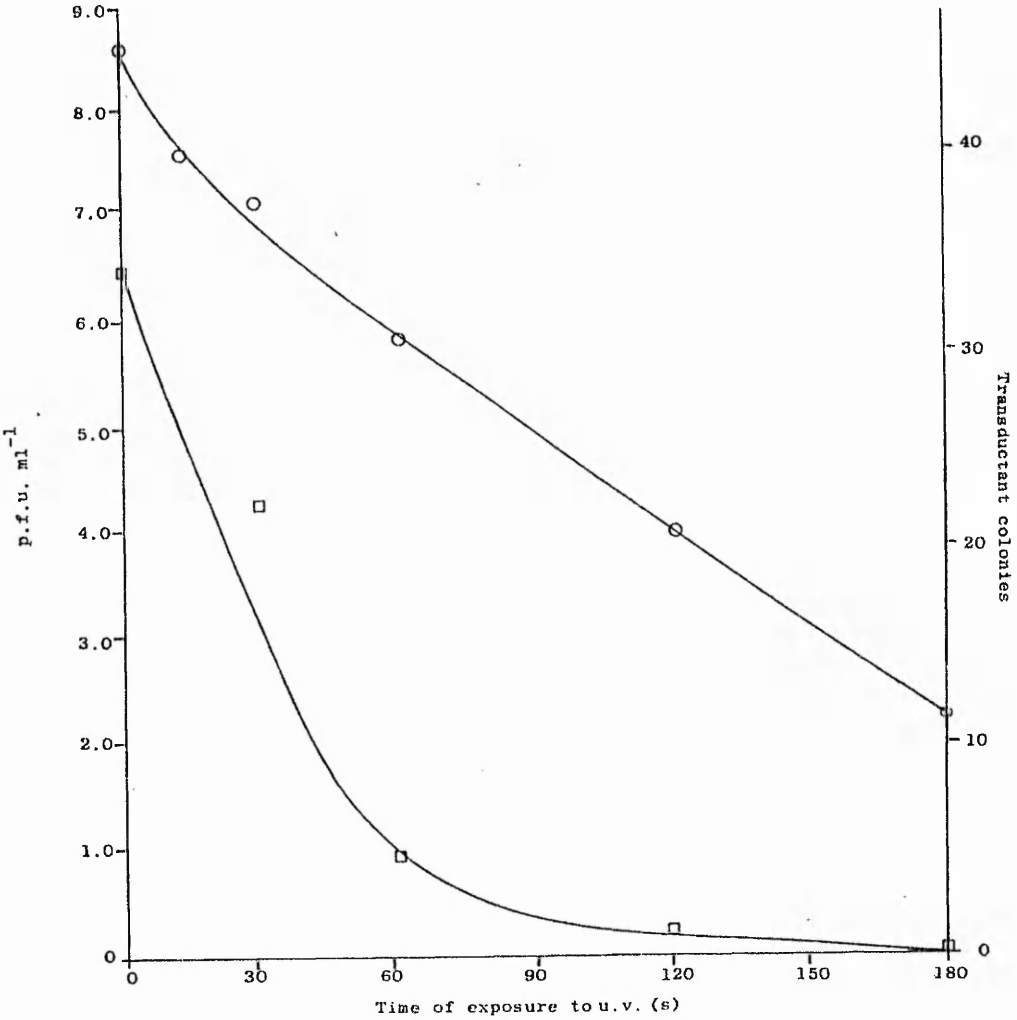


Legend

A stock concentration (10^9 p.f.u. ml^{-1}) of phage JS 017 was diluted and used to transduce a thymine auxotroph of B. caldotenax in Cal II minimal medium. Three samples of the culture taken at different OD were examined for transduction.

Figure 8.3

The effect of u.v. irradiation of phage JS 017 on the p.f.u. and transduction of thymine auxotrophs



Legend

A suspension of phage JS 017 was irradiated with a u.v. dose of 6.8 J m^{-2} s^{-1} for 180 s. Samples were examined for p.f.u. and transducing ability on thymine auxotrophs of B. caldotenax. ○, p.f.u.; □, transductant colonies.

Table 8.3

Analysis of B. caldotenax thy$^+$ transductants and revertants

	Number of transductant and revertant thy$^+$ colonies on initial Cal II minimal plates	Thymine mutant group	Replica plating media			
			Cal II minimal	Cal II minimal + thymine	Cal II minimal + thymine + trimethoprim	TSBA
1)	22 transductants	1	22	22	0	22
	2 revertants		2	2	0	2
2)	20 transductants	8	20	20	0	20
	1 revertant		1	1	0	1
3)	65 transductants	5	65	NT	0	65
	107 trevertants		100	NT	0	100
4)	23 transductants	7	20	NT	0	20
	15 revertants		15	NT	0	15
5)	73 transductants	High thy requirer	50	55	21	69
	40 revertants*		0	30	NT	0
6)	150 transductants	High thy requirer	103	140	NT	NT
	50 revertants		1	50	NT	NT

Legend

Table 8.3 presents the data from six independent transduction experiments using five different thymine auxotrophs of B. caldotenax. The colonies appearing on the transduction test plates and the revertant control plates (Cal II minimal agar) were replica plated to four new plates to assess the thy genotype of the colonies.

NT; not tested.

*The revertant count was ten colonies after 2 days incubation and 40 colonies after 3 days.

Why unstable revertants should appear is not clear, although more stable reversions do occur as shown in other studies (1, 2, 3 and 4, Table 8.3). In these cases the revertants behaved as wild type cells, capable of growth without exogenous thymine but sensitive to the presence of trimethoprim.

8.3.2 Cell morphology of transductants

Cells from 17 thy$^+$ transductants and two revertant colonies from Cal II minimal agar were Gram stained and examined microscopically. The revertants and six of the transductants had the normal wild type cell morphology. The remaining 11, showed evidence of thymine starvation with cell elongation and filament formation. Examination of replica plated transductant colonies showed no evidence of cell elongation or filament formation.

8.3.3 The effect of DNase on transducing ability

Pre-incubation of phage with (2, 10 and 25 mg ml^{-1}) DNase had no effect on the number of transductants (Table 8.5).

8.3.4 Examination of transductants for the presence of lysogenic phage

Overlayering of sub-cultured transductants on TSBA with wild type B. caldotenax indicated a hazy halo around the transduced colonies. Plating of supernatant from 10 ml cultures of transductants (inoculated following three successive transfers on TSBA) indicated the presence of phage in over 50% of the transductant colonies examined (Table 8.4). It appears that at least half of the transductants retain a close association with phage JS 017 as stable or unstable lysogens.

8.3.5 Transduction using phages from a thymine auxotrophic strain of B. caldotenax

Transduction was not evident using phage suspensions prepared from lysates of a thymine auxotroph of B. caldotenax (Table 8.6).

8.3.6 Transduction using phage isolated from a thy$^+$ transductant

Transduction using phage JS 017 re-isolated from a thy$^+$ transductant did not appear to enhance the yield of thy$^+$ transductants (Table 8.7).

8.3.7 Transduction with different groups of thy mutants

Selection of thymine requiring mutants on agar supplemented with thymine and trimethoprim produced mutants requiring different conc. of thymine for normal growth (Table 7.1). Mutants from four groups were examined for transduction to the thy$^+$ state. The results (Table 8.8) show that transduction occurred with each type of mutant examined. Since the phage titres were not identical and cultures were

Table 8.4

Isolation of phage JS 017 from 21 thy$^+$ transductants

Transductant	p.f.u. 0.1 ml^{-1} of supernatant
1	1
2	0
3	12
4	0
5	0
6	uncountable
7	1
8	2
9	0
10	0
11	uncountable
12	0
13	uncountable
14	0
15	0
16	uncountable
17	0
18	3
19	0
20	0
21	uncountable
B. caldotenax (wild type)	0
B. caldotenax (JS 017 lysogen)	uncountable

Legend

Following three successive sub-cultures of 21 thy$^+$ transductants on TSBA, colonies were inoculated to 10 ml of L broth and incubated at 60 ^{o}C overnight. 0.1 ml of the filtered culture supernatant was dropped to the surface of TSBA plates and overlaid with trypticase soft agar seeded with wild type B. caldotenax. B. caldotenax wild type and a B. caldotenax JS 017 lysogen were included as controls.

Table 8.5

The effect of pre-treatment of transducing phage with DNase

Phage preparation	p.f.u. ml^{-1}	thy$^+$ transductants
JS 017 + 2 mg ml^{-1} DNase	1×10^9	44
JS 017 + 10 mg ml^{-1} DNase	1×10^9	41
JS 017 + 25 mg ml^{-1} DNase	1×10^9	52
JS 017 + No DNase	1×10^9	48

Table 8.6

Transduction using phage harvested from thy$^-$ and thy$^+$ hosts

Phage preparation	p.f.u. ml^{-1}	thy$^+$ transductants
JS 017 harvested from thy$^-$ strain	1×10^9	0
JS 017 harvested from thy$^-$ strain	1×10^8	0
JS 017 harvested from thy$^+$ strain	1×10^9	38
JS 017 harvested from thy$^+$ strain	1×10^8	8

Table 8.7

Transduction using phage isolated from a thy$^+$ transductant

Phage preparation	p.f.u. ml^{-1}	thy$^+$ transductants
JS 017 isolated from thy$^+$ transductant	1×10^9	35
JS 017 isolated from thy$^+$ transductant	1×10^8	6
JS 017 normal preparation	1×10^9	41
JS 017 normal preparation	1×10^8	6

Legend (Table 8.5, 8.6, 8.7)

All transductions were carried out using the method previously described, transductants were plated on Cal II minimal agar.

Table 8.8

Transduction to thy$^+$ of four distinct groups of thymine auxotrophic strains of B. caldotenax

Thymine mutant group	p.f.u. ml^{-1}	Culture OD	Number of transductants
1 (mutant 6A)	1×10^9	1.04	4
	1×10^9	1.2	32
1 (mutant 7A)	4×10^8	1.2	83
	9.2×10^8	1.6	18
1 (mutant 8A)	9.2×10^8	1.15	20
	9.2×10^8	1.35	19
14 (mutant 15A)	1×10^9	1.1	60
15 (mutant 9A)	1×10^9	1.12	20, 30
16 (mutant 1A)	1×10^9	1.12	28, 35
	5×10^9	1.14	22
	1×10^9	1.2	11
	9.2×10^8	1.32	9
	9.2×10^8	1.12	32, 33
16 (mutant 5A)	9.2×10^8	1.76	40

Legend

Transductions were performed as normal using thymine deficient strains which varied in their minimum requirement of thymine for normal growth and in their resistance to trimethoprim.

Group 1 mutants require 0.5 μg ml^{-1} of thymine.

Group 14 mutants grow weakly on 2 μg ml^{-1} of thymine.

Group 15 mutants require 5 μg ml^{-1} of thymine and are resistant to 200 μg ml^{-1} of trimethoprim.

Group 16 mutants require 5 μg ml^{-1} and are sensitive to 200 μg ml^{-1} of trimethoprim.

Table 8.9

Inter strain thy$^+$ transductions

Source of transducing preparation	p.f.u. ml^{-1}	thy$^-$ recipient	Number of transductants
B. caldotenax	1×10^9	B. caldotenax*	28
		B. caldovelox	23
B. caldovelox	1×10^9	B. caldovelox	24
		B. caldotenax	18
B. caldotenax	5×10^9	B. caldotenax*	69
		B. thermocatenulatus	0
B. thermocatenulatus	4×10^9	B. caldotenax*	0
B. stearo. ATCC 8005	6×10^9	B. caldotenax*	0

Legend

Transduction of thymine auxotrophs of B. caldotenax, B. caldovelox and B. thermocatenulatus was examined using phage lysates produced on different hosts. *, Mutant 1A from thymine mutant group 16 (Table 7.1).

grown to differing cell densities it was not clear if transduction frequencies were the same for each mutant although the evidence suggested that they were similar.

8.3.8 Inter strain thy⁻ transduction

Transducing phages harvested from wild type B. caldotenax were able to transduce thymine auxotrophic strains of B. caldotenax and B. caldovelox with a similar frequency (Table 8.9); similar results were obtained using phage harvested from wild type B. caldovelox. Transduction of B. thermocatenulatus was unsuccessful using phage from B. caldotenax. Attempts to use preparations of phage JS 017 harvested from B. thermocatenulatus and B. stearothermophilus ATCC 8005 to transduce thy⁻ B. caldotenax were unsuccessful.

8.4 TRANSDUCTION OF his, ilv, met AND ade AUXOTROPHS OF B. CALDOTENAX

Attempts to transduce his, ilv and ade auxotrophs of B. caldotenax were unsuccessful (Table 8.10). The ilv mutation was too unstable during transduction studies to draw any conclusions. Transduction of his auxotrophy was examined at three levels of cell density and at three m.o.i. The mutation appeared stable and no revertant colonies were visible on control plates although transductant colonies were not observed. Transductions of adenine auxotrophic strains were unsuccessful, these mutants appeared slightly unstable with reversion rates in the region of 5×10^{-6}.

Only thy and met markers were successfully transduced by phage JS 017. The transduction frequencies were in the region of 10^{-7}.

Cotransduction of his/thy and ilv/thy double mutants was unsuccessful (Table 8.11), although met/thy mutations cotransduced with a frequency of >90%. On examination fifteen thymine transductants were found to be prototrophic for methionine, while 38 methionine transductants revealed that 36 were thymine prototrophs.

Table 8.10

Transduction of amino acid and adenine auxotrophs of B. caldotenax

Mutant	p.f.u.	OD	Transductants	Reversion
met$^-$	3×10^9	1.26	10*	6
		1.39	135*	2
		1.55	200*	2
met$^-$ (2)	9.2×10^8	1.46	8, 4	0
met$^-$ (1)	9.2×10^8	1.42	16, 20	1
	9.7×10^9	1.42	8	0
met$^-$ (1)	?	1.3	94	0
met$^-$ (2)	?	1.4	17	0
his$^-$	9.7×10^9	1.13	0	0
	9.7×10^8	1.13	0	0
	9.7×10^7	1.13	0	0
	9.7×10^9	1.2	0	0
	9.7×10^8	1.2	0	0
	9.7×10^7	1.2	0	0
	9.7×10^9	1.38	0	0
	9.7×10^8	1.38	0	0
	9.7×10^7	1.38	0	0
ilv$^-$	1×10^9	1.58	0	90
ade$^-$ (Pur B)	1×10^9	1.12	0	73

Legend

All colonies were counted following 2 days incubation at 60 °C.

Transductant colonies are listed following subtraction of revertants on the control plates. All phage controls were negative.

*Replica plating to fresh minimal agar indicated no surviving methionine prototrophs.

Transduction of *ilv* markers were not observed due to the heavy reversion rates which masked any possible transductants.

Table 8.11

Cotransduction of thy with met, his and ilv

Mutant	Plating media	Transductant colonies	Revertants	Replica plating of transductant colonies									
				Cal II Minimal	Cal II +thy	Cal II +met	Cal II +met/thy	Cal II +his/ilv	Cal II +his	Cal II +ilv	Cal II his/thy	Cal II ilv/thy	TSBA
thy⁻ met⁻	Minimal	14, 15	0 0	14, 15	14, 15	14, 15	14, 15	-	-	-	-	-	-
	thy⁺	7,	15	0 0	-17	-21	-21	-100+	-	-	-	-	-
	met⁺	18, 20	0 0	18, 18	11, 19	18, 19	25, 27	-	-	-	-	-	-
thy⁻ ilv⁻ his⁻	Minimal	0	0										
	his/thy	4	9										
	his/ilv	20	3	0	-	-	-	20	-	-	0	0	20
	ilv/thy	56	206										
thy⁻ ilv⁻ his⁻	his/ilv	63	6	0	-	-	-	56	0	0	-	-	-
thy⁻ ilv⁻	ilv	30	1	0	-	-	-	-	-	28	-	28	28

Legend

All phage control plates were negative. Colonies were counted after 48 h incubation at 60 °C. Transductions were carried out as described in Section 2.21.2. The transduced cells were plated to minimal and defined Cal II agar. Following incubation for 48 h at 60 °C the transductant colonies were replica plated to a range of defined media for evidence of cotransduction. The phage titre was 1×10^9 p.f.u. ml^{-1} and the culture OD was 1.2.

CHAPTER 9

DISCUSSION

9. DISCUSSION

The most recent comprehensive study of thermophilic aerobic sporeformers included many of the isolates reported up to the early sixties (Walker & Wolf, 1971). Since then, several new thermophilic Bacillus isolates have been reported, including the starch negative strains of Daron (1967) and Epstein and Grossowicz (1969); B. acidocaldarius (Darland & Brock, 1971); B. sphaericus, B. thermodenitrificans (Klaushofer & Hollaus, 1970); B. caldotenax, B. caldovelox and B. caldolyticus (Heinen & Heinen, 1972); B. thermocatenulatus (Golovacheva et al., 1975); B. megaterium (Stahl & Olsson, 1977) and B. schlegelii (Schenk & Aragno, 1979). Several of these isolates have been included in this study together with standard reference strains and 80 new isolates from a wide range of localities.

