# IDENTIFICATION OF THE VIRULENCE DETERMINANTS OF THE NEONATAL MENINGITIC BACTERIUM *CRONOBACTER SAKAZAKII*

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## COPYRIGHT STATEMENT

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# ABSTRACT

The *Cronobacter* genus is a member of the family *Enterobacteriaceae*, comprising of seven species *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. dublinensis*, *C. muytjensii*, *C. universalis*, and *C. condimenti*. *Cronobacter* is gaining importance as a pathogen due to the severity of the infection caused such as septicaemia, necrotizing entercolitis and severe infantile meningitis, and the numerous outbreaks reported. Clinical cases are associated with the three species *C. malonaticus*, *C. turicensis* and, in particular, with *C. sakazakii* multilocus sequence type 4. The understanding of its pathogenicity is still not fully understood despite the clinical evidence, resulting in the concern the FAO-WHO.

Therefore, this study aimed to apply the Multiple Locus Sequence Typing scheme (MLST) to three collections of clinical strains which had not been previously profiled by MLST. These were from USA (2011), Israel (2000), and the Czech Republic (2007-2012). The strains from the latter two collections had only been identified as *E. sakazakii*, at that point. Among the Israeli strain collection, isolates from infants ranging from 2 to 36 weeks old, 7/9 strains were identified as belonging to the ST4 clonal complex. Similarly, 10/15 of the *C.sakazakii* strains isolated from US infant cases were found to belong to the ST4 clonal complex. Whereas 11 strains of the Czech isolates were from various age groups and were identified as *C.malonaticus* especially ST7, which is also the most clinically predominant ST of that species among non-infant infections and 6 strains were found to belong ST4. This research reported the first meningitis case by *Cronobacter malonaticus* (CC112) which was from an infant (age <1 month) with severe brain damage which led to their death.

Of particular interest in this research was the finding that *C. sakazakii* and some strains of *C. turicensis* were unique in the *Cronobacter* genus in utilization of exogenous sialic acid as a carbon source which may have a role in the organism's virulence. The presence of sialic acid utilization genes could be relatively recent evolution, as high levels of sialic acid are accessible to bacteria in intestinal mucosa and the brain.

Another important finding was, the presence of a number of key virulence associated genes assessed by laboratory studies in *Cronobacter sakazakii* strains (in particular with ST4). In this study, 36 clinical isolates were analysed that included: two iron acquisition system gene clusters (*eitA* and *iucC*), a pla- like homologue named *Cronobacter* plasminogen activator (*Cpa*) ,and type VI secretion (T6SS) gene cluster. The majority of *C. sakazakii* strains were serum resistant (32/36), and thus, they had the ability to survive in blood by preventing serum-mediated killing. Also, different iron acquisition systems were encoded by *C. sakazakii* 97% to attain iron from the host. Some *C. sakazakii* strains encoding T6SS patterns were from of clinical cases such as NECII and severe meningitis strains

Plasmid profiling experiments were carried out on the *Cronobacter sakazakii* strains sequenced in a parallel study. The high-size plasmid (molecular weight between 138 and 118 kb) was observed as common in (27/34) of all strains which known to encode an assortment of virulence factors. Also, plasmid DNA analysis publicised that there was no specific plasmid profiling among clinical strains. Furthermore, it shown there is no correlation observed between sequence type and present or absent the plasmid.

Furthermore, it was of interest whether the presence of plasmid pESA3 is linked with virulence in *C. sakakzaii*. This research work developed a tool by inserting the plasmid pESA3 into a plasmid-less strain and observeing the significance changes in the phenotypic and virulence associated behaviour. Stains encoding large plasmid were able to invade human intestinal epithelial cells Caco-2, brain endothelial cells HBMEC and rBCEC4. Also have been reported significant observation in siderophore production and serum survival values whereas plasmid less strain and not shown less significant associated virulence.

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## LIST OF ABBREVIATIONS

**ABC** ATP binding cassette

**AP-PCR** Arbitrarily primed polymerase chain reaction

**ATP** Adenosine triphosphate

**BBB** Blood brain barrier

**BLAST** Basic local alignment search tool

**BPW** Buffered peptone water

**CC** Clonal complex

**CDC** Centers for disease control and prevention

**CFU** Colony forming units

**CGH** Comparative genomic hybridization

**CNS** Central nervous system

**COSHH** Control of substances hazardous to health

**CSF** Cerebrospinal fluid

**DDH** DNA-DNA hybridization

**DFI** Druggan-Forsythe-Iversen

**DLV** Double locus variant

**DNA** Deoxyribonucleic acid

**EDTA** Ethylenediamine tetra-acetic acid

**EE** *Enterobacteriaceae* enrichment

**EGA** Estimated gestation age

**ESIA** *Enterobacter sakazakii* isolation agar

**FAO** Food and Agriculture Organization of the UN

**FDA** Food and Drug Administration

**FUF** Follow up formula

**GPA** Gentamicin protection assay

**HBMEC** Human brain microvascular epithelial cells

**ISO** International Organization for Standardization

**ITS** Internal transcribed spacer

**MEGA** Molecular Evolutionary Genetics Analysis

**MFS** Major facilitator superfamily

**MLEE** Multilocus enzyme electrophoresis

**MLSA** Multilocus sequence analysis

**MLST** Multilocus sequence typing

**MLVA** Multi-locus variable-number tandem-repeat analysis

**MPN** Most probable number

**MYA** Million years ago

**NCBI** National Centre for Biotechnology Information

**NCTC** National Collection of Type Cultures

**NEC** Necrotising enterocolitis

**NGS** Next generation sequencing

**NICU** Neonatal intensive care unit

**NTU** Nottingham Trent University

**OD** Optical density

**O-LPS** Oligo-lipopolysaccharide

**OM** Outer membrane

**ORF** Open reading frame

**PBS** Phosphate buffered saline

**PCR** Polymerase chain reaction

**PEP** Phosphenolpyruvate

**PFGE** Pulsed field gel electrophoresis

**PIF** Powdered infant formula

**RAPD** Random amplification of polymorphic DNA

**RFLP** Restriction fragment length polymorphism

**SDS** Sodium dodecyl sulphate

**SLV** Single locus variant

**ST** Sequence type

**TAE** Tris-acetate-EDTA

**TLV** Triple locus variant

**TRAP** Tripartite ATP-independent periplasmic

**TSA** Trypticase soy agar

**TSB** Trypticase soy broth

**UV** Ultraviolet

**VRBGA** Violet red bile glucose agar

**WHO** World Health Organization

# CHAPTER 1

# GENERAL INTRODUCTION

# (OVERVIEW OF *CRONOBACTER* GENUS)

# 1. THE GENUS *CRONOBACTER*

## 1.1 TAXONOMY

*Cronobacter* (formerly known as *Enterobacter sakazakii*) are a rare cause of neonatal Gram-negative infections that can develop into necrotizing enterocolitis, meningitis, and brain abscess formation. Following these infections, one third of infants die and 75% of survivors suffer severe neurological impairment. These infections are opportunistic since they can be part of the natural intestinal flora and not all infants that are colonized become infected. The bacterium has been the source of numerous nosocomial outbreaks in neonatal hospital wards and has been found to contaminate powdered infant formula (PIF). The diverse genus consists of 7 species: *C. sakazakii, C. malonaticus, C. muytjensii, C. turicensis, C. dublinensis, C. universalis,* and *C. condimenti* (Iversen *et al.* 2008; Joseph *et al*. 2011).

Proper classification of an organism is an important requirement for controlling the organism so that it can be differentiated from other closely related organisms that can be also be recovered with the target organism. Hence, regulatory control of bacteria is essentially based on comprehensive and precise bacterial taxonomy for distinguishing the target bacteria. Initial techniques for the detection of *Cronobacter* are not considered to be sufficiently reliable as they were mostly based on a limited number of strains which were poorly characterized or even misidentified (Forsythe *et al*. 2014)

Our knowledge regarding the taxonomy of the genus *Cronobacter* was improved by applying NGS (Next Generation Sequencing). The genus *Cronobacter* is a member of the family *Enterobacteriaceae* and the bacterial class gammaproteobacteria. The recently characterized genus *Kosakonia* together with the well-established genera like *Pantoea* and *Citrobacter* serve to be the most closely related organisms to the *Cronobacter*. Certain isolates like *Enterobacter ludwiggii* and *Enterobacter hormaechei* have been identified incorrectly as *Cronobacter* and this has led to confusion in the literature. Just like most of the *Cronobacter* strains, organisms belonging to the genus *Pantoea* are mainly plant-borne and mostly form yellow pigments. Similarly, *Citrobacter koseri*, an important member of the genus *Citrobacter*, has been found to cause invasive neonatal

meningitis involving brain abscess formation and associated symptoms. These presentations of the infection resemble with the symptoms of meningitis caused by *Cronobacter* species; however, they are different from the symptoms of neonatal meningitis caused by the *E. coli* K1 pathovar (Kucerova *et al*. 2011).

*Enterobacter cloacae*-like bacterial strains which produced yellow pigments were identified as *Enterobacter sakazakii* prior to the first FAO-WHO expert meeting (2004). Upon detailed analysis, all of the strains previously identified as *E. sakazakii* were initially regarded as the *Cronobacter* genus. Iversen *et al* (2007) defined 6 *Cronobacter* species in part based on the sixteen *E. sakazakii* biotypes i.e. *C. sakazakii* (biotypes 1–5, 7, 8, 9, 11, 13 and 14), *C. dublinensis* (biotypes 6, 10 and 12), *C. muytjensii* (biotype 15) and *C. turicensis* (biotypes 16, 16a and 16b). However, this differentiation was later updated (Iversen *et al*. 2008) by adding *C. malonaticus* (biotypes 5, 9 and 14), which was previously characterized as a sub-species of *C. sakazakii*. This was because Iversen *et al* (2007) was unable to differentiate between *C. malonaticus* and *C. sakazakii* on the basis of 16S rDNA sequencing. This differentiation between the above mentioned species proved to be challenging mainly because of two facts: 1) Classification of *Cronobacter* species using biotype profiles was not robust since certain biotype index strains were actually misidentified (Baldwin *et al*. 2009; Joseph *et al*. 2013) 2) *Cronobacter* genome contains 7 copies of rDNA and for that reason, intrageneric variations can cause inconsistent and doubtful base calls resulting in errors in GenBank records.

Joseph *et al* (2012) on the other hand, employed bacterial strains chosen on the basis of seven loci multilocus sequence analysis (MLSA; 3036 bp concatenated length) as representatives for the genus *Cronobacter* and hence came up with a grouping that prevailed over the previous grouping made on the basis of phenotyping. Two additional species of *Cronobacter* i.e. *C. condimentii* and *C. universalis* were characterized as a result of these research studies (Joseph *et al*. 2012). These and other recent developments made in the taxonomy of the genus *Cronobacter* are conveniently adapted with the help of sequence-based MLST method; however, several techniques like PCR probes which have been recently accepted still need to be re-evaluated. In order to re-examine the

taxonomic classification of *Enterobacter turicensis*, *E. pulveris* and *E. helveticus*, Brady *et al*. (2013) executed MLSA using only four loci (*atpD*, *infB*, *rpoB*, and *gyrB*). This resulted in naming of these organisms as *C. zurichensis*, *C. pulveris* and *C. helveticus*, respectively. Since small number of loci (less than the standard five) was used in this specific re-examination, findings of this study were considered to be questionable. Moreover, this particular development resulted in issues relating to control of *Cronobacter* species internationally in powdered infant formula because several techniques for the detection of these strains used the above mentioned *Enterobacter* species as negative controls (Masood *et al*. 2013; Jackson *et al*. 2014). However, Stephan and co-workers later suggested that the latter species should constitute new genera naming *Siccibacter* and *Franconibacter* (Stephen *et al*. 2014) This is evident from Figure 1.1 that 7-loci MLSA validates this update in the *Cronobacter* taxonomy as they do not demonstrate clustering with the 7 species of *Cronobacter*.

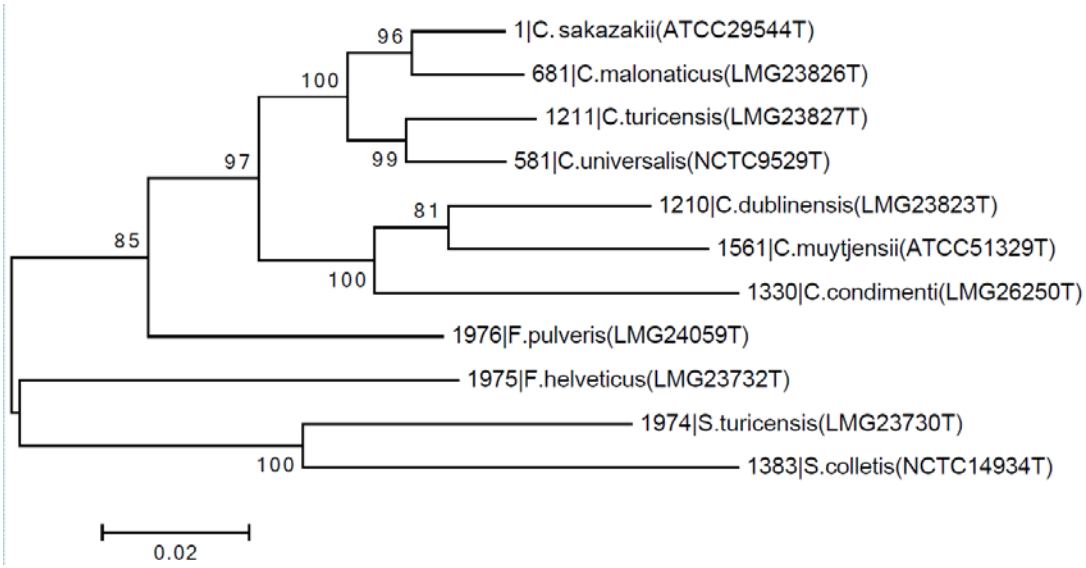


**Figure 1.1** Maximum likehood tree of MLST loci (concatenated length 3036 base pair) of *Cronobacter* genus and related *Enterobacteriaceae (Franconibacter* and *Siccibacter)* genera. The NTU strains IDs are showed at the top of each branch, the tree is drawn to scale using MEGA5, with 1000 bootstap replicates.