Thermophilic aerobic sporeformers were readily isolated from a variety of source materials indicating their widespread occurrence in nature. Preliminary studies indicated a wide variation in colony morphology from flat, rough, erose, irregular colonies (typified by DSM 465) to raised, smooth, circular, entire colonies (typified by strain LO_2) and convex, smooth, circular, entire colonies (typified by B. stearothermophilus NCA 1503 and B. caldotenax). Several isolates showed degrees of spreading varying from strain LUDA T141, where a small number of colonies appeared to produce spreading areas of growth, to strain RS 5 which covered the entire surface of the plate.

Biochemical and physiological tests showed considerable heterogeneity amongst the strains, ranging from those hydrolysing starch, casein, and gelatin, and fermenting a wide range of carbohydrates, to those with no apparent amylolytic or proteolytic activity and fermenting very few carbohydrates. The absence of amylolytic activity in 25% of the strains isolated is significant. Prior to the reports of Daron (1967) and Epstein and Grossowicz (1969), B. stearothermophilus, as classified and described by Gibson and Gordon (1974) in the 8th edition of Bergey's manual, was believed to hydrolyse starch. The collection of 87 thermophilic Bacillus isolates assembled by Klaushofer and Hollaus (1970) contained 15 starch negative strains; Walker and Wolf (1971) isolated 40 starch negative strains from milk and soil samples. The ATCC, NCTC, NCIB and DSM culture collections jointly hold 19 different isolates classified as B. stearothermophilus, none of which are starch negative. Most of the earlier isolates of

thermophilic aerobic sporeformers were spoilage organisms in the canning or dairy industry. Many of the original strains held by the ATCC were initially part of the collection built up by the National Canners Association (NCA) in Washington. Since the minimum and maximum growth temperatures of the starch negative strains (Table 3.8 and 3.9) (Klaushofer & Hollaus, 1970; Walker & Wolf, 1971) are generally lower than starch positive isolates, it suggests that they may be more susceptible to heat sterilisation and thus less likely to occur as spoilage organisms in the canning and dairy industry. No comparative studies of the thermal stability of spores from different taxonomic groups of B. stearothermophilus appear to have been carried out. The 6th edition of Bergey's manual described five isolates of Bacillus able to grow at 65 °C but unable to hydrolyse starch (B. cylindricus, B. calidus, B. robustus, B. non diastaticus, B. thermoalimentophilus).

Numerical taxonomic studies of 102 strains of thermophilic Bacillus (Section 3.2) using the similarity coefficient of Gower (1971) and single linkage analysis, resulted in their allocation to seven clusters:

Cluster (1). This was the largest cluster comprising 32 strains and was divided into two minor sub-groups (1a) and (1b). The major distinguishing characteristics of the strains in sub-group (1a) were; lack of amylolytic activity, little fermentative activity and spreading growth on TSBA. This group appear to correspond with group IA and IB of Klaushofer and Hollaus (1970). The majority (74%) of the strains in sub-group (1b) were starch negative, but they fermented a range of sugars, including fructose (84%), glycerol (74%), inositol (84%), maltose (90%), mannose (74%), mannitol (84%) salicin (79%) and sorbitol (90%). These reactions closely resemble the description given by Walker and Wolf to the organisms in their group 2. Strain LUDA T141 from Walker and Wolf's group 2 clustered with this group, as did the starch negative strain EP 136, isolated by Epstein and Grossowicz (1969). Citrate was not utilised by strains in sub-group (1a), but was utilised by 84% of the strains in sub-group (1b). None of the strains in group IA and IB of Klaushofer and Hollaus utilised citrate; Walker and Wolf did not study citrate utilisation. Strain EP 136 (the isolate of Epstein & Grossowicz, (1969)), which had been reported citrate negative (Gordon et al., 1973), was found to be citrate positive (Table 3.6). The starch negative strain reported by Daron (1967) which closely resembles

the isolate of Epstein and Grossowicz was originally reported to utilise citrate.

Cluster (2). This was the second largest cluster comprising 26 strains allocated to two homogeneous sub-groups (2a) and (2b) and a third group (2c) which comprised a heterogeneous group of strains with characteristics of the other two sub-groups. Sub-group (2a) was composed of seven strains: B. kaustophilus (ATCC 8005) isolated by Prickett (1928); B. caldotenax, B. caldovelox and B. caldolyticus isolated by Heinen and Heinen (1972); and strains RS 7, RS 15 and RS 16; this group corresponds with group 1b3 of Walker and Wolf (1971) and group IIIB of Klaushofer and Hollaus (1970). Two of the major characters differentiating sub-groups (2a) and (2b) are hippurate hydrolysis and citrate utilisation by sub-group (2a). Neither Walker and Wolf nor Klaushofer and Hollaus examined the hydrolysis of hippurate, but the latter reported citrate utilisation was variable in their group IIIB.

Sub-group (2b) included six marker strains, B. stearothermophilus NCA 26 (Donk, 1920) (LUDA T210), NCA 1503, NCIB 8919, NCA 1518 (LUDA T214), ATCC 10149 and NCTC 10003. All members of this group fit the classical definition of B. stearothermophilus (Gordon et al., 1973; Gibson & Gordon, 1974) and correspond to group 3a of Walker and Wolf and to group IIIA of Klaushofer and Hollaus.

Sub-group (2c) included the heterogeneous strains ATCC 12016, DSM 456 (from group IIIA of Klaushofer and Hollaus) LUDA T42 and LUDA T60 (from group 1b1 of Walker and Wolf) and B. thermocatenulatus (Golovacheva et al., 1975).

Cluster (3). The 12 strains in this group were typified by flat, rough, irregular, erose colonies. All strains reduced nitrate and nitrite to gas under aerobic conditions, and most (83%) utilised citrate. The majority of strains (73%) also reduced nitrate to gas under anaerobic conditions. Cluster (3) included two strains of B. thermodenitrificans (DSM 465 and DSM 466), isolated by Klaushofer and Hollaus (1970), and LUDA T22 isolated by Walker and Wolf (1971). Cluster (3) corresponds to group II of Klaushofer and Hollaus and to group 1a of Walker and Wolf. Klaushofer and Hollaus reported the strains in their group II (including DSM 465 and DSM 466) to be citrate negative, however, in this study 83% of strains in cluster (3) utilised citrate, including DSM 465 and DSM 466. This disparity is

due to the fact that Klaushofer and Hollaus incubated their biochemical and physiological tests at 65 °C, whereas in this study and in the studies of Gordon et al. (1973) and of Walker and Wolf (1971), tests were incubated at 55 °C. Re-examination of citrate utilisation by DSM 465 and DSM 466 at 65 °C indicated both the strains to be citrate negative. The strains in cluster (3) appeared similar to the description of Denitrobacterium thermophilum (Ambroz, 1913) (Rods 3.5 - 7 µm by 1 - 1.8 µm, terminal oval spores with slight swelling of the sporangium, colonies on agar resembling those of B. mycoides, nitrate reduced to gas, no hydrolysis of gelatin or starch, temperature range 37 °C to 65 - 70 °C.). Organisms of this description were not examined by Smith et al. (1952) or Gordon et al. (1973). Mishustin (1950) included this organism in his identification key, but re-named it B. thermodenitrificans to fit the accepted nomenclature. Golovacheva et al. (1965) described a similar organism isolated from a hot gas well near Mount Yangan-Tau in the southern Urals of the USSR and called their isolate B. thermodenitrificans. Klaushofer and Hollaus (1970) referred to Ambroz's description of Denitrobacterium thermophilum and considered nine of their isolates to be similar. They accepted the name proposed by Mishustin (1950) and named their isolates B. thermodenitrificans. The isolates of Golovacheva et al. (1965) and Klaushofer and Hollaus (1970) differ from Ambroz's description by their reaction with starch. Ambroz (1913) reported no hydrolysis, whereas Golovacheva et al. (1965) reported weak hydrolysis, while Klaushofer and Hollaus (1970) reported positive hydrolysis. Walker and Wolf's (1971) group 1a, which appears similar to Ambroz's description, showed restricted hydrolysis of starch. Of the ten strains in cluster (3), nine showed restricted hydrolysis and one gave no evidence of starch hydrolysis.

While cluster (3) did not represent all of the denitrifying strains, it did comprise a relatively homogeneous group of strains with little overall similarity with any other cluster. This cluster was considered to represent the species of Denitrobacterium thermophilum initially described by Ambroz (1913) and later re-named B. thermodenitrificans by Mishustin (1950).

Cluster (4). The five strains in this group had strong amylolytic and proteolytic activity and were able to grow at 25 °C. The colonies

were raised, erose, shiny and circular following overnight incubation on TSBA and became mucoid after several days incubation at room temperature. The five isolates which all produced acetoin but did not utilise citrate or propionate at 55 °C, were initially considered to be strains of B. licheniformis; Gordon et al. (1973) reported that acetoin production and utilisation of citrate and propionate were characteristic of B. licheniformis. Re-testing these five isolates for citrate and propionate utilisation at 37 °C showed that all the strains were positive (Table 3.14). Studies at higher growth temperatures indicated that citrate utilisation occurred at 2 - 5 °C below the maximum growth temperature. RS 45 and LO_2, two other isolates examined, had relatively high similarity with members of cluster (4), and it is significant that strain LO_2 was independently identified as a strain of B. licheniformis (R. Berkeley, pers. comm.).

Cluster (5). All five strains in this group grew at 70 °C but did not grow at 37 °C. All strains denitrified nitrite to gas and four strains denitrified nitrate to gas; four strains hydrolysed hippurate and utilised citrate. This group showed closest similarity to the strains in cluster (2a) (the B. kaustophilus group), but had several distinguishing characters: all five strains hydrolysed tyrosine producing a brown pigment throughout the medium, and four strains failed to grow in NB at pH 6.5. This group appears to be similar to sub-group 1b2 of Walker and Wolf (1971) which comprised ten strains; nine of which were isolated from milk by Smith et al. (1952) and one was isolated from compost in Aberdeen (Webley, 1947). The five strains of cluster (5) were soil isolates from: Calcutta (three); Les Eaux, France (one); and Bracknel, UK (one). None of the thermophilic strains studied by Gordon et al. (1973) hydrolysed tyrosine; Gyllenberg (1951), Stark and Tetrault (1952), Allen (1953), Grinsted and Clegg (1955) and Walker and Wolf (1971) did not however include tyrosine hydrolysis in their studies. Golovacheva et al. (1965) did not report the examination of their B. stearothermophilus isolates on tyrosine agar either, although they did report the production of a brown pigment in tyrosine agar by a thermophilic isolate of B. megaterium (later reclassified as B. thermocatenulatus by Golovacheva et al., 1975). Klaushofer and Hollaus (1970) reported the hydrolysis of tyrosine by their group II organisms (B. thermodenitrificans) but this group was also reported to be tolerant to 5% (w/v) NaCl, while all of cluster (5) were

sensitive to 3% (w/v) NaCl. The evidence suggests that cluster (5) represents a distinct taxonomic group of Bacillus thermophiles.

Cluster (6). This group comprised three strains which appear to be mesophilic with growth at 25 °C but no growth at 65 °C. Members of this mesophilic group have not been studied further and are at present unidentified.

Cluster (7). This group comprised three strains, two of which were B. coagulans marker strains.

The seven clusters and sub-groups were defined from the similarity matrix at the level of 80 - 85% similarity; the dendrogram, however, while clearly delineating clusters (1a), (1b), (3) and (4), did not define cluster (2) with such clarity. The formation of long straggly clusters, similar to that shown for cluster (2), using single linkage analysis, was discussed by Sneath and Sokal (1973). The use of a clustering method such as UPGMA (Unweighted Pair Group Method with Averages) based on the average similarities of strains within the clusters may present more clearly defined clusters.

The examination of an alternative method of coding, which scores matching negatives as 0, or the use of an alternative similarity coefficient such as S_{SM} where all data is coded as two state, may prove useful in confirming the trends shown in this present study.

Twelve strains of thermophilic Bacillus from this study were examined by pyrolysis mass spectrometry by Dr. C. Gutteridge. The data was analysed by average linkage cluster analysis and is presented as a dendrogram (Fig. 3.4). Similar relationships were indicated between clusters (1), (2) and (7) as previously found in the numerical taxonomy study (Fig. 3.2). Strains NCA 1503 and NW 10, identified as members of B. stearothermophilus (Donk, 1920) (cluster (2b)) clustered at 98% similarity. B. caldovelox and B. caldotenax, members of the B. kaustophilus (Prickett, 1928) group (cluster (2a)), clustered together at 97% similarity and with strains from cluster (2b) at 95% similarity. B. caldolyticus, a third member of cluster (2a), had 93% similarity with these two groups.

Although the sub-groups of cluster (2) were not clearly defined, cluster (2) as a whole joined at 90% similarity and the two strains examined from cluster (1) joined at 91% similarity. Clusters (1) and (2) joined at the 85% similarity level and joined the B. coagulans group (cluster (7)) at 80% similarity. Pyrolysis mass spectrometry was thus

clearly demonstrated as a useful technique for distinguishing the three clusters included in this pilot study and in co-operation with Dr. C. Gutteridge, it is intended to extend this study to include all the strains examined by numerical taxonomy.

Evidence from constitutive enzyme production data (API ZYM; Table 3.15) and esterase enzyme analysis (Table 3.16) using ten strains, indicated relationships between the strains similar to those demonstrated in the larger numerical study.

Bacteriocin production has been reported in many species and its ability to inhibit the growth of related strains has found application in the identification of several groups of organisms such as Ps. aeruginosa (Govan & Gillies, 1969), Proteus sp. (Cradock-Watson, 1965) and Shigella sonne (Naito et al., 1966). The production of bacteriocins from B. stearothermophilus was first reported by Shafia (1966). He demonstrated bacteriocin production from 12 out of 22 strains of B. stearothermophilus. None of these inhibited mesophilic strains of Bacillus although each inhibited one or more strains of B. stearothermophilus. Of the 80 thermophilic strains of Bacillus examined in this study for the production of thermocin, 67 produced growth inhibitors to one or more of 15 test strains (Table 6.1). Bradley (1967) considered bacteriocins could be divided into two very distinct groups based on their ability to be sedimented by ultracentrifugation or resolved by electron microscopy.

Studies of thermocins from ten different strains indicated that two were large molecules which could be sedimented at 40,000 x g and four were small compounds unable to be sedimented at 40,000 x g; the size of four others remained unresolved. Thermocin 93, which appears to be a small molecular weight protein (13,500 Daltons), is similar to the slightly larger thermocin (20,000 Daltons) produced by B. stearothermophilus NU 10 (Yule & Barridge, 1976). Strain RS 93 was observed to carry a plasmid (Sharp et al., 1979), but it has not been confirmed if this codes for thermocin production. Yule and Barridge (1976) reported no evidence of a plasmid in B. stearothermophilus NU 10. Both these thermocins were sensitive to proteolytic enzymes and had similar heat stability. Their activity range was found to be similar (Table 3.19), although thermocin NU 10 was the only bacteriocin to inhibit the growth of the B. coagulans group.

Bacteriocin typing of the thermophilic strains, examined in the numerical taxonomy study, supported the various taxonomic groups previously established. The two groups of starch negative thermophiles in cluster (1) were insensitive to the majority of the bacteriocins used. In contrast, cluster (2), and in particular sub-group (2a), was sensitive to the majority of the bacteriocins. Sub-group (2a) was sensitive to thermocin RS 53, whereas all of the strains in sub-group (2b) were resistant.

Cluster (3) (B. thermodenitrificans) showed a varied pattern of sensitivity. Cluster (4) (B. licheniformis) was relatively insensitive to all of the thermocins used and no thermocin inhibited more than 40% of the strains. Cluster (5) was the most sensitive group and was very similar to group (2a) in its sensitivity pattern. Bacteriocin typing, using the well diffusion method, showed possibilities as a rapid and relatively simple method for identifying thermophilic strains of Bacillus. The preparation of suitable crude bacteriocin for typing is time consuming and the possibility of freeze drying or deep freezing large stocks is worth consideration. Standardisation of bacteriocin titres was not carried out but this would be desirable to ensure similar levels of activity in new bacteriocin preparations.

The mole % G+C of 27 of the strains examined in the numerical study was determined from an examination of the Tm; the strains examined showed a range of 42 - 71.5% G+C. From theoretical studies, De Ley (1969) showed that DNA sequences differing by 18 - 30% G+C had almost no common nucleotide sequences. Such organisms were considered to be taxonomically unrelated. Differences in the % G+C of strains of over 5% are generally considered to indicate different species, while over 10% indicates different genera (Jones & Sneath, 1970; Bradley & Mordarski, 1976).