## 1.2 EPIDEMIOLOGY

*Cronobacter* spp. has been known to cause various life threatening diseases such as meningitis, necrotising enterocolitis (NEC), septicaemia and pneumonia (Caubilla-Barron *et al*. 2007; Muytjens *et al*. 1983).

*Cronobacter* spp. affects infants, specifically pre-term, low birth weight infants, i.e. <2500 g and less than 28 days of age (Lai *et al*. 2001), which suffer from infection symptoms such as abscess, bacteraemia or sepsis, conjunctivitis, digestive problems, necrotising enterocolitis, meningitis and tonsillitis. Meningitis followed by bacteraemia and necrotising enterocolitis are the most common conditions seen (Healy *et al*. 2009). *Cronobacter* meningitis or bacteraemia was seen in 1 case among 100,000 infants per year according to a survey conducted by the Food borne Diseases Active Surveillance Network (FoodNet) in 2002 (Bowen and Braden 2008). There have been many factors contributed to NEC for example incomplete immune system, hypothermia, hypoxia and consumption of the enteral formula feed as an alternative to breast milk which including constructive contents (Grishin *et al*. 2013). The central nervous system (CNS) infections *Cronobacter* has a tropism near the central nervous system, once it arrives the systemic circulation it may cause sepsis and bacteraemia in babies with higher birth-weight and meningitis specially in low birth-weight babies (Yan *et al*. 2012). According to Jardaat *et al.* 2014, ventricle phenomenon have been happened by abnormal accumulation of CSF which caused cranial pressure on the brain cell.

Elderly or immunocompromised adults older than 50 years (Lai 2001) are also affected by *Cronobacter* and had experienced bacteraemia, sepsis, pneumonia and wound infection but not as serious as the infants. Some adult *Cronobacter* infections have also arisen as secondary infections, due to other underlying problems such as malignancies. Most adult infections have been associated with conjunctivitis, urinary tract infection and environmental source. For example medical tools, water and route of transmission person to person (Friedemann. 2009; Flores *et al*. 2011). No *Cronobacter* meningitis has been reported in any adults (Friedemann. 2009).

## 1.3 OUTBREAKS

- The first *Cronobacter* outbreak was recorded by Muytjens *et al*. (1983) in which eight infants from The Netherlands had neonatal meningitis; five were from the same hospital and the remaining three from three different hospitals. Six infants died, mostly due to contamination of infant formula. Moreover, the organism was also found in the powdered formula tin, dishwashing brush and spoon.

- In 1994, in the NICU of a French hospital, a *Cronobacter* spp. outbreak took place and was reported by Caubilla-Barron *et al*. (2007). They performed a thorough phenotypic and genotypic examination of the 31 *Cronobacter* spp. strains taken from 16 infected infants, (of which 3 infants died) and prepared and unprepared infant formula tins.
- The first outbreak which proved a strong link between *Cronobacter* and NEC cases occurred in Belgium, where twelve infants had *Cronobacter* NEC recorded by Van Acker *et al*. (2001). All the 12 infants were pre-term, low birth weight, formula-fed infants. Two infants eventually died. Three diverse profiles among the patient isolates contained the strains from which were examined by randomly primed polymerase chain reaction (AP-PCR) to reveal three different profiles among the patient isolate.

- Five infants in a hospital in Jerusalem had bacteraemia and meningitis and colonizations recorded by Block *et al*. (2002). These bacterial strains were negative for nitrite reduction. A blender in the hospital, in which the formula was processed, also contained *Cronobacter* strain of the same pulse type as the ones from the infants when tested by pulsed field gel electrophoresis (PFGE). Despite constant decontaminations, the organism was constantly present for up to 5 months, proving the creation of an environmental niche by *Cronobacter* spp. in the blender.

- Himelright *et al*. (2002) recorded the outbreak of *Cronobacter* spp. in a hospital in Tennessee. Forty nine hospitalized infants were tested after a meningitic infant's death, eight infants were found to be positive. Two infants had respiratory illnesses while six

were asymptomatic. Eventually, there was a recall of the product by the manufacturer. The formula was a commercial formula product not proposed for use by neonates, unless advised by the neonatologists.

- In December 2011, in four different US states (Florida, Illinois, Missouri and Oklahoma), the Centres for Disease Control and Prevention (CDC) reported cases of *Cronobacter* spp. infections, in which three of the infants were meningitic, two of which died (in Missouri and Florida). As a result, the supermarket giant Walmart removed infant formula from their shelves. A PFGE analysis of the strains from Illinois and Missouri was undertaken by CDC. In Missouri, an opened PIF tin, prepared PIF and opened nursery water bottle, used to prepare the PIF, all contained the organism; whereas in Illinois, an opened nursery water bottle contained the organism (CDC 2011).

## 1.4 SOURCES OF *CRONOBACTER* SPECIES

Sources of *Cronobacter* spp. are widespread, ranging from various types of herbs, spices, cereals, ready-to-eat products such as salads and confectionery, cheese products, meat and vegetables (Iversen *et al*. 2004; Friedemann 2007; Baumgartner *et al*. 2009), from which many associate with the proposed natural plant habitat for *Cronobacter* spp., and the raw materials cause the contamination of the organism. Among the environmental sources, freshwater contained the species type strain of *C. universalis* (previously *Cronobacter*. genomospecies 1) NCTC 9529 (Iversen *et al.* 2007).

Among animals sources, *Cronobacter* strain has been isolated from the nostril of a stable horse (Holy *et al*. 2011), wild house flies (*Muscadomestica*), filth flies, larvae of the stable fly (*Stomoxys calcitrans*) and the guts of Mexican fruit fly (*Anastrephaludens*) (Kuzina *et al*. 2001; Mramba *et al*. 2006; Butler *et al*. 2010; Pava-Ripoll *et al*. 2012), which leads to another option that flies act as a vector for *Cronobacter* spp.

Blood, cerebrospinal fluid (CSF), sputum, bone marrow, urine, faeces, wound infections have also been shown to contain *Cronobacter* spp (Farmer *et al*. 1980; Muytjens *et al*. 1983; Gallagher and

Ball 1991; Iversen *et al*. 2006; Caubilla-Barron *et al*. 2007). Moreover, a strain of the species *C. malonaticus*, LMG 23826$^{T}$, was isolated from a breast abscess, and *C. sakazakii* from neonatal enteral feeding tubes in intensive care units (Hurrell *et al.* 2009b).

The most consumed source of this organism is baby food, specifically follow up formula (FUF) and PIF, i.e. powdered infant formula (Chap *et al*. 2009), which is often used in place of breast milk for infants.

According to Forsythe *et al*. (2014), the *Cronobacter* MLST database contains records of >1000 isolates indicating the source, geographic and temporal diversity of the genus. *C. sakazakii* NCIMB 8282, the oldest isolate was isolated in 1950 from dried milk powder. Now, its genome has been sequenced and is included in the >100 genomes available in the PubMLST database (Masood *et al*. 2013). Different strains of *Cronobacter* have been isolated from 36 different countries and from various sources including environment (35.15%), infant formula (21.6%), clinical specimens (20.4%), food and related items (14.2%), water and other sources (4%) (Table 1.1). In the past, researchers had reported isolation of *Cronobacter* species from different food items and plants (herbs, rice, spices and wheat) Iversen *et al* (2004).

**Table 1 Summary of *Cronobacter* isolates in the *Cronobacter* PubMLST database**

| Species | Number of strains (%) | Number of STs[a] | Number of genomes | Earliest isolate | Countries | Source | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Clinical | Infant formula | Food and ingredients | Environment | Other |
| *C. sakazakii* | 726 (72.1) | 155 | 73 | 1950 | 33 | 19.8[b] | 23.8 | 32.8 | 16.3 | 7.3 |
| *C. malonaticus* | 136 (13.5) | 53 | 14 | 1973 | 17 | 36.0 | 16.2 | 36.8 | 7.4 | 3.7 |
| *C. dublinensis* | 59 (5.9) | 44 | 9 | 1956 | 11 | 5.1 | 13.6 | 47.5 | 6.8 | 27.1 |
| *C. turicensis* | 41 (4.1) | 26 | 6 | 1970 | 12 | 17.1 | 12.2 | 36.6 | 22.0 | 12.2 |
| *C. muytjensii* | 35 (3.5) | 14 | 2 | 1988 | 10 | 2.9 | 28.6 | 45.7 | 2.9 | 20.0 |
| *C. universalis* | 9 (0.9) | 5 | 2 | 1956 | 6 | 11.1 | 0.0 | 55.6 | 11.1 | 22.2 |
| *C. condimenti* | 1 (0.1) | 1 | 1 | 2010 | 1 | 0 | 0 | 100 | 0 | 0 |
| Total | 1007 | 298 | 107 | | 36 | 20.4[c] | 21.6 | 14.2 | 35.1 | 8.7 |

[a]Sequence type.
[b]Percentage of species total.
[c]Percentage of genus total.

**Table 1.1** Summary of *Cronobacter* isolates in the *Cronobacter* PubMLST database by Forsythe *et al.* (2014).

## 1.5 PHENOTYPIC IDENTIFICATION METHODS AND CULTURE

The development of efficient techniques to detect *Cronobacter* species has become extremely important considering the diversity of sources and their presence in clinical specimens. Standard methods for isolating *Cronobacter* from PIF have been approved by the U.S. Food and Drug Administration (FDA). In these methods, the organism is isolated on the basis of enrichment and biochemical characterization. Previously, these bacteria were isolated using methods that were similar to the methods of isolating *Salmonella* species from samples of powdered milk (FDA, 2002). These protocols involved a pre-enrichment step carried out using buffered peptone water (BPW) or distilled water. Next step was enrichment overnight at 36°C in the *Enterobacteriaceae* enrichment (EE) broth. This was followed by streaking the sample on Violet Red Bile Glucose (VRBG) agar plates which were incubated at 36°C. After overnight incubation, the plates were then examined for colonies of *Enterobacteriaceae*. In particular, the bacteria form purple colonies on this medium. These colonies are surrounded by purple halo formed due to the precipitation of bile acids. During the next step, TSA plates are inoculated with five presumptively positive colonies and incubated at 25ºC to check for the growth of yellow colonies. Finally the identification of bacteria is verified with the help of API20E strips and determination of Most Probable Number (MPN) is made for the bacteria.

The above protocol had several drawbacks. Since all members of *Enterobcacteriaceae* are enriched when cultured in the EE broth, it is possible that the growth of *Cronobacter* species was suppressed by other bacteria. Hence, the method is not efficiently selective for *Cronobacter* species right from the start. Besides this, the method may lead to false negative identifications as only 80% *Cronobacter* species form yellow colonies. The method was quite time consuming and strenuous as well since it needed almost five days for execution. The latest method approved by FDA which was published in 2012 addresses all these limitations, however, is only useable for the US. In this method, the pre-enrichment is carried out for one day using sterile BPW followed by centrifugation. The pellets thus formed are transferred to phosphate buffered saline (PBS). Aliquots of this suspension are cultured followed by screening to detect *Cronobacter* species. Screening made use of chromogenic media like R&F agar and Druggan-Forsythe-Iversen (DFI) agar, real-time PCR assays and biochemical tests (Rapid ID32E or VITEK 2.0). The whole procedure can be executed in only two days including confirmation of the identification of the organism. (Chen *et al*. 2010; Chen *et al*. 2011a).

International Organization for Standardization (ISO) method is valid in the rest of the world for the detection of *Cronobacter* spp. in PIF isolates. In this method, the pre-enrichment step with BPW is followed by the inoculation of 100 µl of the culture to 10 ml of a selective enrichment medium called the modified lauryl sulphate (mLST) broth and incubation at 44 $^{o}$C for 24 hours. Followed by streaking on the chromogenic *Enterobacter sakazakii* isolation agar (ESIA) for growth at 44 $^{o}$C, and subsequent sub-culture on TSA plates for yellow pigmentation at 25 $^{o}$C. The yellow pigmented colonies are then selected for biochemical test (Anonymous 2006).

## 1.6 PHYSIOLOGY

A number of unique physiological characteristics are demonstrated by *Cronobacter* species relating to their growth on powdered infant formula (PIF). Formation of huge quantities of capsular polysaccharides is one of these characteristics. Capsule production may enable the organism to resist desiccation. It has been demonstrated by Caubilla-Barron and Forsythe (2007) during their

study which made use of 27 strains of *Enterobacteriaceae* that capsule forming *Cronobacter* species can be isolated from dehydrated PIF even after two and a half years. Besides resistance to desiccation, capsule formation is also involved in formation of biofilms which have been detected in enteral feeding tubes of neonates in NICUs (Hurrel *et al*. 2009a). The ability of these bacteria to resist dryness is also an important characteristic. It has been found that they are more tolerant to osmotic stress as compared to other bacterial strains found in PIF like *Citrobacter* species, *Salmonella* species and *E. coli* (Breeuwer *et al*. 2003). Researchers have proposed different mechanisms for this tolerance to osmotic stress. For instance, Riedel and Lehner (2007) have identified 53 protein groups that take part in the response of *Cronobacter* species to the osmotic stress. Trehalose synthesis has also been reported to be involved in resistance to osmotic stress as trehalose is capable of functioning as molecular chaperon offering protection to cellular membranes and proteins from denaturation. This role of trehalose was seen in *E. coli* strains (Breeuwer *et al*. 2003; Horlacher and Boos. 1997).

Another important characteristic of *Cronobacter* species that has been extensively researched is their thermotolerance. Variations in the ability of different *Cronobacter* species to resist heat have been reported. Nevertheless, pasteurization temperatures (72$^{o}$C) inactivate all of these bacteria (Nazarowec-White and Farber 1997; Iversen *et al*. 2004b; Breeuwer *et al*. 2003; Nazarowec-White *et al*. 1999). The guidelines for preparation of PIF in order to minimize the risk of growth of these bacteria in PIF were reviewed on the basis of the above mentioned finding (FAO-WHO, 2004; 2006). According to Adekunte *et al.* (2010), numbers of *C. sakazakii* in reconstituted PIF was significantly reduced through a combination of ultrasound parameters and temperature. However, this combination requires optimization and validation by additional studies. Iversen *et al*. (2004) has reported that *Cronobacter* is capable of surviving at a broad temperature range of 6-47$^{o}$C in reconstituted PIF. Laboratory studies showed that *Cronobacter* species are conveniently grown in various media like MacConkey agar and Trypticase Soy Agar (TSA) at 37$^{o}$C in 16-18 hours. Furthermore, 80% strains of *Cronobacter* form yellow pigment when cultured at 25$^{o}$C on TSA. It has been observed that the number of pigmented strains decreased when cultured at 37$^{o}$C indicating that the pigment production is dependent on temperature (Iversen and Forsythe. 2007).

Iversen and Forsythe (2003) hypothesized that plants are the natural habitats of *Cronobacter* as they demonstrate characteristics like resistance to desiccation, pigment formation and formation of polysaccharides. Formation of pigments is a characteristic that allows protection against oxygen radicals formed by radiations of sun. Moreover, this characteristic can also be associated with existence of these bacteria in starches that serve to be the main constituent of different products like PIF. On the other hand, capsule production helps the organism to attach to any surface. Lastly, the bacterium's ability to resist desiccation allows it to survive in extreme environmental conditions.

Resistance of *Cronobacter* strains to acidic conditions has also been reported. Kim and Beuchat (2005) have reported that *Cronobacter* species can survive for up to two days at 25$^{o}$C in several vegetables and fruits which are acidic in nature. According to Edelson-Mammel *et al*. (2006), certain strains of *Cronobacter* demonstrate moderate resistance to pH values up to 3.0; however, pre-exposure to non-lethal pH values can cause improvement in this resistance.

In context of food preservation, acids can act as antimicrobial agents as they can inactivate bacteria like *Cronobacter* species, especially in baby food items like PIF. Both survival and growth of *Cronobacter* species in fluids like baby food and fruit juices have been demonstrated to be inhibited by organic acids, acetic acid and propionic acid (Back *et al*. 2009). According to Al-Holy *et al*. (2010), *Cronobacter* species are also shown to be inactivated by sub-lethal concentrations of copper used in combination with lactic acid. Similarly, Kim *et al*. (2010) proposed that the antimicrobial action of red muscadine (*Vitis rotundifolia*) juices against *C. sakazakii* is due to the combined action of acids (tannic acid, tartaric acid and malic acid) present in them.

## 1.7 IDENTIFICATION METHODS AND MOLECULAR TYPING

Significant developments have been made in last few years regarding *Cronobacter*. Identification and characterization of different strains of *Cronobacter* have been improved with the development of innovative molecular typing techniques. Just like the methods for identification of *Enterobacteriaceae*, most of the molecular techniques for the identification of *Cronobacter* initially made use of the 16S rDNA region. Researchers have devised several different probes

which analyse various genome sequences for identification of isolates or for detection of organisms (Keyser *et al*. 2003; Lehner *et al*. 2004; Iversen *et al*. 2007). The TaqMan real-time PCR assay is one of these methods (Malorny and Wagner. 2005). The 16S rDNA and hsp60 sequences were used for the analysis of diversity and phylogenetic relationships of *Cronobacter* species (though it was regarded as *E. sakazakii* at that time). As per the findings of the analysis, the strains were grouped into four different phylogenetic clusters. Moreover, these findings strongly indicated that these strains constitute a separate genus (Iversen *et al*. 2004). In another study involving utilization of Artifical Neural Networks (ANN), sequences specific to *E. sakazakii* were identified in the partial 16S rDNA sequence. In particular, it is a 528bp long sequence that can be utilized for identification of *Cronobacter* (Iversen *et al*. 2006). In contrast to this, entire length of 16S rDNA sequences (over 1300bp) is targeted for phylogenetic analysis. Researchers have been using variations in 16S rDNA sequences in order to specify genus (5%) and species (3%) boundaries in case of prokaryotes. Still, the 16S rDNA sequence analysis has some limitations when subjected to closely related bacteria as variations in the sequences are minimal. Additionally, multiple copies of 16S rDNA operon are found in most of the organisms and variation in their sequences may lead to erroneous results (Acinas *et al*. 2004). Same is the case with *Cronobacter* species, especially when distinguishing between *C. malonaticus* and *C. sakazakii*. 16S rDNA of these two strains demonstrate 99.7% resemblance, although as per the findings of DDH (DNA-DNA hybridization) studies, they are over 70% related. When *E. sakazakii* species were being reclassified as the genus *Cronobacter*, *C. malonaticus* was first classified as a subspecies of *C. sakazakii* (Iversen *et al*. 2007). Later, its classification as a separate species was made (Iversen *et al*. 2008).

Researchers have used TaqMan Real-time PCR probes and oligonucleotide arrays to study internal transcribed spacer (ITS) regions in order to detect *Cronobacter* in PIF (Liu *et al*. 2006a & 2006b). ITS is the region present between 16S and 23S rDNA genes of bacterial cells. Seo and Brackett (2005) have developed a specific real-time PCR assay for detecting the bacterium in PIF by using the partial macromolecular synthesis operon, particularly the genes encoding rpsU and primase (dnaG). It is worth mentioning that in these techniques, the probes were developed on the basis of small number of *E. sakazakii* strains. For that reason, these probes still need to be validated,

especially considering the updated taxonomy of *Cronobacter*.

Different molecular techniques were used during the research that caused classification of *E. sakazakii* as a separate genus *Cronobacter*. These techniques allowed detection of variations within this strain. Besides the standard biochemical tests which are performed for validation of the phenotypic features of the species, some other schemes like full length 16S rDNA sequencing, f-AFLP employing EcoRI and TaqI, DDH and ribotyping were carried out to characterize the *Cronobacter* genus (Iversen *et al*. 2007 & 2008).

The methodology of PFGE is based on a PulseNet approved protocol developed for *Salmonella* species (Swaminathan *et al*. 2001) that makes use of the most extensively employed restriction endonuclease i.e. *XbaI*. This technique served to be the most reliable method for surveillance of the organism, source tracking and outbreak analysis (Caubilla-Barron *et al*. 2007; Nazarowec-White & Farber. 1997; Craven *et al*. 2010; CDC. 2011). It has been demonstrated through several studies that PFGE is efficient enough to differentiate *Cronobacter* species. Still, there are some drawbacks of this method which limit its utilization. Moreover, it is laborious and time consuming too. PFGE has recently been modified specifically for *Cronobacter* strains. During a study conducted by Caubilla-Barron *et al*. (2007), thirty *Cronobacter* strains were subjected to PFGE analysis. All these strains were isolated from a fatal NICU outbreak that occurred in France. The analysis indicated that strains isolated from single infant demonstrated multiple pulsetypes. It was therefore recommended that multiple colonies should be analysed from single sample. PFGE has also been carried out in several research studies for characterizing *Cronobacter* species isolated from powdered milk and processing plants for infant formula so that distribution of these isolates can be traced in the manufacturing area or in the entire factory (Craven *et al*. 2010; Jacobs *et al*. 2011).

Phenotypic and genotypic techniques have been used for a number of *Cronobacter* species (*E. sakazaki)* by Nazarowec-White and Farber (1999) in order to determine their efficiency in characterizing. This method included PFGE, random amplification of polymorphic DNA (RAPD) and ribotyping. As per the findings, PFGE and RAPD proved to be efficient for differentiating between the strains.

Mullane *et al*. (2008a) put forward a multi-locus variable-number tandem-repeat analysis (MLVA)

scheme for *Cronobacter* which was evaluated using 112 strains of the organism. This technique used genome sequence of *C. sakazakii* BAA-894. At the same time when this study was being carried out, it was proposed that *E. sakazakii* should be classified as a separate genus *Cronobacter* (Iversen *et al*. 2007). Moreover, findings of the mentioned study indicated significant diversity which was in accordance with the reclassification. Nevertheless, the technique was not re-analysed after this taxonomic revision.

Gram-negative bacteria possess O-antigen, which is found in their cell walls. Production of O-antigen is considered to be an important characteristic as different serotypes have been identified on the basis of variation in O-antigen. In case of *Enterobacteriaceae*, genes encoding for O-antigen are found at the *rfb* locus that codes for dTDP-D-glucose 4,6 dehydratase between the *gnd* and *galF* genes coding for gluconate-6-phosphate dehydrogenase and UDP-glucose pyrophosphorylase respectively. Because of variation in these genes, several molecular typing techniques make use of these genes. For instance, the PCR base O-serotyping methods have been specifically devised for the *Cronobacter* species (Mullane *et al*. 2008b; Jarvis *et al*. 2011; Sun *et al*. 2011). Using the PCR probes that targeted *wehI* and *wehC* genes, the O2 and O1 serotypes were identified for the *Cronobacter* species (then *E. sakazakii*) (Mullane *et al*. 2008b). Later, these were named as serotypes of *C. sakazakii* (Jarvis *et al*. 2011). The research was extended to cover different strains of the genus *Cronobacter* resulting in identification of 10 additional sertoypes including four serotypes O3, O4, O5, O6 and O7 for *C. sakazakii*, two serotypes O1 and O2 for *C. malonaticus*, two serotypes O1 and O2 for *C. turicensis* and one serotype O1 for *C. muytjensii*.

Several PCR probes are available for *Cronobacter* which have been developed on the basis separate housekeeping genes like *rpoB* and *gyrB* (Dauga & Breeuwer. 2008; Stoop *et al*. 2009). The method using the *rpoB* gene has been revised recently considering the taxonomic updates so that all seven species of *Cronobacter* can be covered. This method offers the ease of employing only one target gene for identifying the species as different sets of primers with different amplification conditions are used for each species (Lehner *et al*. 2012). Another probe which has been designed for detection of *Cronobacter* in infant formula targets the *ompA* gene. This gene

encodes for an outer membrane protein which is found associated with virulence of the bacterium (Nair and Venkitanarayanan, 2006). PFGE and BOX-PCR technique have been analysed in another study for detecting *Cronobacter* by using 27 *Cronobacter* spp. strains. BOX-PCR is basically a PCR-RFLP sequencing technique targeting the flagellin gene *fliC*. The results indicated that *fliC* gene is not a suitable target gene for detecting the variation among strains. Still, the efficiency of both the techniques proved to be quite comparable for differentiating between the isolates (Proudy *et al*. 2008). A multiplexed PCR assay has recently been developed which targets the di-guanylate cyclise gene, cdgc1. This technique is capable of differentiating 6 out of 7 species of *Cronobacter*. (Carter *et al*. 2012)

In case of *Enterobacteriaceae,* examinations carried on the basis of multiple housekeeping genes like those using multilocus sequence analysis (MLSA) were found to generate good results (Lacher *et al*. 2007; Ibarz Pavón and Maiden. 2009). During a research, MLSA targeting the *thdF*, *rpoA* and *recN* genes was performed for *Cronobacter* and *Enterobacteriaceae* related to *Cronobacter*. This method also employed multiple primers with different amplification conditions for every target gene. Findings of the analysis could be used to describe the phylogenetic linkages between different species (Kuhnert *et al*. 2009).