The % G+C of the genus Bacillus ranges from 32% in B. cereus to 69% in B. thermocatenulatus. If the % G+C of organisms within a genus fall within a range of 10%, then the genus Bacillus requires division into a minimum of three or four genera. On the basis of numerical taxonomic studies, Priest (1981) arranged the genus Bacillus into seven taxons of which the mole % G+C of strains within each taxon fell within a range of 12%. Priest allocated B. stearothermophilus and B. thermodenitrificans with mole % G+C of 41 - 53% to taxon 2; B. coagulans with a % G+C of 44 - 55% to taxon 6 and B. thermocatenulatus and

B. acidocaldarius with % G+C of 61 - 67% to taxon 7.

Comparison of the clusters established in the numerical study (Fig. 3.1) with the % G+C of representative strains, indicated a relatively high level of homogeneity in clusters (1b), (2a) and (2b). Cluster (1b) represented by the three strains EP 136, RS 93 and LUDA 141 had a mole % G+C range of 42 - 46%. Cluster (2a) (B. kaustophilus group) represented by the four strains B. caldotenax, B. caldovelox, B. caldolyticus and ATCC 8005 had a % G+C range of 60 - 65%: Frank et al. (1975) independently estimated the % G+C of B. caldotenax to be 67.2%. Cluster (2b) (B. stearothermophilus, Donk) was the most homogeneous group comprising the four strains NCA 1503, NW 10, LUDA 210 (NCA 26) and LUDA 214 (NCA 1518) with a % G+C of 58 - 59%: Frank et al. (1975) reported the % G+C of NCA 1503 to be 56.5%. The heterogeneous group of strains in cluster (2c) showed similar heterogeneity with the % G+C ranging from 51 - 70%. This group included B. thermocatenulatus with a % G+C of 70%, close to the figure of 69% reported by Golovacheva et al. (1975). Strain RS 222, distinguished by its yellow colonies, had a similarly high % G+C of 69%. Three strains in cluster (2c) (EP 240, RS 85 and ATCC 12016) had % G+C values within the range of the B. kaustophilus group and one (LUDA T42) was much lower at (51%). Three strains examined from cluster (3) (B. thermodenitrificans) had % G+C values in the range 59 - 71.5%. Phenotypically this cluster appears relatively homogeneous; determinations of the % G+C content of other members of the cluster are necessary to establish the taxonomic validity of this group.

Strain LO_2, although not a member of cluster (4) (B. licheniformis), showed considerable homogeneity with this group and had a % G+C of 43%; this is within the range of 43 - 47% G+C reported for B. licheniformis (Gibson & Gordon, 1974). Two strains of B. coagulans examined were within the range of 47 - 48% G+C reported by Gibson and Gordon (1974).

Determinations of the mole % G+C were made before the completion of the numerical taxonomy study and consequently, none of the strains in clusters (1a), (5) or (6) were examined. The standing of cluster (5), as representing an independent group of obligate Bacillus thermophiles, still requires the support of mole % G+C data, as does the starch negative group (1a) which had little fermentative ability.

The DNA hybridisation data from 12 thermophilic strains of Bacillus gave support to the groups established by numerical methods.

Two strains in cluster (2a) (B. caldotenax and B. caldovelox) showed a high level of hybridisation, but a third member (B. caldolyticus) appeared to hybridise more closely with the DNA from NCA 1503 (cluster (2b)). DNA from NCA 1503 and strain NW 10 showed a high level of hybridisation supporting their presence together in cluster (2b). Other strains in cluster (2) (ATCC 12016 and EP 240) had high levels of DNA hybridisation with NCA 1503. The two strains examined from cluster (1) (RS 93 and EP 136) showed different associations. EP 136 indicated a relatively high level of hybridisation with DNA from B. coagulans, while RS 93 showed a high level of hybridisation with DNA from NCA 1503. Since these results do not fully support the data obtained from numerical taxonomic studies, bacteriocin typing, pyrolysis mass spectrometry and % G+C determinations, a more detailed hybridisation study of the 102 strains is required. The use of only two labelled obligate thermophilic reference DNA samples was restrictive and future examination must include labelled DNA from each of the taxonomic groups indicated by the numerical study.

The average size of the bacterial genome ranges from $1 - 5 \times 10^9$ Daltons representing $2 \times 10^6 - 1 \times 10^7$ nucleotide pairs (Bachman et al., 1976), enough to code for 2,000 - 5,000 structural proteins and enzymes (Sanderson, 1976). Plasmids vary in size from $1.5 \times 10^6 - 4 \times 10^8$ Daltons and carry the genetic information to code for up to 200 proteins (Duggleby et al., 1977). It is evident, therefore, that a pair of strains, with identical chromosomal genetic compliments, may differ phenotypically due to the characters determined by plasmid DNA. Two strains (LO_2 and RS 93) examined in the numerical study are known to carry plasmid DNA; strain LO_2 carries a Tc^r determinant (Bingham et al., 1980); RS 93 produces thermocin 93, but it has not been determined if this is a plasmid mediated characteristic. The presence of extrachromosomal DNA has not been reported to be a problem during determinations of mole % G+C of chromosomal DNA and both strain LO_2 and RS 93 appear to have values within the expected mole % G+C range of their particular taxonomic group.

The presence of plasmids in several species of Bacillus has been reported, although a conjugation system appears absent. Reanney (1976) considered that many Bacillus sp. were polylysogens, harbouring up to ten different prophages. Since phages are able to survive for long

periods in soil, he suggested that in species of Bacillus, selection pressures may have acted to promote genetic transfer through phage particles which become easily dispersed through the soil. Bacteriophages able to infect several groups of thermophilic Bacillus were readily isolated from soil and compost (Table 5.1); the examination of culture supernatant following the growth of thermophilic species of Bacillus (Table 5.2) indicated evidence of bacteriophage activity in over 60%.

Bacteriophage typing data (Table 3.20) and phage host range data (Table 5.5) indicated considerable group specificity in phage/host interactions. Phages infecting the organisms in group (1) were unable to infect representatives of groups (2a) or (2b) and vice versa. The only notable exception was strain RS 93 which was sensitive to phages generally considered specific to cluster (2a). Recent attempts to isolate from compost a bacteriophage, able to infect members of group (3) (B. thermodenitrificans), have been successful. Preliminary host range data have shown the phage able to lyse only two strains from cluster (3) (DSM 465 and LUDA T22) and unable to infect B. caldotenax and B. stearothermophilus NCA 1503.

Reanney (1976) considered that many of the reports regarding the specificity of the bacteriophage host range were possibly misleading, since the test cultures generally used were internationally recognised stock cultures, whose origins were ecologically and geographically distinct from those of the phage under examination. He reported studies of seven phages which were each able to infect strains of B. pumilus, B. sphaericus, B. stearothermophilus and B. subtilis, B. licheniformis and B. circulans. Reanney (1976) postulated that the genus Bacillus constitutes a single genospecies (Jones & Sneath, 1970) in which genes are interchanged through a complex series of host and bacteriophage interactions.

Genetic exchange between thermophilic strains of Bacillus has not previously been reported. Phage JS 017 was found able to transduce thymine auxotrophs of B. caldotenax (Section 8). The transducing frequency was similar whether the phages were prepared following lysis of B. caldotenax or B. caldovelox. These two strains can, therefore, be considered to be members of the same genospecies. Phage preparations from B. caldolyticus, B. kaustophilus and B. thermocatenulatus have so far proved unable to transduce the thymine marker of B. caldotenax, but studies are continuing. Three other strains from cluster (2a) (RS 7,

RS 15 and RS 16) have not yet been examined for sensitivity to phage JS 017.

Transformation of adenine prototrophs of B. caldotenax, using a plate transformation system, was examined using DNA from 25 thermophilic strains of Bacillus (Munster et al., 1982). As expected, the highest frequency of transformation (2×10^{-4}) was found using DNA from B. caldotenax (Table 9.1), but this was closely followed by DNA from B. caldovelox, B. caldolyticus, B. kaustophilus and unexpectedly from B. coagulans (ATCC 12245). Transformants were found at much lower frequencies using DNA from strains NCA 1503, ATCC 12016, NCTC 10003, B. thermocatenulatus, DSM 465 and RS 93. This work merits further study and it is intended to re-examine the transformation of thymine auxotrophs of B. caldotenax and B. caldovelox using DNA from all seven strains in cluster (2a) and representatives from each of the other taxonomic groups.

The seven major clusters and three sub-groups identified by numerical taxonomy are supported by the evidence from esterase analysis, pyrolysis mass spectrometry, % G+C, bacteriocin typing and bacteriophage typing. Two clusters, (4) and (6), were mesophilic strains, cluster (7) were facultative thermophiles and clusters (1), (2), (3) and (5) were obligate thermophiles. Cluster (1) was characterised by not hydrolysing starch; Epstein and Grossowicz (1969) named their starch negative isolate as B. stearothermophilus var. nondiastaticus; however the starch negative strains appear phenotypically and genetically distinct to the description of B. stearothermophilus (Donk, 1920). Five thermophilic starch negative species of Bacillus were described in the 6th edition of Bergey's manual (Breed et al., 1948); B. cylindricus, B. calidus, B. robustus (Blau, 1905); B. non diastaticus, and B. thermoalimentophilus (Bergey et al., 1919). Whilst the three isolates of Blau (1905) appear to take priority as the earliest descriptions of starch negative obligate thermophiles, the name B. non diastaticus proposed by Bergey et al. (1919) appears more suitable to describe this group and might aptly be modified to B. thermonondiastaticus. Cluster (2a) was denoted as the B. kaustophilus group since it contained the original isolate of Pricket (1928), the strains reported by Heinen and Heinen (1972), B. caldotenax,

Table 9.1

Transformation of B. caldotenax ade-5 mutant using DNA isolated from other thermophilic Bacillus species

Donor DNA	Transformation frequency
B. caldotenax	1×10^{-7} to 2×10^{-4}
B. caldovelox	2×10^{-5}
B. caldolyticus	2×10^{-5}
RS 1	0
RS 6	0
RS 57	0
RS 85	0
RS 88	0
RS 93	5×10^{-9}
RS 222	0
RS 240	0
LUDA T22	0
LUDA T42	0
LUDA T60	0
LUDA T141	0
B. subtilis NCTC 3610	0
B. subtilis IG 20	0
B. coagulans ATCC 12245	6×10^{-4}
B. coagulans ATCC 8038	0
B. thermodenitrificans DSM 465	2×10^{-8}
B. thermodenitrificans DSM 466	0
B. thermocatenulatus DSM 730	4×10^{-7}
B. licheniformis LO_2	0
B. stearothermophilus NCA 1503	5×10^{-8}
B. stearothermophilus NCTC 10003	1×10^{-6}
B. stearothermophilus ATCC 12016	2×10^{-7}
B. stearothermophilus ATCC 8005	7×10^{-5}

B. caldovelox and B. caldolyticus, do not appear from this study to merit the rank of species and would more accurately be described as strains resembling B. kaustophilus. Cluster (2b) appears to represent strains fitting the original description of B. stearothermophilus (Donk, 1920). The heterogeneous group of strains in cluster (2c) require closer examination and appear to represent strains similar to both clusters (2a) and (2b). Cluster (3) was denoted as B. thermodenitrificans following the descriptions of Ambroz (1913), Mischustin (1950), Golovacheva et al. (1965), Klaushofer and Hollaus (1970) and Walker and Wolf (1971). Cluster (5) fits the description of B. thermodenitrificans by Walker and Wolf (1971). The % G+C content of strains in this group has not yet been determined, however the strains in this cluster are distinguished from those in cluster (3) by their colony morphology and in hydrolysing tyrosine with the production of a brown pigment. None of the early descriptions listed in the 6th edition of Bergey's manual appear to include studies of tyrosine hydrolysis. A suitable name for this group reflecting its ability to hydrolyse tyrosine would be B. thermotyrovorans. Strains in cluster (4) appear to resemble B. licheniformis and cluster (7) represents the facultative thermophile B. coagulans.

Genetic studies of thermophilic microorganisms were restricted due to the lack of available mutants and the difficulties in establishing a suitable procedure for their isolation. The use of u.v. irradiation for the isolation of B. caldotenax mutants was only successful in producing streptomycin resistance mutations. The dose response curve for u.v. irradiation of B. caldotenax (Fig. 7.7), indicated a decrease of three logs in the viable count following exposure to 24.8 J m^{-2}. Since there was no evidence of sporulation in the culture, B. caldotenax appears to have some inherent resistance to u.v. irradiation. Attempts to obtain satisfactory u.v. death curves of B. caldotenax have been unsuccessful (Munster, pers. comm.). There appear to be no reports of studies into DNA repair in strains of B. stearothermophilus, this is not surprising since there are so few reports of mutation studies, or mutant isolation from these organisms. The high reversion frequencies of auxotrophic mutations in B. caldotenax following exposure to u.v. irradiation, NTG or EMS, together with the relatively high resistance to u.v. irradiation

compared to E. coli, indicates the presence of some, as yet, unknown mechanism for DNA repair. The isolation of a mutant with a defective DNA repair system and its use for subsequent mutant isolation, would probably lead to greater success in the isolation of stable auxotrophic mutants.

The main effect of u.v. on the chromosome is to produce pyrimidine dimers which are formed when the double bonds between the 5' and 6' carbon atoms in two adjacent pyrimidines open and form a four carbon ring. This draws the two bases together and disrupts the hydrogen bonding to the complementary bases on the opposite strand (Howard-Flanders, 1981). Dimers are most frequently found between adjacent thymine bases, but may be formed between adjacent cytosines or between thymine and cytosine bases; the relatively high mole % G+C (65%) of B. caldotenax and conversely low % A+T may contribute to the increased u.v. resistance. One of the most widely studied u.v. resistant microorganisms is Micrococcus radiodurans (Anderson et al., 1956; Sweet & Moseley, 1974) with a mole % G+C of 60 - 74% (Baird-Parker, 1974).

Hansen (1978) reported the presence of four genome equivalents of DNA per cell in resting phase cells of M. radiodurans and up to ten in exponentially growing cells; Evans and Moseley (1980) found that each genome was capable of independent segregation: they accounted for the organism's high resistance to radiation damage by postulating that recombinational repair could occur between homologous regions of the chromosomes in this configuration. The presence of multi-copy genomes in B. caldotenax may account for the high resistance to u.v., although there appears to be no reported studies of chromosome numbers in B. stearothermophilus or B. caldotenax cells.

Other possible mechanisms of DNA repair in B. caldotenax may include the production of a photo-reactivation enzyme which utilises pyrimidine dimers as substrate and requires light for activation energy; in E. coli, the enzyme, which is present as 5 - 25 molecules per cell, cuts the cyclobutane ring and reforms the original configuration (Moseley, 1982). This enzyme has not been found in B. subtilis and there appears to be no report of its occurrence in B. stearothermophilus.

Where more extensive DNA damage occurs, it may be repaired by a sophisticated excision repair system, like that observed in E. coli and many other organisms which involves incision of the damaged DNA,

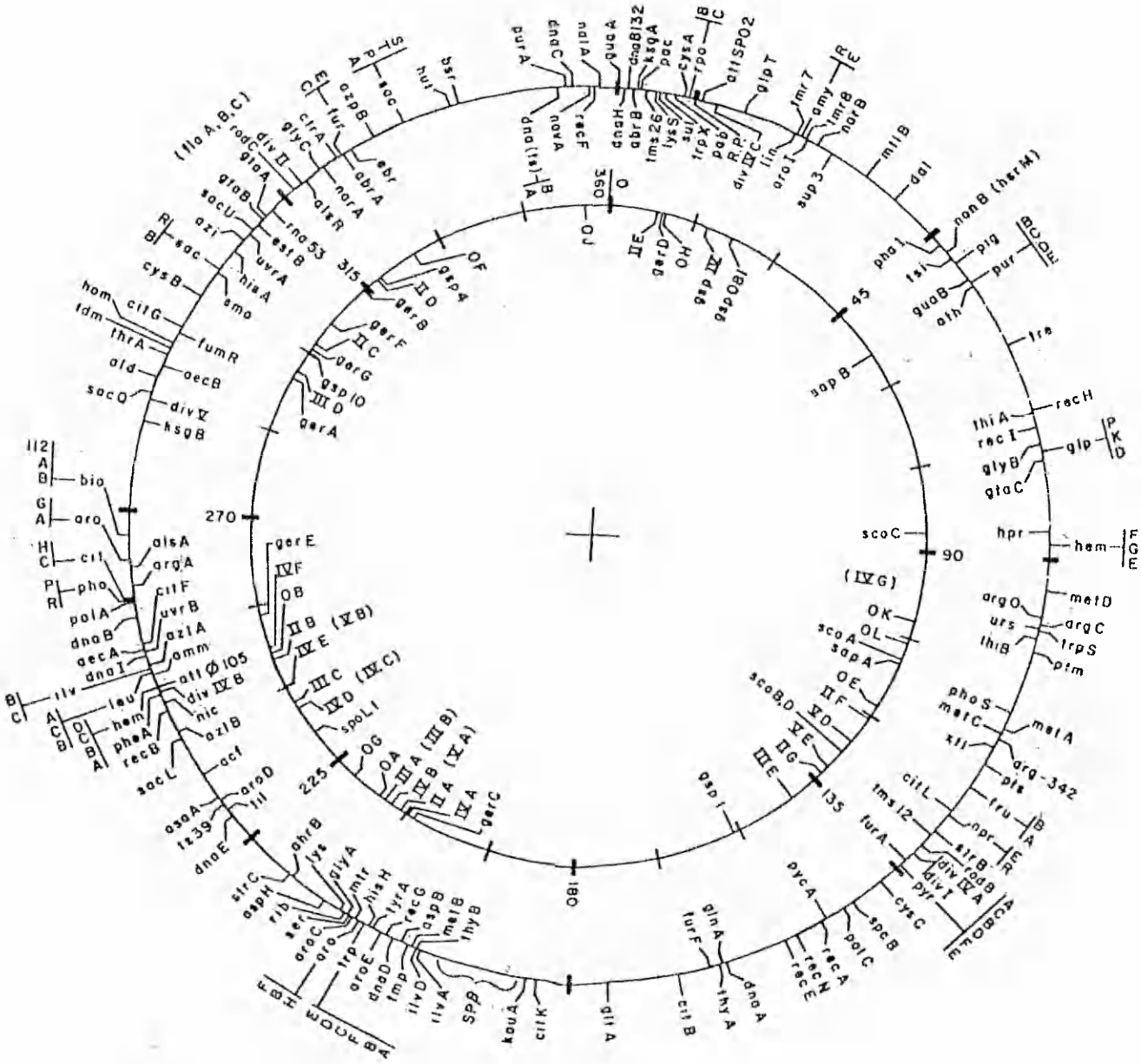
filling of the gap by synthesis and sealing of the strands (Moseley & Williams, 1977; Hanawalt et al., 1979) using the enzymes from uvrA, uvrB, uvrC, DNA polymerase I and DNA ligase. Other mechanisms include "post replication repair", using the gene products of recA, recB and recC controlled by lexA, and "induced error prone repair", involving recA and lexA and probably, although it remains to be confirmed, a DNA polymer which inserts bases at random. This latter system is not present in Haemophilus sp., Proteus mirabilis and Diplococcus sp. (Moseley, 1982). B. subtilis, which is taxonomically the closest organism to B. stearothermophilus and B. caldotenax, has four uvr sites (A, B, C and D) involved in DNA repair and six rec sites (A, B, C, E, F and G) involved in DNA recombination and repair (Henner & Hoch, 1980).

During melting temperature studies of DNA isolated from obligate thermophiles, the Tm values did not show the expected decrease following a ten fold dilution of SSC buffer. Schildkraut and Lifson (1965) predicted a reduction of 16.6 ^{o}C; Silvestri and Hill (1965) predicted a reduction of 15.4 ^{o}C; Cantoni (1966) reported 15.55 ^{o}C; Dove and Davidson (1962) 18.5 ^{o}C and Mandel et al. (1970) 16.3 $\pm$ 0.5 ^{o}C. Table 3.23 indicates differences of 13.3, 14.2 and 15.2 ^{o}C respectively for DNA from E. coli, salmon sperm and B. licheniformis LO_2. Examination of DNA from five obligately thermophilic strains of Bacillus indicated differences in the region of 0 - 5 ^{o}C in Tm values determined in SSC and 0.1 x SSC buffer. Treatment of the DNA with RNase and pronase had no effect on the Tm values. While this inherent stabilisation of DNA from obligate thermophiles has not been reported, stabilisation of DNA following the addition of spermines and spermidines has been reported (Mahler et al., 1961) but normally these would either be removed during the phenol deproteinisation step in the extraction, or be digested by pronase.

Use of penicillin enrichment methods to isolate mutants of B. caldotenax after exposure to NTG and u.v. irradiation were unsuccessful. B. caldotenax was sensitive to ampicillin in complex media both on plates (10 $\mu g\ ml^{-1}$) and in liquid media (50 $\mu g\ ml^{-1}$), but was relatively insensitive in liquid minimal medium. The reason for this is not apparent, but may be the result of changes in the cell wall composition following growth in minimal medium.

Figure 9.1

Detailed map of the B. subtilis genome



Legend

Reproduced from Henner and Hoch (1980).

Mutants deficient in thymidylate metabolism were isolated relatively easily by selection with trimethoprim. Exogenous thymine is not incorporated into the DNA of wild type cells of E. coli, however the existence of thymine requiring mutants indicates that the enzymes for the conversion of thymine to thymidine triphosphate must exist. Thymine requiring mutants undergo thymineless death when incubated in the absence of thymine, thus penicillin or bacitracin enrichment methods cannot be used in their isolation. Okada et al. (1961) found that thy$^-$ mutants could be isolated in the presence of thymine or thymidine as mutants which were resistant to the folate analogues, aminopterin or trimethoprim. Mutants deficient in thymidylate metabolism have been isolated, using aminopterin or trimethoprim as selective agents from many organisms including; E. coli, Salmonella typhimurium, B. subtilis, Enterobacter aerogenes, Serratia marcescens, Lactobacillus fermenti and Pseudomonas acidovorans (O'Donovan, 1978); these mutants are deficient in the production of thymidylate synthetase. Aminopterin and trimethoprim inhibit dihydrofolate reductase which catalyses the conversion of dihydrofolate to tetrahydrofolate (Fig. 9.2). Thymidylate synthetase is unique amongst enzymes catalysing C1 transfer reactions since tetrahydrofolate is consumed (oxidised) in the reaction. The continued activity of thymidylate synthetase, in the presence of trimethoprim or aminopterin, reduces the pool of available tetrahydrofolate which is required in a variety of biosynthetic pathways and for the initiation of protein synthesis. Cells which lack thymidylate synthetase are therefore at a selective advantage when grown in the presence of folate antagonists, provided that they are able to metabolise exogenous thymine. The ability of thy$^-$ cells to grow in these conditions indicates that the inhibition of dihydrofolate reductase is not complete and the synthesis of catalytic amounts of tetrahydrofolate required for other biosynthetic reactions is still possible (O'Donovan, 1978).

Mutants defective in thymine metabolism (thy$^-$) were isolated from a number of thermophilic strains of Bacillus including, B. caldotenax, B. caldovelox, B. caldolyticus, B. stearothermophilus ATCC 8005, B. thermocatenulatus and B. stearothermophilus RS 93, at frequencies in the region of 1×10^{-5}. These mutants are considered to be thy$^-$ mutants, defective in thymidylate synthetase production. Wilson et al. (1966) found that in B. subtilis the requirement for thymine was the result of mutations in two unlinked genes which they

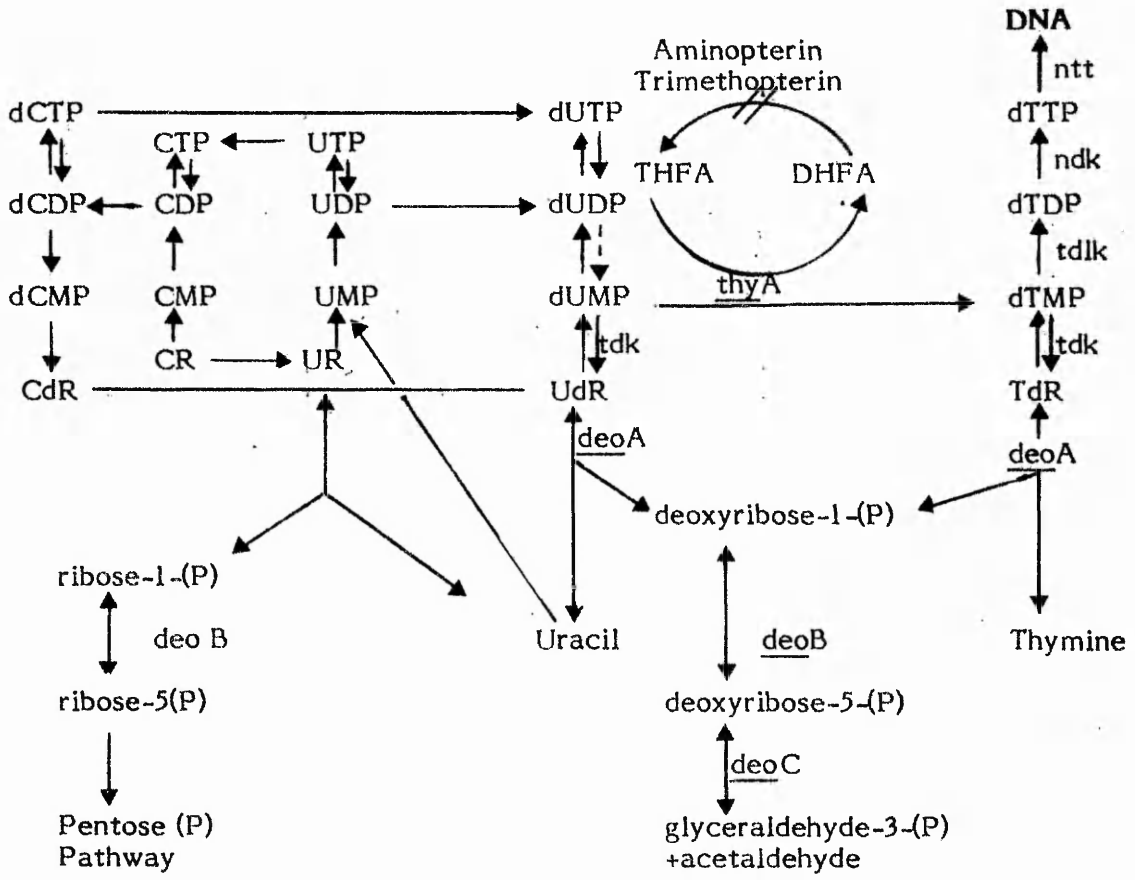
designated as thyA and thyB. ThyA mutants were found to be defective in thymidylate synthetase, but they did not identify the product of the thyB gene. Neuhard et al. (1978) isolated two thymidylate synthetases from B. subtilis grown at 37 °C (TSase A and TSase B) whose activity was dependent upon the presence of functional thyA and thyB genes. The two enzymes had similar requirements for activity, but differed in their kinetic and physiochemical properties; when the cells were grown at 46 °C, only TSaseA activity was present. Mutants which were thyA$^-$, thyB$^+$ or thyA$^+$, thyB$^-$ are phenotypically wild type in their requirement for thymine, but they could be distinguished by their resistance to trimethoprim. Mutants which were thyA$^-$, thyB$^+$ were found to be slightly more resistant to trimethoprim (up to 3 μg ml^{-1}). The thyA gene maps at position 160, between the glnA and furF genes, while thyB maps at position 200, between ilvD and ilvA and adjacent to metB and tmp genes (Fig. 9.1).

The isolation of thy$^-$ mutants of Bacillus thermophiles does not indicate whether these organisms have one or two thy genes. The evidence from transduction studies with B. caldotenax and phage JS 017 indicates that a transducible thy marker is closely linked to a co-transducible met marker; comparison with the genetic map of B. subtilis suggests, that if regions of homogeneity are present in the chromosomes of Bacillus sp. (at present only B. subtilis has been examined in detail) it may be reasonable to assume that the transducible thy marker of B. caldotenax corresponds to the thyB marker of B. subtilis.

Two classes of thymine auxotrophic mutants can be isolated from E. coli and Salmonella typhimurium; one requires approximately 20 μg ml^{-1} of thymine and the other requires 2 μg ml^{-1} (O'Donovan & Neuhard, 1970; O'Donovan, 1978). Low thymine (2 μg ml^{-1}) requiring strains (Tlr$^-$) of E. coli in addition to having a mutation in thyA, (only one thy gene specifying thymidylate synthetase has been found in E. coli) have a second mutation in either deoC or deoB (Breitman & Bradford, 1967) coding for deoxyriboaldolase and deoxyribomutase respectively. The lack of thymidylate synthetase in thy$^-$ mutants provides the cell with deoxyribose-1-phosphate required for the incorporation of thymine. Deoxyribose-1-phosphate is readily degraded to acetaldehyde and glyceraldehyde by the two inducible enzymes, deoxyribomutase and deoxyriboaldolase. A mutation in either of these genes (deoC or deoB) increases the pool of deoxyribose-1-phosphate and it was postulated that this would reduce the requirement for thymine

Figure 9.2

Relevant pathways in the synthesis of dTTP in E. coli and Salmonella typhimurium



Legend

ntt, DNA nucleotidyl-transferase; ndk, nucleoside diphosphate kinase; tdlk, thymidylate kinase; thyA, thymidylate synthetase; tdk, thymidine kinase; deoA, thymidine phosphorylase; deoB, deoxyribomutase; deoC, deoxyriboaldolase; TdR, thymidine; UdR, uridine, CdR, cytidine; dTMP deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; dCMP, deoxycytidine monophosphate.

(O'Donovan, 1978).

A third class of E. coli mutants have been described as "super low thymine requirers" (0.2 μg ml^{-1}) (Ahmad & Pritchard, 1969). In addition to the thyA$^-$ and deoB$^-$ mutations, a third mutation (deoR) (Ahmad & Pritchard, 1969) is present which results in the constitutive synthesis of thymidine phosphorylase, deoxyribomutase and deoxyriboaldolase. Constitutive production of thymidine phosphorylase results in the rapid conversion of deoxyuridine to deoxyribose-1-phosphate and, since there is a mutation in deoB, high pool levels of deoxyribose-1-phosphate are maintained. As a consequence of high levels of deoxyribose-1-phosphate and thymidine phosphorylase, the cells require very low levels of exogenous thymine.

Two types of thy$^-$ mutants of B. stearothermophilus RS 93 were isolated which appear to correspond to the "high thymine (20 μg ml^{-1}) requiring" mutants of E. coli or B. subtilis (thyA$^-$, thyB$^-$) and the "low thymine (2 μg ml^{-1}) requiring" mutants of E. coli (thyA$^-$, deoB$^-$). Based on the levels of thymine required for normal growth, thy$^-$ mutants of B. caldotenax were isolated for all three categories described by O'Donovan (1978). The activities of thymidylate synthetase, thymidine phosphorylase, deoxyriboaldolase, deoxyribomutase and deoxyribose-1-phosphate in the B. caldotenax thy$^-$ mutants have not yet been determined, although this information would be useful in making a comparison of thymine metabolism in B. caldotenax and E. coli.

Transducing phages have been reported for many organisms including E. coli and Staphylococcus, Pseudomonas and Bacillus sp. Although the isolation of thermophilic bacteriophages able to infect strains of B. stearothermophilus have been reported by several groups, (Section 1.4) this is the first report of a thermophilic bacteriophage able to transduce an obligate thermophilic species of Bacillus.

Bacteriophage JS 017, isolated from garden compost in Wiltshire, has been demonstrated to transduce thymine and methionine auxotrophs of B. caldotenax; transduction was optimal at 55 ^{o}C with cells in their log phase of growth. A ten fold dilution of the recipient auxotrophic cells with new growth medium resulted in a ten fold increase in the transduction frequency; perhaps due to growth in fresh medium producing physiologically younger cells. It is also possible that inhibitors of phage adsorption or transduction may have been diluted, or that high cell densities may inhibit phage adsorption. Phage infection

or transduction, may require adsorption or penetration by more than one phage particle, reduction of the cell population would increase the number of possible "multiple" adsorptions or infections.

The exposure of transducing phage populations to u.v. irradiation has been demonstrated to stimulate generalised transduction (Thorne, 1968; Vary, 1979). This procedure reduces the viability of the non-transducing particles, and causes only slight damage to the transducing particles which contain predominantly bacterial DNA. The exposure of phage JS 017 to 3.6×10^4 - 2.16×10^6 J m^{-2} of u.v. irradiation reduced the viability of the phage population by 96% and reduced the transduction frequency. Exposure to DNase did not reduce the yield of transductants, indicating that the colonies appearing as putative transductants were not the result of transfection, or transformation with residual bacterial DNA from the phage lysate.

The isolation of infective phages from most of the transductant clones suggests that the phage lysogenises the cells or retains a close association with the organism in a manner similar to the B. subtilis phage SP 10 (Kawakami & Landman, 1968). The DNA of phage SP 10 does not integrate with the B. subtilis chromosome, but is maintained in the population by frequent re-infection (Hemphill & Whiteley, 1975).

As previously discussed, phage JS 017 can cotransduce met and thy markers of a mutant of B. caldotenax implying close linkage of these two markers. On the B. subtilis chromosome thyB and metB map close to each other at position 200; adjacent to this site is the attachment site for phage SPβ. Phage SPβ transduces kauA and citK which map at position 185 on the other side of the phage SPβ insertion site (Rosenthal et al., 1979). A comparison of the genetic map of B. subtilis with two projected markers and a possible prophage DNA attachment site on the B. caldotenax genome, must be treated with caution and may in the end prove misleading. (The mole % G+C of B. subtilis and B. caldotenax are 43% and 64% respectively.) B. subtilis contains four ilv genes, two of which, ilvA and ilvD, are adjacent to the thyB and metB genes. Attempts to cotransduce ilv, thy auxotrophs of B. caldotenax have proved unsuccessful due, in part, to the high reversion rate of the ilv mutation. At present the ilv marker of B. caldotenax has not been characterised.