Pagotto and Farber used the multi-locus sequence typing (MLST) technique for *Cronobacter* for the first time. Ten genes namely *rpoB, rplB, recA, recG, pyrG, lepA, ileS, gyrB* and *fusA* were used (Fanning & Forsythe. 2008). Yet, the research publication lacks information about primers, target sequences and results and has never been published in a jurnal article.

In order to identify and characterize *C. malonaticus* and *C. sakazakii*, a seven-loci MLST method was devised (Baldwin *et al*. 2009). It involved partial sequence analysis of seven housekeeping genes namely *atpD*, *fusA*, *gltB, glnS, infB*, *gyrB* and *ppsA*. These seven genes are constituted from 3036 nucleotides when taken together. The method proved to be efficient enough to distinguish between the above mentioned two species phylogenetically though they were not differentiable by other techniques like 16S rDNA sequencing. According to the researchers, the two species were not reliably differentiable before because of the misleading speciation of certain biotype index strains. It can therefore be stated that there can be errors in the characterization of species through

phenotyping. The MLST scheme for *Cronobacter* can be accessed freely at http://www.pubmlst.org/cronobacter.

It is worth mentioning that certain techniques of detection and typing discussed above were designed during the period when the issue of microbiological safety of PIF was just raised. Moreover, certain techniques made use of poorly characterized strains of bacteria, particularly the *E. sakazakii* strains which were unable to embody all the variations correctly. Moreover, all techniques cannot be used now for the detecting *Cronobacter* species considering the validation required after the taxonomic updates. It is important that the end-detection technique is efficient enough to encompass all known species of *Cronobacter*. This is because each 10g of PIF should be totally free of *Cronobacter* species as per the latest international regulations.

## 1.8 GENOME STUDIES

Recent years have witnessed many developments made in the field of genomics of *Cronobacter*. It was *C. sakazakii* strain BAA-894 whose genome was sequenced first at the Genome Centre, Washington University, USA. During this project, the whole genome shotgun method was used together with end sequencing of fosmid library. The strain BAA-894 has been isolated from a tin of formula that caused a *Cronobacter* NICU outbreak in Tennessee, USA (Section 1.3 in details). As per the results, a 4.4Mb chromosome together with a pair of plasmids (pESA2 31 kb in size and pESA3 131 kb in size) constituted the genome of the organism (Accession No. NC_009778 – 80). Kucerova *et al*. (2010) published the sequence of the entire BAA-894 genome in a whole-genome microarray research for investigating the diversity of the genus *Cronobacter*. This sequence was utilized while constructing a 384,030 probe oligonucleotide tiling DNA microarray. Ten strains from 5 species of the genus (*C. dublinensis*, *C. muytjensii*, *C. turicensis*, *C. malonaticus*, *C. sakazakii*) were included in this probe. As per the findings of the microarray research, the genome comprised of mobile elements together with the 55% core genome for *C. sakazakii* and 43% core genome for the genus *Cronobacter*. Moreover, it was reported that the mobile elements which included the prophage regions gave rise to the diversity seen among the members of the genus.

During follow-up research, it was found that several virulence factors are also present in the genome like fimbrial clusters, type six secretion system and iron acquisition systems (Kucerova *et al*. 2011).

The Institute for Food Safety and Hygiene, Zurich, Switzerland, carried out sequencing of genome of *C. turicensis* strain z3032 soon after the first research study. This strain was isolated back in 2005 from a patient in Zurich's Children's Hospital. According to the results reported, the size of the chromosome of this strain was 4.38 Mb. Besides chromosome, the genome comprise of three plasmids of sizes 22kb, 53kb and 138kb (Accession No. NC_013282 – 85) (Stephan *et al*. 2011).

Bigger plasmids mentioned above (pESA3 131kb and pCTU1 138kb) were compared during a study and it was found that the two plasmids shared a great proportion of content. Moreover, these were regarded as virulence plasmids as they contained genes for filamentous haemagglutinin, plasminogen activator, iron acquisition system and type six secretion systems (Franco *et al*. 2011).

Joseph *et al*. (2012) have conducted an extensive comparative genomic study for analysis of 14 genomes from 7 species of *Cronobacter*. During the study, the researchers determined a larger pan-genome consisting of more than 6000 genes and a shorter core genome containing 2000 genes. The presence of genes coding for virulence factors demonstrate significant level of variation among genomes. These virulence factor genes include genes for adhesins, heavy metal resistance such as resistance against silver, copper, tellurite and genes for T6SS other system as well. It was found that genome of *C. sakazakii* is unique in the sense that it contained genes required for the utilization of exogenous sialic acid. Other members of this genus analysed at this time did not have sialic acid utilization genes.

## 1.9 PATHOGENICITY AND VIRULENCE FACTORS

Despite *Cronobacter* spp. infections are not very frequent, it is still emphasized due to severity of infections the organism causes, as well as the sensitive age group of the neonates that are affected by them; therefore research is being done to determine the pathogenesis of *Cronobacter*.

The bacteria have the capability to attack the epithelial cells and also survive within macrophages as shown in the laboratory studies (Townsend *et al*. 2007). The occurrence of invasive meningitis

proves that the organism can translocate across the human blood brain barrier. Moreover, the responsibility of dendritic cells in causing tissue damage during necrotising enterocolitis has been evident in recent *in vitro* studies (Emami *et al*. 2011).

Ever since the *C. sakazakii* BAA-894 and *C. turicensis* z3032 genomes were available, examination of the virulence of the organism has been done at a genomic level (Kucerova *et al*. 2010; Stephan *et al*. 2011). T6SS, iron acquisition, blood-brain barrier (BBB) penetration, enterobactin and aerobactin synthesis are acknowledged as the supposed virulence factors related to biofilm formation according to researchers (Kucerova *et al*. 2011; Hartmann *et al*. 2010; Franco *et al*. 2011).

It is not proven that all *Cronobacter* species are infective in infants. Until now *C. sakazakii* has been the most prevalent among clinical infant cases.

As mentioned before, genomic studies assist in determining the mode of virulence of this organism and also in establishing what differentiates the virulent *Cronobacter* strains from the non-virulent ones. Similarly, the various putative virulence factors have also been mentioned before.

The virulence of *Cronobacter* spp was seen to be associated with the presence of an outer membrane protein A (OmpA) according to various studies, because according to Singamsetty *et al.* (2008) OmpA expression is responsible for *Cronobacter* invasion of HBMEC cells. The role of the OmpA protein in causing meningitis by multiplication in blood and traversal of the BBB was proved by Mittal *et al.* (2009) using *ompA*-mutants of *Cronobacter* spp. Moreover, OmpA was also attributed for starting the invasion of brain microvascular endothelial cells of the BBB as it is the major fibronectin-binding protein in *Cronobacter* spp. (Nair *et al.* 2009).

Zinc-containing metalloprotease (*zpx*) factor was found to contribute to *Cronobacter* spp virulence by Kothary *et al*. (2007). It caused the necrosis and cellular destruction during the NEC in infants. *Cronobacter* plasminogen activator (*Cpa*), which was a plasmid borne outer membrane protease, was present along with *C. sakazakii* in causing the organism's serum resistance. *Cpa* had a great similarity with the *Pla* subfamily of omptins, as it was recognized as a virulence factor in various *Enterobacteriaceae* members (Franco *et al.* 2011). The function of the *sodA* gene in macrophage

survival of the organism by resistance to oxidative stress conditions was shown with the help of microarray analysis by Townsend *et al* (2007).

It has been established that human intestinal cells are invaded by the *Cronobacter* which multiply in macrophages and finally the blood brain barrier is invaded (Townsed *et al*. 2007 ; 2008). Moreover, the infection is possibly established when the bacteria get attached to and invade the intestinal cells. Several possible virulence factors have been identified through whole genome sequencing; however, some of them need to be validated through research studies (Kucerova *et al*. 2011; Joseph *et al*. 2012). Furthermore, BLAST analysis as well as comparative genomic analysis through the PubMLST database can be performed using the current 107 strains of *Cronobacter* including fifty *C. sakazakii* ST4.

Genomes of *Cronobacter* species are reported to contain ten fimbriae clusters (Joseph *et al*. 2012) With a couple of exceptions; several fimbriae clusters are shared by all species. It is only the *C. sakazakii* which encodes for β-fimbriae while curli fimbriae are found in other species. Moreover, members of the genus *Cronobacter* demonstrate iron assimilation through several mechanisms (Grim *et al*. 2012). Because of these mechanisms, these bacteria can utilize iron from infant formula and breast milk. Researchers have detected five Type VI secretion system (T6SS) clusters in the genomes of *Cronobacter* which might have a role in attachment, invasion in host cell, multiplication within macrophages, persistence inside host and cytotoxicity. Penetration of these bacteria in the blood brain barrier has been reported to involve proteins found in the outer membrane i.e. OmpA and OmpX (Kim *et al*. 2010). However, the mechanism (s) through which brain cells are destroyed still needs to be determined. Several haemolysins are also encoded by *Cronobacter* (Joseph *et al*. 2012). Besides these, plasmid-associated characteristics like expressing the outer membrane protease referred to *Cronobacter* plasminogen activator are also demonstrated by *Cronobacter*. Proteins belonging to this subfamily can cause degradation of several serum proteins like the circulating complement. In this way, protection against complement-dependent serum killing is ensured (Franco *et al*. 2011). A well-maintained open access database containing 107 genomes for the genus *Cronobacter* can prove to be helpful in evaluation of known virulence factors as well as identification of more. Utilization of BLAST facility, for instance, indicates that

*C. sakazakii* strains (n=72 genomes) contains *cpa* gene whereas *C. malonaticus* lacks it. Variation in susceptibility of host can partly be justified with this difference in genome.

Genomic analysis of the *Cronobacter* genomes has revealed the existence of several different genes responsible for resistance to heavy metals such as tellurite, zine, silver and copper and genes for capsule formation which can be helpful in protection against disinfectants. Consumption of exogenous sialic acid by *C. sakazakii* can be clinically important. It has been shown by comparative genomic projects that *nanATKR* gene cluster is present in *C. sakazakii* and this enables the bacterium to utilize sialic acid (Joseph *et al*. 2012; 2013). As sialic acid is present in gangliosides, mucin and breast milk, consumption of sialic acid by this bacterium as a source of carbon can be viewed as an important evolutionary adaptation to the host environment. However, powdered infant formula also contains sialic acid as it is required for development of the brain. Researchers have conducted studies to validate consumption of sialic acid by *C. sakazakii* and these studies have demonstrated that the same organism can also grow on ganglioside GM1 using it as a source of carbon (Joseph *et al*. 2013). Conversely, *C. sakazakii* cannot consume malonic acid, though other species of *Cronobacter* are able to use it. It has been revealed by genomic analysis that genes for malonate decarboxylase are present adjacent to the auxin efflux carrier which can be involved in the uptake of malonate. Since malonic acid is present in plant tissues, ability of *Cronobacter* to consume malonic acid indicates their association with plants. Hence, loss of the ability to use malonate and development of the ability to consume sialic acid can be seen as an adaptation of *C. sakazakii* for the new environment and this can be clinically important.

Food and Agricultural Organisation-World Health Organisation (2004 and 2006) was considering the debilitating consequences of *Cronobacter* infection in neonates and its association with the consumption of contaminated powdered infant formula, it is highly recommended that an internationally accepted technique for the recognition and molecular typing of *Cronobacter* should be developed. MLST profiles of strains isolated from outbreaks occurred all across the world have been included in the *Cronobacter* PubMLST database. All known *Cronobacter* species are covered by the MLST technique so that the interspecific as well as intraspecific diversity of *Cronobacter* can be measured efficient and the strains can be characterized as per the source and virulence

factors. The database also allows retrospective analysis of historic outbreaks and cases once the strains have been re-identified. Taxonomic re-examinations can cause loss of these strains.

The seven-loci MLST scheme was later devised to address different limitations of 16S rDNA sequence analysis and phenotyping. In particular, subjective results were generated by phenotypic tests and 16S rRNA gene sequence analysis could not differentiate between all species of *Cronobacter*. This newly developed scheme involves partial sequence analysis of 7 housekeeping genes namely phosphoenolpyruvate synthase A (*ppsA*), translation initiation factor IF-2 (*infB*), glutaminyl tRNA synthetase (*glnS*), elongation factor G (*fusA*), ATP synthase b chain (*atpD*), gyrase subunit B (*gyrB*) and glutamate synthase large subunit (*gltB*). These sequences constitute 3036 nucleotides in total when contatenated together and for that reason their analysis is known as multilocus sequence analysis (MLSA). Figure 1.1 shows a phylogenetic tree for the genus *Cronobacter* and closely related *Siccibacter* and *Franconibacter* genera obtained through MLSA.

## 1.10 FUTURE DIRECTIONS AND PUBLIC HEALTH SIGNIFICANCE

Execution of appropriate strategies for controlling the growth of *Cronobacter* essentially requires identification of different risks to public health caused by this organism, although it does not frequently cause infections. In particular, maintenance of the microbiological safety of baby food items like PIF should be given immense attention considering the fact that *Cronobacter* infection is mostly seen among infants and the main source of infection is mostly PIF. This attention must be given not only in the factories manufacturing these products but also during handing of these products especially preparing infant feeds. Although, PIF is not manufactured as sterile item; microbial count should be kept at minimum to reduce the contamination.