A number of phages have been reported to carry genes involved in thymidylate metabolism; phages T2 and T5 were reported to induce thymidylate synthetase production following infection of thymine

deficient cells of E. coli (Barner & Cohen, 1959). Young (1980) discussed the transformation of a thymine auxotroph of E. coli using the gene for thymidylate synthetase isolated from the B. subtilis phages ϕ3T and β22 and inserted into plasmid pCD1. Markewych et al. (1979) reported a novel means of phage mediated production of deoxythymidylate monophosphate which utilised tetrahydrofolate in the transfer of 1 carbon fragments, but did not oxidise it to dihydrofolate as previously described in the E. coli system (Fig. 9.2). The presence of such a mechanism in phage JS 017 for the production of deoxythymidylate monophosphate, which was not repressed during lysogeny, provided an alternative explanation for the conversion of thy auxotrophs to prototrophy. The lack of transducing ability of phage suspensions prepared from lysis of a thy$^-$ mutant of B. caldotenax, suggested that the phage particles were unable to "pick up" functional thy$^+$ genes from their previous host. It was, nevertheless, conceivable, that phages previously harvested from a thy$^-$ host were unable to synthesise deoxythymidylate monophosphate. The transduction of a met auxotroph of B. caldotenax and the cotransduction of a met, thy auxotroph, presented much clearer evidence for the occurrence of transduction. The transduction of thy auxotrophs with different requirements for thymine, (i.e. high, low and super low) suggests that the thyA (or thyB) genes for thymidylate synthetase production have been restored to prototrophy, although the deoC, deoB or deoR genes probably remain deficient. Further biochemical and genetic analysis of transductants should indicate if this was the case.

Phage JS 017 appears to function as a specialised transducing phage able to transduce thymine and methionine auxotrophs of B. caldotenax. The examination of other thermophilic bacteriophages for their transducing ability was handicapped due to the lack of suitably marked strains. Only phage JS 017 was examined for the enhancement of transducing ability following exposure to u.v., but re-examination of the transducing ability of the other thermophilic phages, following exposure to u.v., may prove worthwhile, particularly those phages which are able to infect B. caldotenax, since more suitably marked strains are now available.

Thermophilic phages infecting a wide range of thermophilic strains of Bacillus were isolated from soil and compost with relative ease. Morphologically they were of three main types; the majority

(JS 002, JS 019, JS 022, JS 024 and JS 025 from lysates of B. caldotenax) had polyhedral heads (60 - 70 nm in diameter) with tails (130 - 180 nm in length). Their morphology closely resembles the B. stearothermophilus phages previously reported: TP 1C from lysates of strain 4S (Welker & Campbell, 1965), TP 84 from lysates of strain NW 10 (Bassel et al., 1971), ϕμ-4 from lysates of NU 10 (Rabussay et al., 1970) and Tϕ3 from lysates of ATCC 8005 (Egbert, 1969). Three phages (JS 004, JS 005 and JS 027), that infected starch negative strains, were distinct in having icosahedral heads, 80 nm in diameter, but with comparatively short tails, 50 - 60 nm; JS 027 had an unusually short stubby tail, 15 nm long. Phage JS 014, which was able to infect B. stearothermophilus NCA 1503, had an icosahedral head 90 nm in diameter, with a flexible tail, 400 - 500 nm in length, showing helical symmetry. Reanney and Wood (1973) described a phage (D5) isolated from compost, which infected B. stearothermophilus NRS T91; a strain derived from the original NCA 1503 isolate (Gordon et al., 1973). Phage D5 was reported to have an icosahedral head, 87 nm in diameter, with a striated tail, 180 nm in length (Reanney & Wood, 1973). Phage JS 007, which infected starch negative strains, had a slightly larger head, 95 - 100 nm in diameter and a flexible tail up to 600 nm in length. Hemphill and Whitely (1975) reviewed the morphology of 16 B. subtilis phages, all had tails of between 30 nm and 250 nm in length, a phage tail over 250 nm in length therefore appears to be an exception.

The cylindrically shaped head of phages JS 017 and JS 026 was morphologically distinct from all previously described thermophilic bacteriophages. JS 017 has shown evidence of morphological variation with phage heads double the normal length of 80 - 90 nm. In some preparations these "variants" numbered between 1 - 5% of the population and it was initially considered that these larger variants might be the transducing particles. Subsequent examination of a number of transducing phage preparations by electron microscopy indicated that the proportion of these "variants" ranged from 0 - 5% while the transducing ability of the individual preparations did not vary significantly.

B. subtilis bacteriophages have been characterised or "finger printed" by examination of their DNA with restriction endonucleases; ϕ3T, SP 02 and ϕ105 were examined by Wilson et al. (1974); ϕ29 and ϕ15 were examined by Ito and Kawamura (1976). Morphologically phages

JS 022, JS 024 and JS 025 were identical (Table 5.10) and all had similar host ranges. Examination of their restriction endonuclease "finger prints" indicated a close similarity between JS 022 and JS 025, with small, but significant differences between these two and JS 024. The "finger printing" of thermophilic bacteriophages by this procedure has not been previously reported, but it does offer a relatively simple means of distinguishing between phage isolates which morphologically and physiologically appear identical.

Buoyant density in percoll gradients ranged from 1.0 g ml^{-1} - 1.1 g ml^{-1}. The occurrence of multiple, or two or three single separate bands from individual phage preparations indicated the occurrence of multiple density classes of phage particles. These were probably the result of damaged phage particles which banded separately in the gradient as phage heads and tails. In the examination of several phage preparations by electron microscopy phage "ghosts" were often predominent; the absence of DNA within the phage heads would result in phage particles with different densities. Karamata (1970) identified multiple density classes of phage P1 which were the result of tetramer formation through binding at phage base plates. In this form the phages were not infective, although they were protected from inactivation by phage antiserum.

The multiple density bands found in some preparations of thermophilic phages have not been examined by electron microscopy, although only one band showed evidence of a high p.f.u. Four close density bands found for JS 017 may be the result of variations in the size of the phage heads and tails as previously observed. A re-examination of the buoyant density of phage JS 017 in a percoll gradient showed two bands with densities of 0.98 g ml^{-1} and 1.017 g ml^{-1}.

Thermal stability studies on eight phage isolates and TP 1C and TP 84 showed few significant differences. The p.f.u. of most phage preparations fell by less than 1 log after 4 - 5 h at 50 ^{o}C; the exceptions were JS 007, JS 024 and JS 025, whose p.f.u. decreased respectively by 2, 2 and 3 logs. After 4 h at 70 ^{o}C the p.f.u. had decreased by between 2 and 7 logs. These results were comparable with those reported for most other thermophilic phages. Phage D5 (Reanney & Wood, 1973) showed little reduction in p.f.u., following 3 h at 60 ^{o}C but was reduced by 3 logs at 70 ^{o}C. Welker and Campbell (1965)

reported a decrease in p.f.u. of phage TP 1C in TYF medium of 52% after 30 min at 65 °C, followed by a plateauing of the survival curve. Phage ϕμ-4 suspended in distilled water, showed little decrease in p.f.u. after 1 h at 75 °C, although the p.f.u. were reduced by 2 logs when the phages were suspended in Tris buffer (Shafia & Thompson, 1964). Onodera (1961) reported the isolation of a thermophilic bacteriophage from compost which was able to infect a spore forming, strictly aerobic, thermophilic, Gram positive Bacillus. The phage was unusual in consisting of spherical particles only 20 nm in diameter, and in resisting heat inactivation at 100 °C for 2 h.

To summarise, preliminary studies comparing the three strains of Bacillus thermophiles described by Heinen and Heinen (1972) with cultures of Bacillus stearothermophilus, were expanded to include a number of new isolates and internationally recognised strains. Examination by numerical taxonomy resulted in their allocation to seven major groups. These taxonomic groupings were supported by other taxonomic data from mole % G+C, bacteriocin typing, phage typing and pyrolysis mass spectrometry.

Auxotrophs of B. caldotenax were isolated following mutation with NTG; high, low and "superlow" mutants deficient in thymidylate metabolism were isolated following selection with trimethoprim.

Twenty-eight thermophilic bacteriophages were isolated and several characterised by "finger printing" with restriction endonucleases, buoyant density estimations, morphology, host range and temperature stability determinations. Many of the phages were examined for their ability to act as transducing phages. Phage JS 017, which had an unusual morphology, functioned as a specialised transducing phage, transducing thymine and methionine auxotrophs of B. stearothermophilus.

REFERENCES

REFERENCES

Adams, M.H. (1959). "Bacteriophages", London: Interscience Ltd.

Adelberg, E.A. Mandel, M. and Chein Ching Chen, G. (1965). Optimal conditions for mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine in E. coli K 12. Biochemical and Biophysical Research Communications, 18, 788-795.

Agris, P.F., Koh, H. and Soll, D. (1973). The Effect of growth temperatures on the in-vivo ribose methylation of Bacillus stearothermophilus transfer R.N.A. Archives of Biochemistry and Biophysics, 154, 227-282.

Ahmad, S.I. and Pritchard, R.H. (1969). A map of Four Genes Specifying Enzymes Involved in Catabolism of Nucleosides and Deoxynucleosides in Escherichia coli. Molecular and General Genetics, 104, 351-359.

Allen, M.B. (1953). The thermophilic aerobic spore forming bacteria. Bacteriological Reviews, 17, 125-173.

Ambroz, A. (1913). Denitrobacterium thermophilum spec. nova. Ein Beitrag zur Biologie der thermophilen Bakterien. Zentralblatt fur Bakteriologie und Parasitenkunde, II, 3-16.

Amelunxen, R.E. and Lins, M. (1968). Comparative thermostability of enzymes from Bacillus stearothermophilus and Bacillus cereus. Archives of Biochemistry and Biophysics, 125, 765-769.

Amelunxen, R.E. and Singleton, R. (1976). Thermophilic glyceroldehyde -3-P dehydrogenases. In "Enzymes and Proteins from thermophilic microorganisms", (Ed. H. Zuber) 107-120. Basel and Stuttgart: Birkhauser Verlag.

Amelunxen, R.E. and Murdock, A.L. (1978). Life at High Temperatures: Molecular Aspects. In "Microbial Life in Extreme Environments" (Ed. D.J. Kushner), 217-278. London and New York: Academic Press.

Anderson, A.W., Nordon, H.C., Cain, R.F., Parrish, G. and Duggon, D. (1956). Studies on a radioresistant micrococcus. Isolation, morphology, cultural characteristics and resistance to gamma radiation. Food Technology, 10, 575-577.

Arca, M., Frontali, L. and Tecce, G. (1965). Lack of specificity in the formation amino-acyl-sRNA as a possible source of coding errors. Biochimica et biophysica acta, 108, 326-328.

Arrhenius S. (1927). Origin of thermophilic micro-organisms. Zeitschrift fur Physikalische Chemie, 130, 516-519.

Ashwood-Smith, M.J. (1965). The genetic stability of bacteria to freezing and thawing. Cryobiology, 2, 39-45.

Atkinson, A., Bradford, P.A. and Selmes, I.P. (1973). The large scale preparation of chromatographic grade hydroxylapatite and its application in protein separation procedures. Journal of Applied Chemistry and Biotechnology, 23, 517-529.

Atkinson, A. (1976). Thermostable enzymes. Journal of Applied Chemistry and Biotechnology, 26, 577-578.

Atkinson, A., Bruton, C.J., Comer, M.J. and Sharp, R.J. (1979). UK patent application.

Bachman, B.J., Low, K.B. and Taylor, A.L. (1976). Recalibrated linkage map of Escherichia coli K-12. Bacteriological Reviews, 40, 116-167.

Baillie, R. and Norris, J.R. (1963). Studies on enzyme changes during sporulation in Bacillus cereus using starch gel electrophoresis. Journal of Applied Bacteriology, 26, 102-108.

Baillie, A. and Walker, P.D. (1968). Enzymes of thermophilic aerobic spore forming bacteria. Journal of Applied Bacteriology, 31, 114-119.

Baird-Parker, A.C. (1963). A classification of Micrococci and Staphylococci based on physiological and biochemical tests. Journal of General Microbiology, 30, 409-427.

Baird-Parker, A.C. (1974). In "Bergey's manual of Determinative Bacteriology" (Ed. R.E. Bughanan & N.E. Gibbons). Baltimore: The Williams and Wilkins Company.

Baker, H., Frank, O., Pasher, I., Black, B., Hutner, S.H. and Sobotka, H. (1960). Growth requirements of 94 strains of thermophilic Bacilli. Canadian Journal of Microbiology, 6, 557-563.

Baker, H., Sobotka, H. and Hutner, S.H. (1953). Growth Requirements of some Thermophilic and Mesophilic Bacilli. Journal of General Microbiology, 9, 485-493.

Barner, H.D. and Cohen, S.S. (1959). Virus-induced acquisition of metabolic function. Journal of Biological Chemistry, 234, 11, 2987-2991.

Barritt, M.M. (1936). The intensification of the Vogues-Proskauer reaction by the addition of α-naphthol. Journal of Pathological Bacteriology, 42, 441.

Bassel, A., Shaw, M. and Campbell, L.L. (1971). Dissociation by Chelating agents and substructure of the thermophilic bacteriophage TP-84. Journal of Virology, 7, (5), 663-672.

Belehradek, S. (1931). Le mechanisme physico-chemique de l'adaptation thermique. Protoplasma, 12, 406-434.

Belly, R.T. and Brock, T.D. (1974). Widespread occurrence of acidophilic strains of B. coagulans in hot springs. Journal of Applied Bacteriology, 37, 175-177.

Bendich, A. (1957). Methods for characterisation of nucleic acids by base composition. In "Methods of Enzymology", 3 (Ed. S.P. Colowick and N.O. Kaplan). New York: Academic Press.

Berg, P., Baltimore, B., Boyer, H.W., Cohen, S.N., Davis, R.W., Hogness, D.S., Nathan, D., Roblin, R.O., Watson, J.D., Weissman, S. and Zinder, N.D. (1974). Potential Biohazards of Recombinant DNA molecules. Science, 185, 303.

Bergey, D.H. (1919). Journal of Bacteriology, 4, 304.

Bergey, D.H., Harrison, F.C., Breed, R.S., Hammer, B.W., Huntoon, F.M. (1923). Manual of Determinative Bacteriology. 1st edition. Baltimore: The Williams and Wilkins Company.

Bernhard, K., Schrempf, H. and Goebel, W. (1978). Bacteriocin and antibiotic resistance plasmids in Bacillus cereus and Bacillus subtilis. Journal of Bacteriology, 133, (2), 897-903.

Berry, R.N. (1933). Some new heat resistant acid tolerant organisms causing spoilage in tomato juice. Journal of Bacteriology, 25, 72-73.

Billing, E. and Garrett, C.M.E. (1980). Phages in the Identification of Plant Pathogenic Bacteria. In "Microbiological Classification and Identification" (Ed. M. Goodfellow and R.G. Board), 319-338. London, New York, Toronto, Sydney, San Francisco: Academis Press.

Bingham, A.H.A., Sharp, R.J. and Atkinson, A. (1977). Plasmids in thermophilic bacteria. N.A.R.S.M., N.A.R. 77.

Bingham, A.H.A., Bruton, C.J. and Atkinson, T. (1979). Isolation and partial characterisation of four plasmids from antibiotic-resistant thermophilic Bacilli. Journal of General Microbiology, 114, 401-408.

Bingham, A.H.A. (1980). Plasmids from thermophilic Bacilli. Ph.D. thesis. Imperial College of Science and Technology, University of London.

Bingham, A.H.A., Bruton, C.J. and Atkinson, T. (1980). Characterisation of Bacillus stearothermophilus plasmid pAB 124 and construction of deletion variants. Journal of General Microbiology, 119, 109-115.

Blau, (1905). Cent. f. Bakt., II Abt., 15, 126.

Bradley, D.E. (1967). Ultrastructure of bacteriophages and Bacteriocins. Bacteriological Reviews, 31, 230-314.

Bradley, S.G. and Mordarski, M. (1976). Association of polydeoxyribonucleotides of deoxyribonucleic acids from nocardioform bacteria. In "The Biology of the Nocardiae" (Ed. M. Goodfellow, G.H. Brownell and J.A. Serrano), 310-336. London: Academic Press.

Bradley, S.G. (1980). DNA re-association and base composition. In "Microbiological Classification and Identification" (Ed. M. Goodfellow and R.G. Board), 11-26. London, New York, Toronto, Sydney, San Francisco: Academic Press.

Bramucci, M.G. and Lovett, P.S. (1977). Selective plasmid transduction in Bacillus pumilus. Journal of Bacteriology, 131, 3, 1029-1032.

Breed, R.S., Prickett, P.S. and Yale, M.W. (1929). The significance of thermophilic spore forming bacteria in pasteurized milk. Journal of Bacteriology, 17, 37-38.

Breed, R.S., Murray, E.G.D. and Parker-Hitchens, A. (1948). "Bergey's Manual of Determinative Bacteriology", 6th edition. London: Bailliere, Tindall and Cox.

Breed, R.S., Murray, E.G.D. and Smith, N.R. (1957). Manual of Determinative Bacteriology. 7th edition. London: Bailliere, Tindall and Cox.

Breese, M.D. and Sharp, R.J. (1980). Storage of Escherichia coli strains containing plasmid DNA in liquid nitrogen. Journal of Applied Bacteriology, 48, 63-68.