Considering these and other risks associated with consumption of infant formula, WHO has recommended that infants younger than six months should be fed on breast-milk. However, when infant formula is needed for some reason, some measures must be taken both during the manufacturing process as well as during the reconstitution process. In particular, all possible efforts must be made at the manufacturing unit to ensure maintenance of aseptic conditions and to prevent exposure of these products to temperatures which permit growth of the bacteria. Moreover, medical

personnel, caregivers and mothers must be given appropriate training to deliver information regarding possible risks associated with reconstitution of the infant formula and how these risks can be minimized. It has been suggested that a temperature of 70ºC is suitable for reconstitution of milk as bacteria (if present) are inactivated at this temperature. Additionally, the milk must be taken within half an hour of preparation or alternatively it can be refrigerated for only one day (Iversen and Forsythe 2003; WHO 2007).

The microbiological criteria of the Codex Alimentarius Commission remained unchanged till 2008, even though the risk assessment meeting of WHO/FAO were initiated back in 2004. Currently, these are applicable on PIF for infants with age up to 6 months. Still, the criteria were not recommended for infant formula which is usually termed as follow-up formula used for infants with age above six months (weaning stage). Literature contains evidences indicating isolation of *Cronobacter* species from infant formula and other food items given at weaning stage, additional microbiological testing has not been recommended to manufacturers as there are not sufficient epidemiological data which could support such strategies (FAO/WHO, 2008).

Above all, the committee of WHO/FAO (2004) meeting has emphasized on the requirement of execution of further studies as these studies would improve our knowledge related to the taxonomy and virulence of the organism. It is evident from this literature review that taxonomic classification of newly discovered as well as known bacteria is an ongoing process and it will keep on making progress in the future.

## AIMS & OBJECTIVES

Despite *Cronobacter* spp. infections being infrequent, it is still of high concern due to the severity of the infections the organism causes, as well as the sensitive age group of the neonates that are affected by them. In the last two decade the researchers have been caught the attention particular in *Cronobacter sakazakii* which have particular lineage called clonal complex 4. This lineage has been associated with neonatal meningitis which have been established by our group at NTU previously. There have been numerous outbreaks of this emerging food borne pathogen *Cronobacter* that claimed the lives of babies. The overall aim of this research project was to identify the virulence determinants of the neonatal meningitic bacterium *C. sakazakii*. This was achieved according to the following objectives:

I. Studying the diversity of the *Cronobacter* strains obtained from outbreaks, as analysed by multilocus sequence typing scheme (MLST) as a reliable detection and molecular typing methods developed for the control of *Cronobacter* spp..

II. Describing the variation in growth by members of the *Cronobacter* genus in sialic acid, genomic structure and the variation in the gene content of *Cronobacter* associated with sialic acid utilization.

III. Investigation the pathogenesis of *Cronobacter sakazakii* by determining the presence of a number of key virulence associated genes which included: two iron acquisition system gene clusters (*eitA* and *iucC*), *Cronobacter* plasminogen activator (*Cpa*), and type IV secretion (T6SS) gene cluster using laboratory studies in *Cronobacter sakazakii* strains, particularly with ST4 strains regarding the location on plasmid and total DNA.

IV. Developing a tool in order to show the insertion of the well characterized plasmid pESA3 into plasmid less strain (NTU 6) and observing any changes in its phenotypic and virulence associated behaviour including serum resistance, siderophore and tissue culture.

# CHAPTER 2

# MATERIALS AND METHODS

# 2. MATERIALS & METHODS

## 2.1 SAFETY CONSIDERATIONS

Suitable COSHH forms were completed and all the materials and protocols were analysed and assessed methodically. All the experiments were conducted according to the Health and Safety Code of practice for Microbiology Containment Level 2. While handling microbes, media and chemicals and good microbiological laboratory practices were followed. Also good laboratory practices were followed while operating laboratory equipment. All material was disposed according to the instructions that were stated in material safety data sheets.

## 2.2 BACTERIAL STRAINS

All the bacterial strains that were used in this study were from the culture collection of *Cronobacter* spp. of Nottingham Trent University (NTU). Table 2.1 lists the details of the strains that have been isolated and used in this study.

**Table 2.1** List of *Cronobacter* strains used in this study

| Species | NTU ID | Country | Source | Year of Isolation | Comments* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *C. malonaticus* | 1826 | CR** | Clinical- Canula | 2007 | A | B | C | D | E | F |
| *C. malonaticus* | 1827 | CR** | Clinical- Canula | 2007 | √ | | | | | |
| *C. malonaticus* | 1829 | CR** | Clinical-Nose Swab | 2007 | √ | | | | | |
| *C. malonaticus* | 1830 | CR** | Clinical-Throat Swab | 2007 | √ | | | | | |
| *C. malonaticus* | 1831 | CR** | Clinical-Throat Swab | 2007 | √ | | | | | |
| *C. malonaticus* | 1832 | CR** | Clinical-Throat Swab | 2009 | √ | | | | | |
| *C. malonaticus* | 1833 | CR** | Clinical-Stool Dissection | 2010 | √ | | | | | |
| *C. malonaticus* | 1834 | CR** | Clinical-Throat Swab | 2010 | √ | | | | | |
| *C. malonaticus* | 1835 | CR** | Clinical-Throat Swab | 2012 | √ | | | | | |
| *C. sakazakii* | 1836 | CR** | Clinical-Wound Swab | 2012 | √ | | | | | |
| *C. sakazakii* | 1837 | CR** | Clinical-Wound Swab | 2012 | √ | | | | | |
| *C. muytjensii* | 1838 | CR** | Clinical-Sputum | 2012 | √ | | | | | |
| *C. sakazakii* | 1839 | CR** | Clinical-Smear from curtaneas | 2012 | √ | | | | | |
| *C. sakazakii* | 1840 | CR** | Clinical-Sputum | 2012 | √ | | | | | |

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|---|---|---|---|---|---|---|---|---|---|---|
| *C. sakazakii* | 1841 | CR** | Clinical-Sputum | 2012 | √ | | | | | |
| *C. sakazakii* | 1842 | CR** | Clinical-Sputum | 2012 | √ | | | | | |
| *C. sakazakii* | 1901 | CR** | Clinical-Sputum | 2012 | √ | | | | | |
| *C. sakazakii* | 1902 | CR** | Clinical-Sputum | 2012 | √ | | | | | |
| *C. sakazakii* | 1903 | CR** | Clinical-Sputum | 2012 | √ | | | | | |
| *C. malonaticus* | 1914 | CR** | Clinical-Sputum | 2012 | √ | | | | | |
| *C. sakazakii* | 1915 | CR** | Clinical-Sputum | 2012 | √ | | | | | |
| *C. sakazakii* | 1916 | CR** | Clinical-Sputum | 2012 | √ | | | | | |
| *C. malonaticus* | 1917 | CR** | Clinical-Sputum | 2012 | √ | | | | | |
| *C. sakazakii* | 1580 | Israel | Clinical-Faecal | 2000 | √ | | | | | |
| *C. sakazakii* | 1581 | Israel | Infant Formula | 2000 | √ | | | | | |
| *C. sakazakii* | 1582 | Israel | Environment | 2000 | √ | | | | | |
| *C. sakazakii* | 1583 | Israel | Clinical-Faecal | 2000 | √ | | | | | |
| *C. sakazakii* | 1584 | Israel | Clinical-Faecal | 2000 | √ | | | | | |
| *C. sakazakii* | 1585 | Israel | Clinical-Blood | 1999 | √ | | √ | | √ | |
| *C. sakazakii* | 1586 | Israel | Clinical-Blood | 1998 | √ | | √ | | √ | |
| *C. sakazakii* | 1587 | Israel | Clinical-CSF | 2000 | √ | | √ | √ | √ | |
| *C. sakazakii* | 1588 | Israel | Clinical-Blood | 2012 | √ | | √ | | √ | |
| *C. sakazakii* | 1565 | USA*** | Clinical-CSF | 2011 | √ | | | | | |
| *C. sakazakii* | 1566 | USA*** | Clinical-CSF | 2011 | √ | | | | | |
| *C. sakazakii* | 1567 | USA*** | Clinical- Faecal | 2011 | √ | | | | | |
| *C. sakazakii* | 1568 | USA*** | Infant formula | 2011 | √ | | | | | |
| *C. sakazakii* | 1570 | USA*** | Clinical-CSF | 2011 | √ | | | | | |
| *C. sakazakii* | 1571 | USA*** | Infant formula | 2011 | √ | | | | | |
| *C. sakazakii* | 1572 | USA*** | Infant formula | 2011 | √ | | | | | |
| *C. sakazakii* | 1573 | USA*** | Infant formula | 2011 | √ | | | | | |
| *C. sakazakii* | 1574 | USA*** | Clinical-Faecal | 2011 | √ | | | | | |
| *C. sakazakii* | 1575 | USA*** | Clinical- Faecal | 2011 | √ | | | | | |
| *C. sakazakii* | 1576 | USA*** | Clinical-Tracheal Secretion | 2011 | √ | | | | | |
| *C. sakazakii* | 1577 | USA*** | Clinical-CSF | 2011 | √ | | | | | |
| *C. sakazakii* | 1578 | USA*** | Water | 2011 | √ | | | | | |
| *C. malonaticus* | 1569 | USA*** | Clinical-Blood | 2011 | √ | | | | | |

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|---|---|---|---|---|---|---|---|---|---|---|
| *C. sakazakii* | 1 | USA*** | Clinical-Thorat | 1980 | | | √ | √ | √ | |
| *C. sakazakii* | 4 | Canada | Clinical | 2003 | | | √ | √ | √ | |
| *C. sakazakii* | 5 | Canada | ††Unk | 1990 | | | √ | √ | √ | |
| *C. sakazakii* | 12 | CR** | Clinical- Faecal | 2004 | | | √ | √ | √ | |
| *C. sakazakii* | 20 | CR** | Clinical- Faecal | 2003 | | | √ | √ | √ | |
| *C. sakazakii* | 140 | ††Unk | Spice | 2005 | | | √ | √ | √ | |
| *C. sakazakii* | 150 | Korea | Spice | 2005 | | | √ | √ | √ | |
| *C. sakazakii* | 553 | ††Unk | ††Unk | - | | | √ | √ | √ | |
| *C. sakazakii* | 555 | Netherlands | Clinical | 1979 | | | √ | | √ | |
| *C. sakazakii* | 658 | USA*** | Infant Formula | 2001 | | √ | √ | √ | √ | |
| *C. sakazakii* | 680 | USA*** | Clinical-CSF | 1977 | | | √ | √ | | |
| *C. sakazakii* | 696 | France | Clinical- Faecal | 1994 | | | √ | √ | √ | |
| *C. sakazakii* | 701 | France | Clinical | 1994 | | | √ | √ | √ | |
| *C. sakazakii* | 1220 | USA*** | Clinical-CSF | 2003 | | | √ | √ | √ | |
| *C. sakazakii* | 1221 | USA*** | Clinical-CSF | 2003 | | | √ | √ | √ | |
| *C. sakazakii* | 1225 | USA*** | Clinical-Blood | 2007 | | | √ | √ | √ | |
| *C. sakazakii* | 1231 | - | Clinical- Faecal | - | | | | √ | √ | |
| *C. sakazakii* | 716 | France | Infant formula | 1994 | | | | √ | | |
| *C. sakazakii* | 978 | UK | Clinical | 2007 | | | | √ | | |
| *C. sakazakii* | 709 | France | Clinical | 1994 | | | | √ | | |
| *C. sakazakii* | 377 | France | Milk powder | 1950 | | | | √ | | |
| *C. sakazakii* | 1105 | UK | Weaning food | 2008 | | | | √ | | |
| *C. sakazakii* | 690 | France | Clinical | 1994 | | | | √ | | |
| *C. sakazakii* | 693 | France | Clinical | 1994 | | | | √ | | |
| *C. malonaticus* | 510 | CR** | Food | 1985 | | | | √ | | |
| *C. sakazakii* | 1218 | USA*** | Clinical | 2001 | | | | √ | | |
| *C. sakazakii* | 1249 | UK | Clinical | 2010 | | | | √ | | |
| *C. malonaticus* | 685 | USA*** | Clinical | 1977 | | | | √ | | |
| *C. dublinensis* | 583 | UK | Enviromental | 1956 | | | | √ | | |
| *C. malonaticus* | 687 | CR** | Clinical | 2004 | | | | √ | | |
| *C. sakazakii* | 694 | France | Clinical | 1994 | | | | √ | | |
| *C. sakazakii* | 708 | France | Clinical | 1994 | | | | √ | | |

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|---|---|---|---|---|---|---|---|---|---|---|
| *C. sakazakii* | 711 | France | Clinical | 1994 | | | | √ | | |
| *C. sakazakii* | 712 | France | Infant formula | 1994 | | | | √ | | |
| *C. malonaticus* | 1545 | CR** | Clinical | - | | | | √ | | |
| *C. sakazakii* | 700 | France | Clinical | 1994 | | | | √ | | |
| *C. dublinensis* | 1556 | USA*** | Clinical | 1979 | | | | √ | | |
| *C. malonaticus* | 1558 | CR** | Clinical | - | | | | √ | | |
| *C. dublinensis* | 1560 | CR** | Food | - | | | | √ | | |
| *C. sakazakii* | 1533 | Germany | Environmental | 2006 | | | | √ | | |
| *C. sakazakii* | 1536 | Germany | Environmental | 2009 | | | | √ | | |
| *C. sakazakii* | 1537 | Germany | Environmental | 2009 | | | | √ | | |
| *C. sakazakii* | 1542 | Germany | Environmental | 2009 | | | | √ | | |
| *C. sakazakii* | 691 | France | Clinical | 1994 | | | | √ | | |
| *C. sakazakii* | 692 | France | Clinical | 1994 | | | | √ | | |
| *C. sakazakii* | 698 | France | Clinical | 1994 | | | | √ | | |
| *C. sakazakii* | 699 | France | Clinical | 1994 | | | | √ | | |
| *C. sakazakii* | 702 | France | Clinical | 1994 | | | | √ | | |
| *C. sakazakii* | 703 | France | Clinical | 1994 | | | | √ | | |
| *C. sakazakii* | 705 | France | Clinical | 1994 | | | | √ | | |
| *C. sakazakii* | 706 | France | Clinical | 1994 | | | | √ | | |
| *C. sakazakii* | 707 | France | Clinical | 1994 | | | | √ | | |
| *C. sakazakii* | 713 | France | Infant formula | 1994 | | | | √ | | |
| *C. sakazakii* | 714 | France | Infant formula | 1994 | | | | √ | | |
| *C. sakazakii* | 715 | France | Infant formula | 1994 | | | | √ | | |
| *C. malonaticus* | 1569 | USA*** | Clinical | 2011 | | | | √ | | |
| *C. sakazakii* | ES15 | Korea | Whole grain | - | | | | √ | | |
| *C. malonaticus* | 1846 | CR** | Ingredient | 2010 | | | | √ | | |
| *C. sakazakii* | SP291 | Ireland | Environmental | | | | | √ | | |
| *C. sakazakii* | ES713 | USA*** | Infant formula | - | | | | √ | | |
| *C. sakazakii* | ES35 | Israel | Clinical | - | | | | √ | | |
| *C. sakazakii* | 2089 | France | Clinical | 2004 | | | | √ | | |
| *C. sakazakii* | 2106 | Belgium | Clinical | - | | | | √ | | |

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|---|---|---|---|---|---|---|---|---|---|---|
| *C. sakazakii* | 2107 | Belgium | Clinical | - | | | | √ | | |
| *C. malonaticus* | 2109 | Canada | ††Unk | - | | | | √ | | |
| *C. dublinensis* | 2030 | France | - | - | | | | √ | | |
| *C. malonaticus* | 2045 | France | Environmental | - | | | | √ | | |
| *C. malonaticus* | 2046 | France | Environmental | - | | | | √ | | |
| *C. sakazakii* | 2051 | France | Environmental | | | | | √ | | |
| *C. sakazakii* | 2161 | Mexico | Environmental | 2010 | | | | √ | | |
| *C. sakazakii* | 2064 | France | Environmental | - | | | | √ | | |
| *C. sakazakii* | 2048 | France | Environmental | - | | | | √ | | |
| *C. sakazakii* | NBRC102416 | USA*** | Clinical | 1980 | | | | √ | | |
| *C. malonaticus* | 507 | CR** | Clinical | 1984 | | | √ | √ | | |
| *C. malonaticus* | 681 | USA*** | Clinical | 1977 | | | √ | √ | | |
| *C.universalis* | 581 | †UK | Fresh water | 1956 | | | | √ | | |
| *C.turicensis* | 564 | USA*** | Clinical-Blood | 1970 | | | | √ | | |
| *C.turicensis* | 1211 | Switzerland | Clinical-Blood | 2005 | | | √ | √ | √ | |
| *C. muytjensii* | 530 | Denmark | Infant Formula | 1988 | | | | √ | | |
| *C.dublinesis* | 582 | †UK | ††Unk | Unk | | | | √ | | |
| *C.condiementi* | 1330 | Slovakia | Food | 2010 | | | | √ | | |
| *C. sakazakii* | 721 | USA*** | Clinical-CSF | 2003 | | | √ | √ | √ | |
| *C. sakazakii* | 1219 | USA*** | Clinical-CSF | 2002 | | | √ | √ | √ | |
| *C. sakazakii* | 557 | Netherlands | ††Unk | 1997 | | | √ | √ | √ | |
| *C. sakazakii* | 730 | France | Clinical | 1994 | | | √ | √ | √ | |
| *C. sakazakii* | 1222 | USA*** | Clinical-Blood | 2003 | | | √ | | √ | |
| *C. sakazakii* | 1240 | USA*** | Clinical-CSF | 2008 | | | √ | √ | √ | |
| *C. sakazakii* | 1241 | USA*** | Clinical | 2009 | | | √ | | √ | |
| *C. sakazakii* | 520 | CR** | - | 1983 | | √ | √ | √ | √ | |
| *C. sakazakii* | 6 | Canada | ††Unk | 1990 | | √ | √ | √ | √ | |
| *C. sakazakii* | 1242 | USA*** | Clinical Brain | - | | | √ | | √ | |
| *C. sakazakii* | 1223 | USA*** | Clinical-Blood | 2004 | | | √ | | √ | |
| *C. sakazakii* | 558 | Netherlands | ††Unk | 1983 | | | √ | √ | √ | |
| *C. sakazakii* | 767 | France | Clinical- Trachea | 1944 | | | √ | √ | √ | |
| *C.sakazakii* | 695 | France | Clinical-Trachea | 1944 | | | √ | √ | √ | |
| *C.dublinesis* | 1210 | Ireland | Environment-Milk powder manufacturing plant | 2004 | | | | √ | | √ |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *C.dublinesis* | LMG23823 | Ireland | Environment | 2004 | | | | √ | | √ |
| *C.dublinesis* | LMG23825 | Switzerland | Water | 2004 | | | | √ | | √ |
| *C.dublinesis* | LMG23825 | Zimbabwe | Environment | 2003 | | | | √ | | √ |
| *C.malonaticus* | LMG23826 | USA*** | Clinical | 1977 | | | | √ | | √ |
| *C.malonaticus* | CMCC45402 | China | Milk | 2014 | | | | √ | | √ |
| *C.turicensis* | 92 | ††Unk | Herb | 2004 | | | | √ | | √ |
| *C.turicensis* | 1553 | Slovakia | ††Unk | - | | | | √ | | √ |
| *C.turicensis* | 1554 | Slovakia | ††Unk | - | | | | √ | | √ |
| *C.turicensis* | 1880 | CR** | Herb-Bay leaf | 2011 | | | | √ | | √ |
| *C.muytjensii* | ATCC51329 | ††Unk | ††Unk | - | | | | √ | | √ |
| *Siccibacter turicensis* | 1974 | Switzerland | Fruit powder | 2007 | | | | √ | | √ |
| *Franconibacter helveticus* | 1975 | Switzerland | Fruit powder | 2007 | | | | √ | | √ |
| *Francobibacter pulveris* | 1978 | Switzerland | Infant Formula | 2008 | | | | √ | | √ |
| *Francobibacter pulveris* | 601 | Switzerland | Fruit powder | 2007 | | | | √ | | √ |
| *Francobibacter pulveris* | 1160 | Switzerland | Fruit powder | 2007 | | | | √ | | √ |
| *Siccibacter colletis* | 1383 | †UK | Ingredient | 2011 | | | | √ | | √ |
| *Francobibacter pulveris* | 1991 | †UK | Food | 2013 | | | | √ | | |
| *Franconibacter helveticus* | 1387 | †UK | Spice | 2011 | | | | √ | | |
| *Franconibacter helveticus* | 1392 | †UK | Ingredient | 2011 | | | | √ | | |
| *Franconibacter helveticus* | 1208 | Portugal | Follow up formula | 2009 | | | | √ | | |
| *E.coli K12* | 1230 | - | HB101 | - | | √ | | | | |
| *Salmonella enterica server* Enteritidis | 358 | - | NCTC3046 | - | | √ | | | | |
| *Ed.Trada* | 1926 | - | - | - | | | | √ | | |
| *Citrobacter Frendii* | 1927 | - | - | - | | | | √ | | |
| *Citrobacter kosri* | 48 | USA*** | Clinical-CSF | Unk | | √ | | √ | √ | |
| *Citrobacter kosri* | CK-BAA89 | USA*** | | | | | | √ | | |

*** Comments: A –** MLST laboratory experiments and sequence data analysis performed as part of this project**; B –** Transfer of the virulence associated plasmid pESA3 into the plasmid less *C. sakazakii* isolate and its characterization data analysed as part of this project**; C –** PCR Screening of virulence detection & serum resistance data analysed as part of this project**; D –**Sialic acid growth data analysed as part of this project**; E-** Plasmid profile data analysed as part of this project**; F-**Iron acquision data analysed as part of this project.

**CR**:** Czech Republic**; USA***:** United States of America**; †UK:** United Kingdom**††Unk:** Unknown.

**Table 2.2** Plasmid used in this study:

| Plasmids | Features | Source |
|---|---|---|
| **pAJD434** | 6566bp Trimethoprim TmR plasmid | McNally,UK |
| **pKD4** | 3267 bp kanamycin KR plasmid | Datsenko & Wanner, 2000 |
| **pESA3** | The larger plasmid of *C.sakazakii* ATCC BAA-894 131 KB 56% GC-encoding 127 genes. | Kucerova *et al.* 2010 |
| **pESA3K** | pESA3 after insertion of a kanamycin resistance cassette | This study |

## 2.3 BACTERIAL STORAGE AND CULTURE

All the *Cronobacter* strains of both agar plates TSA and liquid culture TSB were incubated for 24 hours at 37°C under aerobic conditions, and stored in TSB/ glycerol (80%) at -20°C and -80 °C. When it was deemed suitable, culture strains were stored at 4°C for short periods.

## 2.4 PREPARATION OF MEDIA AND BUFFERS

### 2.4.1 TRYPTICASE SOY AGAR (TSA)

Five hundred millilitre of distilled water was used to re-suspend 20 grams of TSA (Themo Fisher,UK) base. The re-suspended solution was then autoclaved at 121°C for 15 minutes. The agar was allowed to cool down to a temperature of 50°C. When the agar had cooled approximately 15-20 ml of media was poured in to each sterile Petri dish. The plates were then stored for up to three weeks at 4°C.

### 2.4.2 DRUGGAN-FORSYTHE-IVERSEN (DFI) AGAR FORMULATION

Five hundred millilitre of distilled water was used to re-suspend 21.5 grams of DFI agar base (Lab M, UK) and autoclaved at 121ºC for 15 minutes. When cooled down to 50ºC, 15-20 ml of media was poured into each sterile Petri dish. These plates were then stored at 4ºC for maximum three weeks.

### 2.4.3 LURIA-BERTANI AGAR (LBA)

The manufacturer's directions were followed to prepare Luria-Bertani Agar (Merck KgaA, Darmstad, Germany; 1102830). Thirty-seven grams was dissolved in 1 L of $H_2O$, mixed and then sterilized for 15 min at 121 °C.

### 2.4.4 TRYPTICASE SOY BROTH (TSB)

For re-suspension of 15 grams of TSB (Oxoid, UK) base, 500 ml of distilled water was used. The re-suspended solution was then autoclaved at 121$^{o}$C for 15 minutes.

### 2.4.5 BRAIN HEART INFUSION BROTH (BHI)

One liter of distilled water was used to re-suspend 37 grams of BHI (CM1135, Oxoid Thermo Fisher; UK). The mixture was then heated with mixing until the BHI was completely dissolved. The solution was then dispensed into universal bottles in 20 ml volumes, and autoclaved at 121°C for 15 minutes. The bottles were stored in the refrigerator at 4°C until required.

### 2.4.6 LURIA-BERTANI BROTH (LB)

The directions by the company were followed in the preparation of Luria-Bertani (Sigma Aldrich, UK; L3022). The LB of 25 g was mixed into 1 litre of condensed water. In 50 ml volumes, the mixture was distributed to 100ml bottles and sterilized for 15 min at 121 °C.

### 2.4.7 PHOSPHATE BUFFERED SALINE (PBS)

Two tablets of PBS (Sigma Aldrich, UK) were dissolved in 400ml of distilled water and autoclaved.

### 2.4.8 SALINE SOLUTION (0.85 %)

One tablet of saline (Fisher Scientific, UK) was added to 99ml of $dH_2O$ and autoclaved then stored at room temperature.

### 2.4.9 TRITON X-100 (1%)

One millilitre of Triton X-100 (Fisher Scientific, UK) was added to 99 ml of $dH_2O$ and autoclaved then stored at room temperature.

### 2.4.10 IRON III SOLUTION

To prepare iron III solution, 9 ml of HCl (100 ml of distilled water added to 830 µl HCl) was mixed with 1 ml of $FeCl_3\,6H_2O$ solution (1L distilled water mixed with 2.73g of $FeCl_3\,6H_2O$).

### 2.4.11 CHROME AZUROL SULPHATE (CAS) SOLUTION

ChromeazurolS (199532, Sigma) was prepared by mixing 100ml of sterile distilled water with 0.0121g of CAS.