Breitman, T.R. and Bradford, R.M. (1967). The absence of deoxyriboaldolase activity in a thymineless mutant of E. coli strain 15: A possible explanation for the low thymine requirement of some thymineless strains. Biochimica et biophysica acta, 138, 214-217.

Brock, T.D. (1967). Life at high temperatures, Science; New York, 158, 1012.

Brock, T.D. and Freeze, H. (1969). Thermus aquaticus gen. n and sp. n. a non-sporulating extreme thermophile. Journal of Bacteriology, 98, (1) 289-297.

Brock, T.D. (1978). "Thermophilic microorganisms and life at high temperatures". New York: Springer Verlag.

Buswell, J.A. and Twomey, D.G. (1975). Utilization of Phenol and Cresols by Bacillus stearothermophilus strain PH24. Journal of General Microbiology, 87, 377-379.

Cameron, E.J. and Esty, J.R. (1926). The examination of spoiled canned foods, 2. Classification of flat sour organisms from non-acid foods. Journal of Infectious Diseases, 39, 89-105.

Cantoni, C. (1966). Composizione delle basi constituenti gli acidi desossiribonucleinici di lattobacilli isolati da diversi materiali organici. Boll. Inst. Sieroterap. Milan, 45, 95-101.

Cazzulo, J.J., Sundaram, T.K. and Kornberg, H.L. (1969). Regulation of pyruvate carboxylase formation from the apo-enzyme and biotin in a thermophilic Bacillus. London: Nature, 223, 1137.

Chapman, D.J. (1974). Taxonomic position of Cyanidium caldarium. The Porphyridiales and Goniotrichales. Nova Hedwig, 25, 673-682.

Cho, K.Y. and Salton, M.J.R. (1964). Fatty acid composition of the lipids of membranes of gram-positive bacteria and walls of gram-negative bacteria. Biochimica et biophysica acta, 84, 773-775.

Cohn, F. (1876). Untersuchung en uber Bakterien IV Beitrage zur Biologie der Bacillen. Beitrage zur Biologie der Pflanzen, 2, 249-276.

Comer, M.J., Bruton, C.J. and Atkinson, T. (1979). Purification and Properties of Glycerokinase from Bacillus stearothermophilus. Journal of Applied Biochemistry, 1, 259-270.

Coolhass, C. (1928). Zur Kenntnis der Dissimilation fettsaurer Salze und Kohlenhydrate durch thermophile Bakterien, Zentr. f. Bakt. II, 75, 344-360.

Cowan, S.T. and Steel, K.J. (1974). Manual for the Identification of Medical Bacteria. Cambridge: Cambridge University Press.

Cradock-Watson, J.E. (1965). The production of bacteriocins by Proteus species. Zentr. Bakteriol. Parasitenk. Abt. I Orig., 196, 385-388.

Curtiss, R. (1976). Genetic manipulation of microorganisms: Potential benefits and biohazards. Annual Review of Microbiology, 30, 507-533.

Dallinger, W.H. (1887). The President's address (9th February, 1887). Journal of the Royal Microscopical Society, 185-199.

Daniels, L., Fuchs, G., Thauer, R.K. and Zeikus, J.G. (1977). Carbon monoxide oxidation by methanogenic bacteria. Journal of Bacteriology, 132, (1), 118-126.

Darland, G. and Brock, T.D. (1971). Bacillus acidocaldarius sp. nov., an acidophilic thermophilic spore-forming bacterium. Journal of General Microbiology, 67, 9-15.

Daron, H.H. (1967). Occurrence of isocitrate lyase in a thermophilic Bacillus species. Journal of Bacteriology, 101, 145-151.

Daron, H.H. (1970). Fatty acid composition of lipid extracts of a thermophilic Bacillus species. Journal of Bacteriology, 101, 145-151.

Davis, B.D. (1948). Isolation of biochemically deficient mutants of bacteria by penicillin. Journal of the American Chemical Society, 70, 4267.

Delente, J.J., O'Connor, R.J. and Kuo, M.J. (1974) US patent, 3, 846, 239.

De Ley, J. (1969). Compositional nucleotide distribution and the theoretical prediction of homology in bacterial DNA. Journal of Theoretical Biology, 22, 89-116.

Denhardt, D.T. (1966). A membrane-filter technique for the detection of complementary DNA. Biochemical Biophysical Research Communications, 23, 641-646.

Deranleau D.A. and Zuber, H. (1977). Thermophilic Aminopeptidase. IV. Cooperative Effects in ANS Binding by the Thermophilic Aminopeptidase I from B. stearothermophilus. International Journal of Peptide and Protein Research, 9, 258-268.

De Rosa, M., Gambacorta, A., Lama, L. and Nicolaus, B. (1981). Immobilization of thermophilic microbial cells in crude egg white. Biotechnology Letters, 3, (4), 183-187.

Devanathan, T., Akagi J.M. and Hersh, R.T. (1969). Ferredoxin from two thermophilic Clostridia. Journal of Biological Chemistry, 244, 2846-2853.

Doemel, W.N.R. and Brock, T.D. (1970). The upper temperature limit of Cyanidium caldarium. Archives fur Microbiology, 72, 326-332.

Donk, R.J. (1920). A highly resistant thermophilic organism. Journal of Bacteriology, 5, 373-374.

Dove, W.F. and Davidson, N. (1962). Cation effects on the denaturation of DNA. Journal of Molecular Biology, 5, 467-478.

Duggleby, C.J., Bayley, S.A., Worsey, M.J., Williams, P.A. and Broda, P. (1977). Molecular sizes and relationships of TOL plasmids in Pseudomonas. Journal of Bacteriology, 130, 1274-1280.

Egbert, L.N. and Mitchell, H.K. (1967). Characteristics of Tϕ3, a bacteriophage for Bacillus stearothermophilus. Journal of Virology, 1, 610-616.

Egbert, L.N. (1969). Characteristics of the DNA of Tϕ3, a bacteriophage for B. stearothermophilus. Journal of Virology, 3, (5), 528-532.

Endo, S. (1962). Studies on protease produced by thermophilic bacteria. Journal of Fermentation Technology, 40, 346-353.

Epstein, I. and Grossowicz, N. (1969). Prototrophic Thermophilic Bacillus: Isolation, Properties and Kinetics of Growth. Journal of Bacteriology, 99, (2), 414-417.

Epstein, I. and Campbell, L.L. (1975). Production and Purification of the Thermophilic Bacteriophage TP-84. Applied Microbiology, 29, 219-223.

Esser, A.F. (1978). The influence of growth temperature and lipid state on the planer distribution of lipids and proteins in Bacillus stearothermophilus membranes. In "Biochemistry of Thermophily" (Ed. S.M. Friedman), 45-60. New York, San Francisco, London: Academic Press.

Evans, D.M. and Moseley, B.E.B. (1980). In "Tirgari, S. and Moseley, B.E.B. (1980)". Transformation in Micrococcus radiodurans: Measurement of various parameters and evidence for multiple independently segregating genomes per cell. Journal of General Microbiology, 119, 287-296.

Farrell, J. and Campbell, L.L. (1969). Thermophilic Bacteria and Bacteriophages. Advances in Microbial Physiology, 3, 83-109.

Fisher, H.M., Watson, J. and Luthy, P. (1980). Discrimination of ccc and oc plasmid conformations by 2-dimensional gel-electrophoresis, demonstrated with B. thuringiensis Experientia, 36, 1452.

Frank, G., Haberstich, H.U., Schaer, H.P., Tratschin, J.D. and Zuber, H. (1975). Thermophilic and Mesophilic Enzymes from B. caldotenax and B. stearothermophilus, Properties, Relationships and Formation. In "Enzymes and Proteins from thermophilic microorganisms" (Ed. H. Zuber), 375-390. Basel and Stuttgart: Birkhauser Verlag.

Friedman, S.M. and Weinsten, I.B. (1964). Lack of fidelity in the translation of synthetic polyribonucleotides. Proceedings of the National Academy of Science, USA, 52, 988-996.

Friedman, S.M. (1978). Studies on heat-stable ribosomes from thermophilic bacteria. In "Biochemistry of Thermophily" (Ed. S.M. Friedman), 151-168. New York, San Francisco, London: Academic Press.

Friedman, S.M. and Mojica-a, T. (1978). Transformants of Bacillus subtilis capable of growth at elevated temperatures. In "Biochemistry of Thermophily" (Ed. S.M. Friedman), 117-126. New York, San Francisco, London: Academic Press.

Forrester, I.T. and Wicken, A.J. (1966). The chemical composition of the cell walls of some thermophilic Bacilli. Journal of General Microbiology, 42, 147-154.

Galesloot, Th. E. and Labots, H. (1959). Thermophilic bacilli in milk. Netherlands Milk and Dairy Journal, 13, 155-179.

Gaughran, E.R.L. (1947). The Thermophilic Microorganisms. Bacteriological Reviews, 11, 189-255.

Gibson, T. and Gordon, R.E. (1974). Bacillus. In "Bergey's Manual of Determinative Bacteriology" (Ed. R.E. Buchanan and N.E. Gibbons), 529-555. Baltimore: The Williams and Wilkins Co.

Golikowa, S.M. (1926). Zur Frage der Thermobiose. Zentralblatt fur Bakteriologie und Parasitenkunde II, 69, 178-184.

Golovacheva, R.S., Egorova, L.A. and Loginova, L.G. (1965). Ecology and systematics of aerobic obligate thermophilic bacteria isolated from thermal localities on Mount Yangan-Tau and Kunashir isle of the Kuril chain. Microbiology (USSR) English edition, 34, 693-698.

Golovacheva, R.S., Loginova, L.G., Salikhov, T.A., Kolesnikov, A.A. and Zaitzeva, G.N. (1975). A new thermophilic species Bacillus thermocatenulatus nov. sp. Mikrobiologya, 44, 265-268.

Gordon, R.E. and Smith, N.R. (1949). Aerobic spore forming bacteria capable of growth at high temperatures. Journal of Bacteriology, 58, 327-341.

Gordon, R.E., Haynes, W.C. and Pang, C. H-N. (1973). The Genus Bacillus. United States Department of Agriculture, Washington D.C.

Gorini, L. (1971). Ribosomal discrimination of tRNAs. Nature, New Biology, 234, 261-264.

Govan, J.R.W. and Gillies, R.R. (1969). Further studies in the pyocine typing of Pseudomonas pyocyanea. Journal of Medical Microbiology, 2, 17-25 (1969).

Gower, J.C. (1971). A general coefficient of similarity and some of its properties. Biometrics, 27, 857-874.

Grinsted, E. and Clegg, L.F.L. (1955). Spore-forming organisms in commercial sterilised milk. Journal of Dairy Research, 22, 178-190.

Grootegoed, J.A., Lauwers, A.M. and Heinen, W. (1973). Separation and partial purification of Extracellular amylase and protease from Bacillus caldolyticus. Archives for Microbiology, 90, 223-232.

Guerry, P., Le Blanc, D.J. and Falkow, S. (1973). General Method for the isolation of plasmid DNA. Journal of Bacteriology, 116, 1064-1066.

Guicciardi, A., Biffi, M.R., Manachim, P.L., Craveri, A., Scolastico, C., Rindone, B. and Craveri, R. (1968). Novo termofilo del genere Bacillus. Annali di Microbiologia ad Enzymologia, 18, 191-205.

Gutteridge, C.S. and Norris, J.R. (1979). The Application of Pyrolysis Techniques to the Identifiction of Micro-organisms. Journal of Applied Bacteriology, 47, 5-43.

Gyllenberg, H. (1951). Studies on thermophilic bacteria of the genus Bacillus Cohn. Acta Agraria Fennica, Helsinki, 73, 1-88.

Haberstich, H.U. and Zuber, H. (1974). Thermoadaption of Enzymes in thermophilic and mesophilic cultures of B. stearothermophilus and B. caldotenax. Archives of Microbiology, 98, 275-287.

Hammer, B.W. (1915). Bacteriological studies on the coagulation of evaporated milk. Iowa Agricultural Experiment station Research Bulletin, 19, 119-131.

Hanawalt, P.C., Cooper, P.K., Ganeson, A.K. and Smith, C.A. (1979). DNA repair in bacteria and mammalian cells. Annual Review of Biochemistry, 48, 783-836.

Hansen, M.T. (1978). Multiplicity of genome equivalents in the radiation-resistant bacterium Micrococcus radiodurans. Journal of Bacteriology, 134, 71-75.

Hase, T., Ohmy, N., Matsubara, H., Mullingen, R.N., Rao, K.K. and Hall, D.O. (1976). Amino acid sequences of a 4-iron 4-sulphur ferredoxin from B. stearothermophilus. Biochemical Journal, 159, 55-63.

Heilbrun, L.V. (1924). The colloid chemistry of protoplasm. IV. The heat coagulation of protoplasm. American Journal of Physiology, 69, 190-199.

Heinen, W. (1971). Growth conditions and temperature-dependent substrate specificity of two extremely thermophilic bacteria. Archiv fur Mikrobiologie, 76, 2-17.

Heinen, W., Klein, H.P. and Volkman, C.M. (1970). Fatty acid composition of Thermus aquaticus of different growth temperatures. Archiv fur Mikrobiologie, 72, 199-202.

Heinen, U.J. and Heinen, W. (1972). Characteristics and properties of a caldoactive bacterium producing extracellular enzymes and two related strains. Archiv fur Mikrobiologie, 82, 1-23.

Hemphill, H.E. and Whiteley, H.R. (1975). Bacteriophages of Bacillus subtilis. Bacteriological Reviews, 39, (3), 257-315.

Henner, D.J. and Hoch, J.A. (1980). The Bacillus subtilis chromosome. Microbiological Reviews, 44, (1), 57-82.

Hindle, E. (1932). Some new thermophilic organisms. Journal of the Royal Microscopical Society, 52, 123-133.

Hishinuma, F., Tanaka, T. and Sakaguchi, K. (1978). Isolation of Extrachromosomal Deoxyribonucleic acids from extremely thermophilic bacteria. Journal of General Microbiology, 104, 193-199.

Hocking, J.D. and Harriss, J.I. (1976). Glyceraldehyde 3-phosphate dehydrogenase from an extreme thermophile, Thermus aquaticus. In "Enzymes and Proteins from Thermophilic Microorganisms" (Ed. H. Zuber), 107-120. Basel: Birkhauser, Verlag.

Howard-Flanders, P. (1981). Inducible Repair of DNA. Scientific American, 245, (5), 56-64.

Huang, P.C. and Rosenberg, E. (1966). Determination of DNA base composition via depurination. Analytical Biochemistry, 16, 107.

Hussong, R.V. and Hammer, B.W. (1928). A thermophile coagulating milk under practical conditions. Journal of Bacteriology, 15, 179-188.

Imsenechi, A. and Solnzeva, L. (1945). The growth of aerobic thermophilic bacteria. Journal of Bacteriology, 49, 539-546.

Irwin, G.C., Akagi, J.M. and Himes, R.H. (1973). Ribosomes, polyribosomes and deoxribonucleic acid from thermophilic, mesophilic and psychrophilic Clostridia. Journal of Bacteriology, 113, 252-262.

Isono K. (1970). Transformation of amylase producing ability in Bacillus stearothermophilus. Japanese Journal of Genetics, 45, 285-291.

Ito, J. and Kawamura, F. (1976). Use of restriction endonucleases in analysing the genomes of bacteriophages ϕ29 and ϕ15. In "Microbiology-1976" (Ed. D. Schlessinger), 367-379. American Society for Microbiology, Washington.

Ito, K.A. (1981). Thermophilic organisms in food spoilage: flat-sour aerobes. Journal of Food Protection, 44, (2), 157-163.

Jacobsen, G. (1918). On factors influencing efficient pasteurisation. Abstracts of bacteriology, 2, 215.

Jones, C.W. (1980). Cytochrome Patterns in Classification and Identification Including their Relevance to the Oxidase Test. In "Microbiological Classification and Identification" (Ed. M. Goodfellow and R.G. Board), 127-138. London, New York, Toronto, Sydney, San Francisco: Academic Press.

Jones, D. and Sackin, M.J. (1980). Numerical Methods in the Classification and Identification of Bacteria with Especial Reference to the Enterobacteriaceae. In "Microbiological Classification and Identification" (Ed. M. Goodfellow and R.G. Board), 73-106. London, New York, Toronto, Sydney, San Francisco: Academic Press.

Jones, D. and Sneath, P.H.A. (1970). Genetic Transfer and Bacterial Taxonomy. Bacteriological Reviews, 34, 40-81.

Kahan, D. (1969). The fauna of hot springs. Verh. Int. Verein. Theo. Angew Limnol, 17, 811-816.

Karamata, D. (1970). Multiple density classes of phage P1 due to tetramen formation. Molecular and General Genetics, 107, 243-255.

Karbassi, A. and Vaughn, R.H. (1974). Characteristics of polygalacturonic acid trans-eliminase produced by a thermophilic Bacillus. Abstracts of the Annual Meeting of the American Society of Microbiology. Abstract No. P136, 167.

Kawakami, M. and Landman, O.E. (1968). Nature of the carrier state of bacteriophage SP-10 in Bacillus subtilis. Journal of Bacteriology, 95, 1804-1812.

Kelsey, J.C. (1961). The testing of sterilizers. 2 Thermophilic spore papers. Journal of Clinical Pathology, 14, (3), 313-319.

Kersters, K. and De Ley, J. (1980). Classification and Identification of Bacteria by Electrophoresis of their Proteins. In "Microbiological Classification and Identification" (Ed. M. Goodfellow and R.G. Board), 273-298. London, New York, Toronto, Sydney, San Francisco: Academic Press.

Klaushofer, H. and Hollaus, F. (1970). Zun Taxonomie der hochthermophilen, in Zuckerfabrikssaften verkommenden aeroben sporenbild ner. Zeiteshrift fur die Zuckerindustrie, 20, (a) 465-470.

Klaushofer, H., Hollaus, F. and Pollach, G. (1971). Microbiology of beet sugar manufacture. Process Biochemistry, 6, (6), 39-41.

Kluyer, A.J. and Baars, J.K. (1932). On some physiological artifacts. Konink. Akad. Wetenschnappen, Amsterdam, Proc., 35, 370-378.

Koffler, H. (1957). Protoplasmic differences between mesophiles and thermophiles. Bacteriological Reviews, 21, 227-240.

Koffler, H. and Gale, G.O. (1957). The relative thermostabilities of cytoplasmic proteins from thermophilic bacteria. Archiv fur Biochemistry and Biophysics, 67, 249-251.

Koser, S.A. (1926). Action of Bacteriophage on a Thermophilic Bacillus. Proceedings of the Society of Experimental Biological Medicine, 24, 109-111.

Kovacs, N. (1956). Identification of Pseudomonas pyocyanea by the oxidase reaction. London: Nature, 178, 703.

Laxa, O. (1900). Bakteriologische Studien uber die Produkte des mormalen Zuckerfabriksbetriebes. Zentralblatt fur Bakteriologie und Parasitenkunde II, 6, 286-295.

Lee, A.G. (1977). Lipid phase transitions and phase diagrams. Biochimica et biophysica acta, 472, 237-281.

Levy, P.L., Pangborn, H.K., Bernstein, Y., Ericsson, L.H., Neurath, H. and Walsh, K.A. (1975). Evidence of a homologous relationship between thermolysin and neutral protease A of Bacillus subtilis. Proceedings of the National Academy of Science, USA, 72, 4341-4345.

Lindsay, J.A. and Creaser, F.H. (1975). Enzyme thermostability is a transformable property between Bacillus sp. Nature, 225, 650-652.

Ljungdahl, L. and Sherod, D. (1976). Proteins from thermophilic microorganisms. In "Extreme Environments: Mechanisms of Microbial Adaption" (Ed. M.R. Heinrich), 147-188. New York and London: Academic Press.

Ljungdahl, L., Sherod, D., Moore, M.R. and Adreesen, J.R. (1976). Properties of enzymes from Clostridium thermoaceticum and Clostridium formicoaceticum. In "Enzymes and Proteins from Thermophilic Microorganisms" (Ed. H. Zuber), 237-249. Basel and Stuttgart: Birkhauser Verlag.

Ljungdahl, L. (1979). Physiology of thermophilic bacteria. Advances in Microbial Physiology, 19, 149-243.

Logan, N.A. and Berkeley, R.C.W. (1981) Classification and Identification of Members of the Genus Bacillus using API tests. In "The Aerobic Endospore-forming Bacteria" (Ed. R.C.W. Berkeley and M. Goodfellow), 105-140. London, New York, Toronto, Sydney, San Francisco: Academic Press.

Long, M.E. and Lee, C.K. (1979). US patent, 4, 179, 335.

Lovett, P.S. and Bramucci, M.G. (1975). Plasmid DNA in Bacillus subtilis and Bacillus pumilus. Journal of Bacteriology, 124, (1), 484-490.

Lund, B.M. (1965). A comparison by the use of gel electrophoresis of soluble protein components and esterase enzymes of some Group D Streptococci. Journal of General Microbiology, 40, 413-419.

Magsanaga, A. and Nosoh, Y. (1974). Conformational change with temperature and thermostability of glutamine synthetase from Bacillus stearothermophilus. Biochimica et biophysica acta, 365, 208-211.

Mahler, H.R., Mehrotva, B.D. and Sharp, C.W. (1961). Effects of Diamines on the thermal transition of DNA. Biochemical and Biophysical Research Communications, 4, (1), 79-82.

Mandell, J. and Greenberg, J. (1960). A new chemical mutagen for bacteria, 1-methyl-3-Nitro-1-Nitrosoguanidine. Biochemical and Biophysical Research Communications, 3, (6), 575-577.

Mandel, M., Levi, I., Bergendahl, J., Dodson, M.L. and Scheltgen, E. (1970). Correlation of Melting Temperature and Caesium Chloride Buoyant Density of Bacterial Deoxyribionucleic Acid. Journal of Bacteriology, 101, (2), 333-338.

Manning, G.B. and Campbell, L.L. (1961). Thermostable amylase of Bacillus stearothermophilus. Crystallization and some general properties. Journal of Biological Chemistry, 236, 2952-2957.

Marahiel, M.A., Lurz, R. and Kleinkauf, H. (1981). Characterisation of a plasmid from B. brevis ATCC 9999. Advances in Biotechnology, Vol. III. (Ed. Murray Moo-Young). Toronto, Oxford, New York, Sidney, Paris, Frankfurt: Pergamon Press.

Markewych, O., Casella, E., Dosmar, M. and Witmer, H. (1979). Deoxythymidine Nucleotide Metabolism in Bacillus subtilis W23 Infected with Bacteriophage SP10c: Preliminary Evidence that dTMP in SP10c DNA is Synthesized by a Novel, Bacteriophage-Specific Mechanmism. Journal of Virology, 29, (1), 61-68.

Marmur, J. (1960). Thermal denaturation of deoxyribose nucleic acid isolated from a thermophile. Biochimica et biophysica acta, 38, 342-343.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. Journal of Molecular Biology, 3, 208-218.

Marmur, J. and Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. Journal of Molecular Biology, 5, 109-118.

Marmur, J., Rownd, R. and Schildkraut, C.L. (1963). Denaturation and renaturation of deoxyribonucleic acid. Progress in Nucleic Acid Research, 1, 231.

McDonald, W.C. and Matney, S.T. (1963). Genetic transfer of the ability to grow at 55 ^{o}C in Bacillus subtilis. Journal of Bacteriology, 85, 218-220.

McElhany, R.N. (1976). The biological significance of alterations in the fatty acid composition of microbial membrane lipids in response to changes in environmental temperatures. In "Extreme Environments: Mechanisms of microbial adaption" (Ed. M.R. Heinrich). New York, San Francisco, London: Academic Press.

Medda, S. and Chandra, A.K. (1980). New strains of Bacillus licheniformis and Bacillus coagulans producing thermostable α-amylase active at alkaline pH. Journal of Applied Bacteriology, 48, 47-58.

Miehe, H. (1907). In "Die Selbsterhitzung des Heues". G. Fischer, Jena.

Miquel, P. (1888). Monographie d'un bacille vivant au-dela de 70 °C. Annales Micrographic, 1, 3-10.

Militzer, W., Sonderegger, T.B., Tuttle, L.C. and Georgi, C.E. (1949). Thermal enzymes. Archiv fur Biochemistry, 24, 75-82.

Minnikin, D. and Goodfellow, M. (1980). Lipid Composition in the Classification and Identification of Acid-fast Bacteria. In "Microbiological Classification and Identification" (Ed. M. Goodfellow and R.G. Board), 189-256. London, New York, Toronto, Sydney, San Francisco: Academic Press.

Minnikin, D.E. and Goodfellow, M. (1981). Lipids in the Classification of Bacillus and Related Taxa. In "The Aerobic Endospore-Forming Bacteria". (Ed. Berkeley and Goodfellow), 59-99. London, New York, Toronto, Sydney and San Francisco: Academic; Press.

Mishustin, E.N. (1950). Quoted by Golovacheva et al. (1965).

Moseley, B.E.B. and Williams, E. (1977). Repair of Damaged DNA in Bacteria. In "Advances in Microbial Physiology" 16, 99-156. London, New York, San Francisco: Academic Press.

Moseley, B.E.B. (1982). DNA Repair, an overview. At 94th Ordinary meeting of the S.G.M. (Genetics group symposium). "DNA recombination and repair". Cambridge, UK.

Munster, M.J., Sharp, R.J., Ahmad, S., Vivian, A., Atkinson, T., (1982). Transformation and Transduction in a Bacillus thermophile. Abstracts of the XIIIth International Congress of Microbiology. Boston, USA.

Naito, T., Kono, M., Fujise, N., Yakushigi, Y. and Aoki, Y. (1966). Colicine typing of Shigella sonnei. Principle, technique, selection of indicator strains and foundations for a typing scheme. Japanese Journal of Microbiology, 10, 13-22.

Neuhard, J., Price, A.R., Schack, L. and Thomassen, E. (1978). Two thymidylate synthetases in Bacillus subtilis. Proceedings of the National Academy of Science, USA, 75, (3), 1194-1198.

Norris, J.R. and Wolf, J. (1961). A study of the aerobic sporeforming bacteria. Journal of Applied Bacteriology, 24, (1), 42-56.

Norris, J.R. and Burgess, H.D. (1963). Esterases of crystalliferous bacteria pathogenic for insects; epizootiological applications. Journal of Insect Pathology, 5, 460-466.

Norris, J.R. (1964). The classification of Bacillus thuringiensis. Journal of Applied Bacteriology, 27, 439-447.

Novitsky, T.J., Chan, M., Himes, R.H. and Akagi, J.M. (1974). Effect of temperature on the growth and cell wall chemistry of a facultative thermophilic Bacillus. Journal of Bacteriology, 117, 858-865.

O'Brien, W.E., Brewer, J.M. and Ljungdahl, L.G. (1976). Chemical, Physical and Enzymatic comparisons of Formyltetrahydrofolate synthetases from thermophilic and mesophilic Clostridia. In "Enzymes and Proteins from Thermophilic Microorganisms", (Ed. H. Zuber), 249-262. Basel and Stuttgart: Birkhauser Verlag.

O'Donnell, A.G. and Norris, J.R. (1981) Pyrolysis Gas-liquid Chromatographic Studies in the Genus Bacillus. In "The Aerobic Endospore-forming bacteria" (Ed. R.C.W. Berkeley and M. Goodfellow), 141-180. London, New York, Toronto, Sydney, San Francisco: Academic Press.

O'Donovan, G. and Neuhard, J. (1970). Pyrimidine Metabolism in Microorganisms. Bacteriological Reviews, 34, (3), 278-343.

O'Donovan, G.A. (1978). Thymidine metabolism in bacteria. In "DNA synthesis: Present and Future" (Ed. I. Molineux and M. Kohlyama). Plenium Publishing Corporation.

Ogasahara, K., Imanishi, A. and Isemura, T. (1970). Studies on thermophilic α-amylase from Bacillus stearothermophilus. Some general and physiochemical properties of thermophilic α-amylase. Journal of Biochemistry (Tokyo), 67, 65-75.

Okada, T., Yanagisawa, K. and Ryan, F.J. (1961). A method for securing thymineless mutants from strains of E. coli. Zeitschrift fur Vererbungslehre, 92, 403-412.

Onodera, N. (1961). On some characteristics of a newly isolated thermophilic bacteriophage and consideration of its thermostability. Journal of Electronmicroscopy, 10, (2), 91-102.

Outtrup, H. (1976). US patent, 3, 979, 261.

Pace, B. and Campbell, L.L. (1967). Correlation of maximal growth temperature and ribosome heat stability. Proceedings of the National Academy of Sciences, USA, 57, 1110-1116.

Parker, M.T. (1972). Phage typing of Staphylococcus aureus. In "Methods in Microbiology", vol. 7B. (Ed. J.R. Norris and D.W. Ribbons). London: Academic Press.

Perutz, M.F. and Raidt, H. (1975). Deletion of globin genes in haemoglobin-H disease demonstrates multiple globin structural loci. Nature, London, 225, 256-259.

Pfueller, S.L. and Elliot, W.K. (1969). The extracellular amylase of Bacillus stearothermophilus. Journal of Biological Chemistry, 244, 48-54.

Prickett, P.S. (1928). Thermophilic and thermoduric microorganisms with special reference to species isolated from milk. Description of spore forming types. New York State Agricultural Experimental Station Technical Bulletin 147.

Priest, F.G. (1977). Extracellular enzyme synthesis in the genus Bacillus. Bacteriological Reviews, 41, (3), 711-753.

Priest, F.G. (1981). DNA Homology in the Genus Bacillus. In "The Aerobic Endospore-forming Bacteria" (Ed. R.C.W. Berkeley and M. Goodfellow), 33-58. London, New York, Toronto, Sydney, San Francisco: Academic Press.

Rabussay, D. Zillig, W. and Herrlich, P. (1970). Characterisation of the Bacillus stearothermophilus phage $\phi\mu$-4 and its DNA. Virology, 41, 91-100.

Radloff, R., Bauer, W. and Vinograd, J. (1967). A Dye-Buoyant-Density Method for the detection and isolation of closed circular DNA in Hela cells. Proceedings of the National Academy of Sciences, USA, 57, 1514-1520.

Ramaley, R.F. and Hixon, J. (1970). Isolation of a non-pigmented, thermophilic bacterium similar to Thermus aquaticus. Journal of Bacteriology, 103, 527-528.

Ray, H.R., White, D.C. and Brock, T.D. (1971). Effect of temperature on the fatty acid composition of Thermus aquaticus. Journal of Bacteriology, 106, (1), 25-30.

Reanney, D.C. and Wood, M. (1973). Phages for facultatively thermophilic strains of Bacillus stearothermophilus: Phage D-5. New Zealand Journal of Science, 16, 681-695.

Reanney, D. (1976). Extrachromosomal elements as possible agents of adaption and development. Bacteriological Reviews, 40, 552-590.

Renco, P. (1942). Ricerche su un fermento lattico sporigeno (B. thermoacidicans). Annale Microbiologia, 2, 109-114.

Robertson, A.H. (1927). New York State Agricultural Experimental Station Bulletin, 130.

Robinson, K. (1966). An examination of Corynebacterium sp. by gel electrophoresis. Journal of Applied Bacteriology, 29, 179-184.

Robson, E. and Pain, R.H. (1971). Analysis of the code relating sequence to conformation in proteins: Possible implications for the mechanism of formation of helical regions. Journal of Molecular Biology, 58, 237-259.

Romig, W.R. and Brodetsky, A.M. (1961). Isolation and preliminary characterisation of bacteriophages of B. subtilis. Journal of Bacteriology, 82, 135-141.

Rosenthal, R., Toye, P.A., Korman, R.Z. and Zahler, S.A. (1979). The prophage of SPβ c2d $citK_1$, A defective specialised transducing phage of Bacillus subtilis. Genetics, 92, 721-739.

Rowe, J.J., Goldberg, I.D. and Amelunxen, R.E. (1973). Isolation of mutants of Bacillus stearothermophilus blocked in catabolic function. Canadian Journal of Microbiology, 19, 1521-1523.

Rowe, J.J., Goldberg, I.D. and Amelunxen, R.E. (1975). Development of defined and minimal media for the growth of Bacillus stearothermophilus. Journal of Bacteriology, 124, 279-284.

Ruger, H.T. and Richter, G. (1979). Bacillus globisporus subsp. marinus subsp. nov. International Journal of Systematic Bacteriology, 29, (3), 196-203.

Sanderson, K.E, (1976). Genetic relatedness in the family Enterobacteriaceae. Annual Reviews of Microbiology, 30, 327-349.

Sargeant, K., East, D.N., Whitaker, A.R. and Ellsworth, R. (1971). Production of Bacillus stearothermophilus NCA 1503 for glyceraldehyde-3-phosphate dehydrogenase. Journal of General Microbiology, 65, iii.

Sattar, S.A., Synek, E.J., Westwood, J.C.N. and Neals, P. (1972). Hazard Inherent in Microbiol. Tracers: Reduction of risk by the use of Bacillus stearothermophilus spores in aerobiology. Applied Microbiology, 23, (6), 1053-1059.

Saunders, G.G. and Campbell, L.L. (1965). Properties of the Deoxyribonucleic acid of the thermophilic bacteriophage TP-84. Biochemistry, 4, (12), 2836-2844.

Saunders, G.F. and Campbell, L.L. (1966). Ribonucleic acid and ribosomes of Bacillus stearothermophilus. Journal of Bacteriology, 91, 332-339.

Schaeffer, A.B. and Fulton, M. (1933). A simplified method of staining endospores. Science, New York, 77, 194.

Schenk, A. and Aragno, M. (1979). Bacillus schlegelii, a new species of thermophilic facultatively chemolithoautotrophic bacterium oxidising molecular oxygen. Journal of General Microbiology, 115, 333-341.

Schildkraut, C.L., Marmur, J. and Doty, P. (1962). Determination of the base composition of deoxy-ribonucleic acid from its buoyant density in CsCl. Journal of Molecular Biology, 4, 430-443.

Schildkraut, C. and Lifson, S. (1965). Dependence of the melting temperature of DNA on salt concentration. Biopolymers, 3, 195-208.