### 2.4.12 HEXADECYLTRIMETHYLAMMONIUM BROMIDE (HDTMA)

Distilled water (80ml) was mixed with 0.1458g of HDTMA (H6268, Sigma).

### 2.4.13 SODIUM HYDROXIDE SOLUTION

Ten gram of 50% w/v NaOH was added to distilled water (20ml).

### 2.4.14 GLYCEROL (80 %)

Eighty millimeter of glycerol (Fisher Scientific, UK) was added to 20ml $dH_2O$, mixed, and autoclaved then stored at room temperature.

### 2.4.15 M9 MINIMAL MEDIUM

M9 Minimal Medium composed of 50ml 5XM9 Medium, 1ml 1M $MgSO_4$ , 5ml 20% Glucose , 50µl 1M $CaCl_2$ , 500ml $dH_2O$. They were autoclaved at 121ºC for 15 minutes.

### 2.4.16 PLASMID PROFILING REAGENTS

The alkaline extraction procedure by Birnboim and Doly (1979) was used for plasmid profiling of *Cronobacter* spp. strains.

**SOLUTION I:**

Lysozyme solution composed of 2 mg/ml lysozyme (Fluka), 50 mM glucose (Sigma, UK), 10mM CDTA/EDTA (Sigma, UK), 25 mM Tris-HCl pH 8.0 (Sigma, UK). The stock or solution I was freshly prepared and stored at 4ºC.

**SOLUTION II:**

Alkaline SDS solution composed of 0.2 N NaOH and 1% SDS (Sigma, UK). Solution was stored at 25ºC and remained stable one week.

**SOLUTION III:**

High salt solution 3 moler of sodium acetate (Sigma, UK).

## 2.5 PLASMID PROFILE

The bacterial culture (2.5 ml) was grown overnight shaking in TSB for 18 hours. An aliquot (0.5 ml) of the culture was transferred to a 1.5 ml eppendorf tube for plasmid extraction, whereas the remaining culture was preserved at -20ºC after glycerol had been added to 40%. Supernatant was carefully removed using a fine tip aspirator after the eppendorf tube has been centrifuged for 15 seconds. The cell pellet was then thoroughly suspended in 100 µl of Solution I. After incubation at 0ºC for 30 min, 200 µl of Solution II was mixed so that the tube was softly vortexes, until the suspension was nearly clear and viscous. Subsequently, the tube was then preserved at 0ºC for a period of 5 min and then 150 µl Solution III was mixed. To concentrate the DNA, the tube was gently mixed by inversion for a few seconds and upheld at 0ºC for 60 min to permit precipitation of protein, fast-paced molecular weight RNA and chromosomal DNA. After that the tube was centrifuged for 5 min to produce a nearly clear supernatant of which 400 µl was removed and transferred to another tube. After 1ml of cold ethanol had been mixed, the tube was kept at -20ºC for 30 min and then centrifuged for 2 min to discard and remove supernatant by aspiration. Once this was done, the pellet was dissolved in 100 µl of 0.1 M sodium acetate/0.05 M Tris-HCl (pH 8) and 2 volumes of cold ethanol were used for precipitating. After being held at -20ºC for 10 min, centrifugation was again used to collect the precipitate as before. The pellet was further dissolved using 40 µl of water and a mixture of 10 µl of 5X sample buffer [25% sucrose (Fisher Scientific, UK), 5 mM sodium acetate (Sigma, UK), 0.1% SDS (Sigma, UK)] was added. The unstained 1% agarose (Fisher Scientific, UK) gel made with 1X TAE buffer (Geneflow, UK) was then loaded with 20 µl of the sample and was run for duration of 50 minutes at 90V in 1X TAE buffer. Using

the InGenius® gel documentation system (Syngene, UK), the gel was stained with in ethidium bromide solution - 25 µl EtBr (Sigma, UK) in 400 ml distilled water – for duration of an hour after which it was observed below the UV light to detect the DNA bands.

## 2.6 DETECTION OF VIRULENCE ASSOCIATED GENES USING PCR

In this work, all primers were purchased from Sigma Aldrich as dry products and rehydrated as directed by the manufacturers' instructions. Primers were designed by different research groups (Franco *et al*. 2011a; Franco *et al*. 2011b) and the presence of investigated genes was obtained following the same denaturation, annealing and extension times and temperatures. All primers are listed in Table 2.3. The PCR was investigated as described in Kim *et al*. (2010), Franco *et al*. (2011a) and Franco *et al*. (2011b).

| Target gene | Primer name | Primer sequence (5'-3) | Amplicon (bp) | Annealing/ extension conditions |
|---|---|---|---|---|
| *Cpa* | cpafw | GACAACCCTGAGTTCTGGTAAC | 306 | 56°-30s/ 72°-30 |
| | cparv | ATGCGTATTTCTGCTGGTAA | | |
| T6SS R end | t6ssfw | CTCAGATTACTGATCGGCGCTG | 338 | 56°-30s/ 72°-40s |
| | t6ssrw$_3$ | GTATGGCATACCGCAATTGCGC | | |
| IntT6SS Right | t6ssfw$^3$ | CTCAGATTACTGATCGGCGCTG | 800 | 56°-30s/ 72°-60s |
| | Δt6ssrv$^3$ | CTGAACAGATGGCCGATCTGGT | | |
| IntT6SS Left | Δt6ssfw | GGAATACGCCTGCGCTGATGAC | 1,168 | 56°-30s/ 72°-90s |
| | t6ssrv | CCAGTAATTTCAGCGGCAGCTC | | |
| *VgrG* | vgrGfw | GGTTTCACTTCCCGCTGATA | 850 | 52°-30s/ 70°-60s |
| | vgrGrv | CCCGCAGTTAATCACCAGTT | | |
| *EitA* | EitAF1 | CCTTTTTCACGGCGTCGAGCTG | 280 | 60°-30s/ 72°-30s |
| | EitAR1 | TCTCTTCTGGTTCTCCAGCGCG | | |
| *Iucc* | iucCF | TGCAGTGCCTGATGTCAGGCCAT | 660 | 58°-30s/ 72°-30s |
| | iucCR | ACGCCAAACATCTCCTGATAGCG | | |

**Table 2.3** Primers used for virulence genes investigations. The sequence of each primer and the amplicon size are listed (Franco *et al*. 2011b).

## 2.7 PHYSIOLOGICAL EXPERIMENTS

### 2.7.1 SERUM RESISTANCE

Bacterial cultures were grown overnight at 37°C in TSB broth 200 rpm and then centrifuged. The pellet was diluted to $l0^6$ cfu/ml in 5ml of PBS. From the suspension cells (0.5 ml) were added to 1.5 ml of undiluted human serum. The bacteria and human serum were mixed and incubated at 37°C with shaking (200 rpm). The viable counts of cells were obtained at the beginning and after 1, 2, 3 and 4 hours of incubation. The Miles and Misra technique was used on TSA plates at 37°C for 18 hours. All strains were analysed in triplicate. All bacterial strains have been assayed in 3 independent assays.

### 2.7.2 SIALIC ACID UTILIZATION

Two modifications of the methods stated by Almagro-Moreno and Boyd (2009) were used. Brain Heart Infusion broth (Oxoid ThermoFisher, UK) was used to grow the bacterial cultures overnight. To remove the carryover of nutrients, the cell suspensions were washed three times in PBS. Absorbance increased (595 nm) when growth in M9 minimal media with 1 mg/ml sialic acid ( Sigma Aldrich, UK), 1 mg/ml GM1 monosialoganglioside (Sigma Aldrich, UK), 1 mg/ml mucin (Sigma Aldrich, UK) as the carbon source was used. Inoculation of M9 without a carbon source was used as a negative control, and glucose was added (1 mg/ml) to the minimal medium as a positive control.

### 2.7.3 IRON SIDEROPHORE DETECTION

For siderophore production the method of Schwyn and Neilands (1987) was used with slight modification. Chrome azurolsulphate (CAS) agar was prepared by using two solutions. The First solution (dark blue liquid) was prepared by using 50 ml of CAS solution (see section 2.4.11), 10ml of iron III solution; section 2.4.10 and 40 ml of HDTMA before autoclaving at 121°C for 15 minutes (100ml in total of dark solution). The second solution was prepared by mixing 900ml of distilled water, 15g agar, 30.24g pipes and 12g NaOH and then autoclaving at 121°C for 15

minutes. After autoclaving, the first solution was mixed with the second solution and then the media was poured into the plates. Immediately before use, 5mm diameter holes were punched into the agar. To prepare the bacterial suspension, five colonies were taken from TSA and inoculated into LB broth containing 200µM of 2, 2´-dipyridyl (31.236mg in 1L of LB broth) and incubated with shaking (170 rpm) at 37°C overnight. After incubation the sample was centrifuged at 5000 rpm for 10 minutes and 70 µl of the supernatant was added into the holes. The agar was incubated at 37°C for 4-8 hours. The observance of an orange zone around the hole indicated that the strain is positive for siderophore production. *Yersinia enterocolitica* strain 1880 was used as a positive control.

## 2.8 GENOMIC COMPARISON FOR THE PRESENCE/ ABSENCE OF THE KEY VIRULENCE GENES

To investigate the presence and the absence of the key genes, genome comparisons were applied to find the key virulence genes of the organism such as iron acquisition, serum resistance, sialic acid utilisation and T6SS. This comparative analysis was performed using Artimes comparative tool (ACT) for genome alignment, which has been developed by Carver *et al*. (2005). Further BLAST searches were performed using *Cronobacter* BLAST research facility at (http://pubmlst.org/perl/bigsdb/bigsdb.pl?db=pubmlst_cronobacter_isolates&page=plugin&name=BLAST).

## 2.9 TISSUE CULTURE INVESTIGATIONS

### 2.9.1 MAMMALIAN CELL LINES

An important experiment of this project is the use of tissue culture experiment. To make use of a cell line in the project, the cell lines were grown at the time needed via detailed procedure. The time period required by the cell lines to grow a complete cell layer that could be used in the project varied from two to three days depending on the time the flask was left for obtaining a single layer of cell. In order to maintain growth continuity, the cell lines were split twice a week. Table 2.4 shows that liquid nitrogen has been used to preserve the cell lines that were needed in the project.

At the time of growing a cell layer, a flask of the cell line desired (for carrying out the project) was removed from liquid nitrogen and placed on ice. Seventy-five millimetres of tissue culture flask and a pre-warmed tissue culture growth medium and measuring 20 ml in volume were mixed together in a flask. After the mixture was obtained, the cell line was quickly defrosted so that it could be added into this culture flask which would be followed by the incubation of the cell line at 37°C for the entire night. After the incubation process completed, the residues of the cell line preservatives needed to be eliminated from the mixture of cell line and culture; for this purpose 20 ml of fresh medium was added in the tube in replacement of the older growth medium. The flask was then kept for a period of two or three days to obtain a confluent monolayer of cells. Five millimetres of TrypLe™ express (Life Technologies, UK) was then used to extract the cells. This was followed by the use of CENTAUR2 centrifuge (MES, UK) for centrifuging the extracted cell for duration of approximately 3 minutes at 1500 rpm. The pellet obtained was once again suspended in the growth medium and the supernatant was discarded.

| Cell line | Source | Passages | Reference No |
|---|---|---|---|
| **Colorectal adenocarcinoma epithelial cells (Caco-2)** | European Collection of Cell Cultures | 37 to 38 | ECACC #86010202 |
| **Human brain microvascular endothelial cells (HBMEC)** | Inooprot, Spain | 18 to 24 | Ref. #P10354 |
| **Macrophage cell line (U937)** | American Type Culture Collection | 8 to 9 | ATCC#CRL-1593.2 |
| **Rat brain capillary endothelial cell line (rBCEC4)** | I. E. Blasig* | 5 to 7 | - |

***:** Forschungsinstitut für Molekulare Pharmakologie – Berlin, Germany.

**Table 2.4** Cell lines used in this study

| Cell line | Growth medium | Infection medium |
|---|---|---|
| **Caco-2** | Minimum Essential Medium (MEM) supplied with 10% (v:v) foetal calf serum (FCS), 1% (v:v) non-essential amino acid (NEAA), and 1% (v:v) penicillin-streptomycin (Sigma Aldrich, UK). | Minimum Essential Medium (MEM) supplied with 10% (v:v) FCS, and 1% (v:v) non- NEAA ( Sigma Aldrich, UK). |
| **HBMEC** | Dulbecco's modified eagle medium (DMEM) with 10% (v:v) FCS and 1% (v:v) penicillin-streptomycin (Sigma Aldrich, UK). | Dulbecco's modified eagle medium (DMEM) with 10% (v:v) FCS (Sigma Aldrich, UK). |
| **rBCEC4** | Dulbecco's modified eagle medium (DMEM) with 10% (v:v) FCS and 1% (v:v) penicillin-streptomycin (Sigma Aldrich, UK). | Dulbecco's modified eagle medium (DMEM) with 10% (v:v) FCS (Sigma Aldrich, UK). |
| **U937** | RPMI medium containing 10% (v:v) FCS, 1% (v:v) NEAA, and 1% (v:v) penicillin-streptomycin (Sigma Aldrich, UK) | RPMI medium containing 10% (v:v) FCS and 1% (v:v) NEAA (Sigma Aldrich, UK). |

**Table 2.5** Tissue culture media used in this study

## 2.9.2 PREPARING BACTERIAL INOCULA

For inoculation of the colony of every test strain, 5 ml of LB (Themo Fisher, UK) was used and then it took a period of 18 hours at 37°C to grow them aerobically with shaking at 200 rpm. For examining, 5 ml of infection medium (Table 2.5) was poured into 120 µl of overnight cultures. This was followed by the incubation at 37°C for approximately 1.5 hours and shaking at 200 rpm for achieving optical density (OD) within the range of 0.3-0.5 at $600_{nm}$ utilizing spectrophotometer (JENWAH, UK). For obtaining $4x10^6$ cfu/ml which is multiplicity of infection (MOI 100).