Schlanger, G., and Friedman, S.M. (1973). Ambiguity in a polypeptide synthesizing extract from Saccharomyces cerevisiae. Journal of Bacteriology, 115, 129-138.

Shafia, F. and Thompson, T.L. (1964). Isolation and preliminary characterisation of bacteriophage $\phi\mu$-4. Journal of Bacteriology, 87, (5), 999-1002.

Shafia, F. (1966). Thermocins of Bacillus stearothermophilus. Journal of Bacteriology, 92, 524-525.

Shah, H.N., Williams, R.A.D., Bowden, G.H. and Hardie, J.M. (1976). Comparison of the biochemical properties of Bacteroides melaninogenicus from human dental plaque and other sites. Journal of Applied Bacteriology, 41, 473-492.

Sharp, R.J., Bingham, A.H.A., Comer, M.J. and Atkinson, A. (1979). Partial Characterisation of a bacteriocin (thermocin) from Bacillus stearothermophilus RS 93. Journal of General Microbiology, 111, 449-451.

Sharp, R.J., Bown, K.J., and Atkinson, A. (1980). Phenotypic and Genotypic characterisation of some thermophilic species of Bacillus. Journal of General Microbiology, 117, 201-210.

Shaver, K.J. and Schiff, T. (1969). 47th General Meeting, Int. Assoc. Dental Res. Houston, Texas.

Shen, P.Y., Coles, E., Foote, J.L. and Stenesh, J. (1970). Fatty acid distribution in mesophilic and thermophilic strains of the genus Bacillus. Journal of Bacteriology, 103, 479-481.

Shilo, M. (1979). In "Thermophilic Microorganisms at High Temperature". Dahlem Konferenzen, Berlin. New York: Springer Verlag.

Shiosaka, M. (1976). US patent, 3, 988, 206.

Sidler, W. and Zuber, H. (1977). The production of extracellular thermostable neutral proteinase and α-amylase by Bacillus stearothermophilus. European Journal of Applied Microbiology, 4, 255-266.

Sidler, W. and Zuber, H. (1980). Isolation Procedures for Thermostable Neutral Proteinases produced by Bacillus stearothermophilus. European Journal of Applied Microbiology and Biotechnology, 10, 197-209.

Silvestri, L.G. and Hill, L.R. (1965). Agreement between deoxyribonucleic acid base composition and taxometric classification of gram-positive cocci. Journal of Bacteriology, 90, 136-140.

Simmons, J.S. (1926). A culture medium for differentiating organisms of typhoid-colon aerogenes groups and for isolation of certain fungi. Journal of Infectious Diseases, 39, 209.

Singleton, R. (1976). A comparison of the amino acid compositions from thermophilic and non-thermophilic origins. In "Extreme Environments: Mechanisms of Microbial Adaption", (Ed. M.R. Heinrich), 189-200. New York and London: Academic Press.

Singleton, R. and Amelunxen, R.E. (1973). Proteins from thermophilic microorganisms. Bacteriological Reviews, 37, 320-342.

Skerman, V.B.D. (1967). A Guide to the Identification of the Genera of Bacteria. Baltimore: The Williams and Wilkins Company.

Smith, D.G., Stein, W.H., and Moore, S. (1963). The sequence of amino acid residues in bovine pancreatic ribonuclease: Revisions and confirmations. Journal of Biological Chemistry, 238, 227-234.

Smith, N.R., Gordon, R.E. and Clark, F.E. (1952). Aerobic sporeforming bacteria. Agricultural Monograph No. 16. United States Department of Agriculture.

Sneath, P.H.A. and Sokal, R.R. (1973). "Numerical Taxonomy." San Francisco: W.H. Freeman and Company.

Sneath, P.H.A. (1978). Identification of Microorganisms (Ed. Norris and Richmond) 1978. Essays in Microbiology.

Snedecore, B. and Cooney, C.L. (1974). Thermophilic mixed culture of bacteria utilising methanol for growth. Applied Microbiology, 27, (6), 1112-1117.

Sokal, R. and Sneath, P.H.A. (1963). "Principles of Numerical Taxonomy". London: W.H. Freeman and Co.

Souza, K.A., Kostiw, L.L. and Tyson, B.J. (1974). Alterations in normal fatty acid composition in a temperature sensitive mutant of a thermophilic Bacillus. Archives of Microbiology, 97, 89-102.

Spackman, D.H., Stein, W.H. and Moore, S. (1960). The disulphide bonds of Ribonuclease. Journal of Biological Chemistry, 235, 648-659.

Spizizen, J. (1958). Transformation of a biochemically deficient strain of Bacillus subtilis by deoxyribonucleic acid. Proceedings of the National Academy of Sciences, USA, 44, 1072-1078.

Stacey, K.A. (1976). The consequences of thymine starvation. In "The Survival of Vegetative Microbes" (Ed. T.R.G. Gray and J.R. Postgate). Cambridge, London, New York, Melbourne: Cambridge University Press.

Stahl, S. and Olsson, O. (1977). Temperature range variants of Bacillus megaterium. Archiv. fur Mikrobiologie, 113, 221-229.

Stark, E. and Tetrault, P. (1952). A determinative study of amylolytic, stenothermophilic bacteria isolated from soil. Scientific Agriculture (Canada), 32, 81-92.

Stenesh, J., Roe, B.A. and Snyder, T.L. (1968). Studies of deoxyribonucleic acid from mesophilic and thermophilic bacteria. Biochimica et biophysica acta, 161, 442-454a.

Stenesh, J. and Roe, B.A. (1972). DNA polymerase from mesophilic and thermophilic bacteria. II Temperature dependence of nearest neighbour frequencies of the product from the DNA polymerase reaction. Biochimica et biophysica acta, 272, 167-178.

Stenesh, J. (1976). Information transfer in thermophilic bacteria. In "Extreme Environments: Mechanisms of microbial adaption" (Ed. M.R. Heinrich), 85-101. New York, San Francisco, London: Academic Press.

Streips, U.N. and Welker, N.E. (1969). Infection of Bacillus stearothermophilus with Bacteriophage Deoxyribonucleic Acid. Journal of Bacteriology, 99. 1, 344-346.

Suekane, M., Kanno, M. and Hasegawa, S. (1974). US patent, 3, 826, 714.

Sundaram, T.K. (1973). Physiological role of pyruvate carboxylase in a thermophilic Bacillus. Journal of Bacteriology, 113, 549-557.

Sweet, D.M. and Moseley, B.E.B. (1974). Accurate repair of ultraviolet-induced damage in Micrococcus radiodurans. Mutation Research, 23, 311-318.

Tajima, M., Urabe, I., Yutani, K. and Okada, H. (1976). Role of calcium ions in the thermostability of thermolysin and Bacillus subtilis var. amylosacchariticus neutral protease. European Journal of Biochemistry, 64, 243.

Tanaka, M., Haniu, M., Matsueda, G., Yasunobu, K.T., Himes, R.H., Akagi, J.M., Barnes, E.M. and Devanathan, T. (1971). The primary structure of the Clostridium tartarivorum ferredoxin, a heat stable ferredoxin. Journal of Biological Chemistry, 246, 3958-3960.

Tansey, M.R. and Brock, T.D. (1978). Microbial life at high temperatures: ecological aspects. In "Microbial life in extreme environments" (Ed. D.J. Kushner), 159-216. London: Academic Press..

Thompson, T.L. and Shafia, F. (1962). Energy Requirement for Adsorption of Bacteriophage ϕμ-4'. Biochemical and Biophysical Research Communications 8, (6).

Thompson, G.A. and Nozawa, Y. (1977). Tetrahymena: A system for studying dynamic membrane alterations within the eukaryotic cell. Biochemica et biophysica acta, 472, 55-92.

Thorne, C.B. (1968). Transduction in Bacillus cereus and Bacillus anthracis. Bacteriological Reviews, 32, 4, 358-361.

Tirgari, S. and Moseley, B.E.B. (1980). Transformation in Micrococcus radiodurans: Measurement of various parameters and evidence for multiple independently segregating genomes per cell. Journal of General Microbiology, 119, 287-296.

Varel, V.H., Isaacson, H.R. and Bryant, M.P. (1977). Thermophilic methane production from cattle waste. Applied and Environmental Microbiology, 33, (2), 298-307.

Vary, P.S. (1979). Transduction in Bacillus megaterium. Biochemical and Biophysical Research Communications, 88, (3), 1119-1124.

Walker, P.D. and Wolf, J. (1971). The taxonomy of Bacillus stearothermophilus. In "Spore Research" (Ed. A.N. Barker, G.W. Gould and J. Wolf.), 247-262. London: Academic Press.

Warner, F.D., Kitos, G.A., Romano, M.P. and Hemphill, H.E. (1977). Characterisation of SPβ A temperate bacteriophage from Bacillus subtilis 168M. Canadian Journal of Microbiology, 23, 45-51.

Webley (1947). Quoted by Smith et al. (1952).

Wedler, F.C. and Hoffman, F.M. (1974). Glutamine synthetase of Bacillus stearothermophilus. II Regulation and thermostability. Biochemistry, 13, 3215-3221.

Wedler, F.C. (1978). Properties and Regulation of Thermophilic Glutamine Synthetases. In "Biochemistry of Thermophily" (Ed. S.M. Friedman), 325-345. New York, San Francisco, London: Academic Press.

Weerkamp, A. and Heinen, W. (1972). The effect of temperature on the fatty acid composition of the extreme thermophiles, B. caldolyticus and B. caldotenax. Journal of Bacteriology, 109, (1) 443-446.

Weerkamp, A. and MacElroy, R.D. (1972). Lactate dehydrogenase from an extremely thermophilic Bacillus. Archiv fur Mikrobiologie, 85, 113-122.

Weigel, J. and Ljungdahl, L.G. (1981). Thermo anaerobacter ethanolicus gen. nov., spec. nov., a new, extreme thermophilic, anaerobic bacterium. Archives of Microbiology, 128, 343-348.

Weimer, P.J. and Zeikus, J.G., (1977). Fermentation of Cellulase and Cellobiose by Clostridium thermocellum in the absence and presence of Methanobacterium thermoautotrophicum. Applied and Environmental Microbiology, 33, (2), 289-297.

Welker, N.E. and Campbell, L.L. (1965). Induction and properties of a temperate bacteriophage from Bacillus stearothermophilus. Journal of Bacteriology, 89, 175-184.

Welker, N.E. (1976). Microbial endurance and resistance to heat stress. In "The survival of vegetative microbes", (Ed. T.R.G. Gray and J.R. Postgate), 241-277. Cambridge, London, New York, Melbourne: Cambridge University Press.

White, R., Georgi, C.E. and Militzer, W.E. (1955). Characteristics of a thermophilic bacteriophage. Proceedings of the Society of Biological Medicine, 88, 373-377.

Williams, R.A.D. and Shah, H.N. (1980). Enzyme Patterns in Bacterial Classification and Identification. In "Microbiological Classification and Identification" (Ed. M. Goodfellow and R.G. Board), 299-318. London, New York, Toronto, Sydney, San Francisco: Academic Press.

Williams, R.A.D. (1975). Caldoactive and thermophilic bacteria and their thermostable proteins. Science Progress, Oxford, 62, 373-393.

Wilson, G.A., Williams, M.T., Baney, H.W. and Young, F.E. (1974). Characterisation of temperate bacteriophages of B. subtilis by the restriction endonuclease EcoRI: Evidence for three different temperate bacteriophages. Journal of Virology, 14, (4), 1013-1016.

Wilson, M.C., Farmer, J.L. and Rothman, F. (1966). Thymidylate synthesis and aminopterin resistance in B. subtilis. Journal of Bacteriology, 92, (1), 186-196.

Wisdon, C. and Welker, N.E. (1973). Membranes of Bacillus stearothermophilus: Factors affecting protoplast stability and thermostability of alkaline phosphatase and reduced nicotinamide adenine dinucleotide oxidase. Journal of Bacteriology, 114, 1336-1345.

Wise, D.L., Cooney, C.L. and Augenstein, D.C. (1978). Biomethanation: Anaerobic fermentation of CO_2, H_2, and CO to methane. Biotechnology and Bioengineering, XX, 1153-1172.

Wolf, J. and Sharp, R.J. (1981) Taxonomic and Related Aspects of Thermophiles within the Genus Bacillus. In "The Aerobic endospore-forming Bacteria" (Ed. R.C.W. Berkeley and M. Goodfellow), 251-296. London, New York, Toronto, Sydney, San Francisco: Academic Press.

Young, F.E. (1980). Impact of Cloning in Bacillus subtilis on Fundamental and Industrial Microbiology. Journal of General Microbiology, 119, 1-15.

Yule, R. and Barridge, B.D. (1976). Isolation and characterisation of a bacteriocin produced by Bacillus stearothermophilus strain NU 10. Canadian Journal of Microbiology, 22, 1743-1750.

Yutani, K. (1976). Role of calcium ions in the thermostability of amylase produced by Bacillus stearothermophilus. In "Enzymes and Proteins from thermophilic microorganisms" (Ed. H. Zuber), 91-103. Basel and Stuttgart: Birkauser Verlag.

Zeikus, J.G. and Wolf, R.S. (1972). Methanobacterium thermoautotrophicus sp. n., an anaerobic, autotrophic extreme thermophile. Journal of Bacteriology, 109, (2), 707-713.

Zeikus, J.G. (1979). Thermophilic bacteria: ecology, physiology and technology. Enzyme Microbiology and Technology, 1, 243-252.

Zuber, H. (1978). Comparative studies of thermophilic and mesophilic enzymes: Objectives, problems, results. In "Biochemistry of thermophily" (Ed. S.M. Friedman), 267-287. New York, San Francisco, London: Academic Press.

APPENDIX I

APPENDIX I

Strains used in the numerical taxonomy study listed in the order determined by single linkage analysis

		Cluster No.
0	RS 5	Ua
1	RS 6	Ua
2	RS 10	Ia
3	RS 23	Ia
4	RS 19	Ia
5	RS 241	Ia
6	RS 13	Ia
7	RS 240	Ia
8	RS 42	Ia
9	RS 43	Ia
10	RS 182	Ia
11	RS 3	Ia
12	RS 11	Ia
13	RS 239	Ia
14	RS 216	Ia
15	RS 9	Ua
16	EP 136 Epstein and Grossowicz (1969)	(1b)
17	LUDA 141 Walker and Wolf (1971)	(1b)
18	RS 242	(1b)
19	RS 244	(1b)
20	RS 249	(1b)
21	RS 18	(1b)
22	RS 21	(1b)
23	RS 53	(1b)
24	RS 98	(1b)
25	RS 99	(1b)
26	RS 70	(1b)
27	RS 22	(1b)
28	RS 47	(1b)
29	RS 57	(1b)
30	RS 217	(1b)
31	RS 37	(1b)
32	RS 210	(1b)
33	RS 93 Sharp et al. (1979)	(1b)
34	RS 209A	(1b)
35	RS 45	Ua
36	B. caldotenax Heinen and Heinen (1972)	(2a)
37	B. caldovelox Heinen and Heinen (1972)	(2a)
38	B. caldolyticus Heinen and Heinen (1972)	(2a)
39	RS 16	(2a)
40	ATCC 8005 (B. kaustophilus), Prickett (1928)	(2a)
41	RS 7	(2a)
42	RS 15	(2a)
43	LUDA T210, NCA 26 Walker and Wolf (1971); Donk (1920)	(2b)
44	NCIB 8919, NCA 1356	(2b)
45	LUDA T214, NCA 1518	(2b)
46	ATCC 10149	(2b)
47	NCTC 10003	(2b)
48	NCA 1503	(2b)
49	NW 10	(2b)
50	DSM 456 Klaushofer and Hollaus (1970)	
51	RS 222	
52	RS 248	
53	LUDA T42 Walker and Wolf (1971)	
54	LUDA T60 Walker and Wolf (1971)	
55	RS 48	
56	DSM 730 B. thermocatenulatus Golovache	
57	ATCC 12016, NCA 2184	
58	EP 240	
59	RS 23	
60	RS 85	
61	RS 86	
62	RS 187	
63	RS 203	
64	LUDA T22 Walker and Wolf (1971)	
65	DSM 466 B. thermodenitrificans, Klaushof	
66	DSM 465 B. thermodenitrificans, Klaushof	
67	RS 51	
68	RS 168	
69	RS 209B	
70	RS 211	
71	RS 204	
72	RS 1	
73	RS 14	
74	EP 262	
75	RS 2	
76	RS 56	
77	RS 4	
78	RS 17	
79	RS 80	
80	RS 44	
81	RS 54	
82	RS 49	
83	RS 8	
84	RS 245	
85	RS 215	
86	DSM 463 B. sphaericus, Klaushofer and Ho	
87	RS 173	
88	RS 174	
89	RS 20	
90	RS 87	
91	RS 166	
92	RS 58	
93	RS 236	
94	RS 237	
95	RS 243	
96	ATCC 12245 B. coagulans	
97	ATCC 8038 B. coagulans	
98	RS 155	
99	RS 12	
100	RS 205	
101	LO_2 Bingham et al. (1980)	