## 2.9.3 GENTAMICIN PROTECTION ASSAY (GPA)

The purpose of the experiment was to test whether the isolates were able to attach and invade the human cell. Gentamicin protection testing was applied for determining this test *in vitro* using

Human colonic carcinoma epithelial cells (Caco-2) passages 37 to 38 acquired from the European Collection of Cell Cultures (ECACC #86010202), human brain microvascular endothelial cells passages 18 to 24 (HBMEC; ref. #HMG030 Inooprot, Spain) and rat brain capillary endothelial cell line (rBCEC4) passages 5 to 7, which was obtained from I. E. Blasig (Forschungsinstitut für Molekulare Pharmakologie - Berlin; Germany). Table 2.5 enlists all the culture media used during the experiment. For HBMEC and rBCEC4 cell lines, *Citrobacter koseri* strain NTU 48 was used as a positive control while for Caco-2 cell line, *Salmonella* Enteritidis strain NTU 358 which was used as positive control. The negative control for all cells was *Escherichia coli* K12 strain NTU 1230.

### 2.9.3.1 ATTACHMENT

Townsend *et al.* (2008) have described this experiment previously. Table 2.5 discusses in detail the growth method of mammalian cells. After the growth the cells were then seeded at $4x10^4$ cell/well in the growth medium at 37°C containing 5% $CO_2$ for a period of 48 hours in order to obtain the monolayer of cells. The wells were then filled with the suspension at $4x10^6$ cfu/well and MOI 100 which were then incubated at 37°C for a period of two hours in the presence of 5% $CO_2$. 1% (v: v) PBS (Sigma Aldrich, UK) was used for washing the wells and Triton X-100 (Fisher Scientific, UK) was used for lysing. The viable count was determined by diluting the lysates and then placing on TSA for overnight at 37°C.

### 2.9.3.2 INVASION

The above method was used as per bacterial attachment mentioned earlier. The washing of the well was followed by addition of 0.5 ml of infection medium (Table 6) and 125 μg/ml (v: v) of gentamicin (Sigma Aldrich, UK). This was then incubated at 37°C for a period of two hours in the presence of 5% $CO_2$. Before completing the process of lysing using 1% (v: v) Triton X-100 (Fisher Scientific, UK), the wells were washed once again using PBS (Sigma Aldrich, UK). The final result was diluted and then placed on TSA for overnight at 37°C for determining the viable count.

## 2.9.4 MACROPHAGE ASSAY

As mentioned by Townsend and colleague (2007), section 2.9.1 described in detail the growth media for macrophages and then were treated with 0.1µg/ml of the PMA ( phorbol 12-myristate 13-acetate) (Sigma Aldrich,UK;P8139) at least 24h prior to infection, and they were placed at 37°C into 75$cm^2$ tissue culture flasks under 5% $CO_2$ for stimulation. For cell adhesion the wells were then filled with the bacterial suspension concentration of $4x10^6$ cfu/well at 37 °C for 72 hours in the presence of 5% $CO_2$.

The concentration of overnight bacterial cell were infected by macrophage was $4x10^6$ cfu/ml (MOI 10). This was incubated at 37 °C for 1 hour in 5% $CO_2$. Then the media was replaced by infection media contains 125 µg/ml (v: v) of gentamicin and incubated at 37°C in the presence of 5% $CO_2$. The plates were washed by PBS and supplied with infection media contain 50 µg/ml of gentamicin. This was then incubated, the plates were completed the process of lysing using 1 % (v:v) Triton X-100 (Fisher Scientific, UK) The final result was diluted and then placed on TSA to obtain the intracellular bacteria at different points (uptake, 6 h, 24h, 48h, and 72h). Percentage of uptake and persistence was used for data presentation.

## 2.9.5 STATISTICAL ANALYSIS

Statistical analysis have been undertaken using unpaired t-test and ANOVA one way (GraphPad Prism program Version 5.0) of the independent experiments of the data. The *P* value $p < 0.05$ assigned significantly to the significance variation results. Moreover, those $p < 0.01$ and $p < 0.001$ were considered highly significant, and very highly significant respectively. Also, the heat maps were generated using SPSS statistics software (version 21.0).

# CHAPTER 3

# DIVERSITY OF *CRONOBACTER* STRAINS OBTAINED FROM OUTBREAKS, AS ANALYSED BY MULTILOCUS SEQUENCE TYPING

# 3.1 INTRODUCTION

## 3.1.1 OUTBREAKS

Several cases of neonatal outbreaks of *Cronobacter* spp. infections in neonatal intensive care units (van Acker *et al*. 2001; Block *et al*. 2002; Himelright *et al*. 2002; Caubilla-Barron *et al.* 2007) were described previously in Chapter 1 (Section 1.3). Reconstituted powdered infant formula (PIF) has been held responsible for a large number of these infections, which could have been intrinsically contaminated while they were being prepared or through reconstitution water (Himelright *et al*. 2002). The prepared product could have been affected by the growth of bacteria and this happens because of temperature abuse of the product (Caubilla-Barron *et al.* 2007). Humans can also asymptomatically carry the bacteria (Holy & Forsythe 2013). Effective typing schemes should exist for the *Cronobacter* spp. and studies should be conducted of all the possible sources of the bacteria while an outbreak is taking place.

## 3.1.2 MULTILOCUS SEQUENCE TYPING

Genes involved in maintaining basic cellular functions are known as housekeeping genes and these are scattered through the genome. Multi Locus Sequence Typing (MLST) refers to a molecular technique during which partial sequence analysis of several housekeeping genes is carried out to identify and cluster bacterial isolates. It is actually a modified form of an earlier typing technique, i.e. Multi Locus Enzyme Electrophoresis (MLEE). In the case of MLEE, variations in multiple housekeeping or basic metabolic genes are identified through analysing different gene products by electrophoresis (Maiden *et al*. 1998). In comparison to MLEE, MLST is based on sequencing of genes. Gene sequencing allows us to be sure that alteration in even a single nucleotide is considered while distinguishing between different isolates; MLST has proven later to be more efficient than other approaches.

Since the MLST targets housekeeping genes which are essentially required by the organism, these genes are constitutively expressed and independent of any selective conditions. Moreover, analysis

of more than one gene allows the acquisition of extensive data and improved discrimination as compared to what is offered by analysis of a single gene. This approach also facilitates avoiding the influence of any recombination, which usually takes place at one locus. Hence, targeting multiple genes proves to be beneficial in a number of ways (Maiden *et al.* 1998; Enright & Spratt 1999).

In 1998, Maiden *et al*. used the MLST for the first time to analyse a set of *Neisseria meningitidis* strains. At that time, the method involved analysis of six loci; however, most MLST methods used currently involve seven housekeeping loci thereby allowing greater discrimination and higher efficiency. The MLST method is quite simple in principle as shown in Figure 3.1 The procedure starts with the amplification of target genes followed by sequencing which is done with the help of primers or nested primers (amplification is performed with outer primers and nucleotide sequencing with inner primers). Next step is the trimming of a 400-500bp long region and its analysis for each locus. After this, multiple alleles at each gene locus are arranged and compared. A distinct allelic profile number of the locus is assigned to every variant allele. This profile number is assigned as follows. In a typing technique involving seven loci, each isolate is given a combination of 7 allelic profile numbers. Every distinct combination of these profile numbers denotes a sequence type (ST) for the specific isolate. Isolates demonstrating comparable STs are regarded as isogenic strains or clones of one another since they cannot be differentiated at all using the seven loci. The acquired sequence data is then recorded and stored in a central database so that it can be accessed online and can be reproduced in any laboratory around the world (Maiden *et al*. 1998).

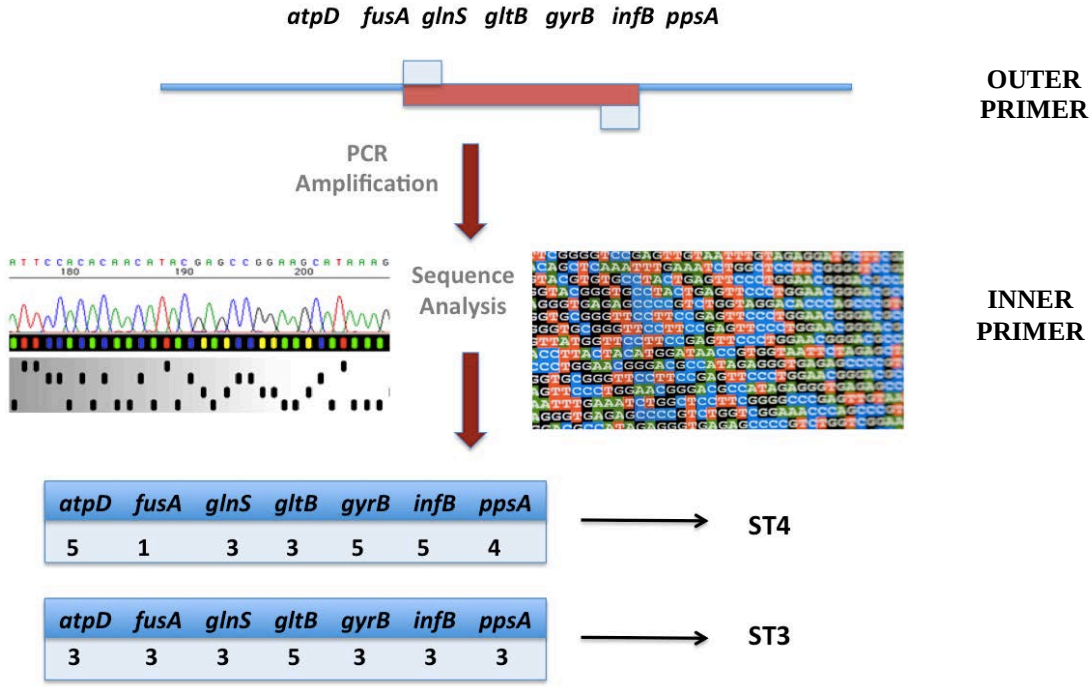


**Figure 3.1** Multi Locus Sequence Typing experimental method. The procedure starts with (1) Amplification of target genes followed by sequencing which is done with the help of primers or nested primers (amplification is performed with outer primers and nucleotide sequencing with inner primers). (2) Trimming of a 400-500bp long region and its analysis for each locus. (3) Multiple alleles at each gene locus are arranged and compared. (4) A distinct allelic profile number of the locus is assigned to every variant allele. This profile number is assigned as follows. (5) In a typing technique involving seven loci, each isolate is given a combination of 7 allelic profile numbers. (6) Every distinct combination of these profile numbers denotes a sequence type (ST) for the specific isolate.

Central analytical facilities support and maintain these databases, which contain a huge amount of data regarding isolates that had been subjected to sequence typing. Besides sequence data, isolation history of the isolate is also recorded in the database. Moreover, the databases contain links to other publications, which have reported that particular strain. Hence, abundant data regarding

different strains are made available at a single platform. This huge amount of data can be accessed freely and easily as no registration or other procedures are required for accessing or downloading the data. Hence, researchers investigating the same strain may compare their findings with those recorded in the database. High quality and authenticity of the data is first ensured before including it in the databases. To make a comparison between alleles and sequence types, MLSA (Multi Locus Sequence Analysis) is used to identify the recombination and relationship in the population, establish links between related STs or isolates through developing minimum spanning trees or phylogenetic trees; relevant links are made available on these databases for evaluating the sequences using different tools. For instance, the database is integrated with the BLAST tools that allow similarity searches of the loci. These tools can also be used to download profiles of all the identified STs. Certain databases like PubMLST are equipped with a tool called "Locus Explorer" that allows visualization of the variable sites in every locus.

The MLST database for *Cronobacter* can be accessed at http://www.pubmlst.org/cronobacter in the PubMLST group of databases. Prof. Keith Jolley maintains the resources for more than forty MLST databases of bacteria including *Yersinia* spp., *Pseudomonas aeruginosa, Neisseria* spp., *Clostridium difficile, Helicobacter pylori, Campylobacter* spp., and *Aeromonas* spp. Each database was divided into two subsections. One subsection contains data related to the technique such as introduction to the MLST, material and methods employed for amplification and sequencing. Second subsection comprises the main data i.e. sequences of alleles, information about the isolate and MLST profiles. Besides typing for epidemiological objectives, sequencing data acquired through MLST can be used in other applications like population genetics and speciation. For that reason, not only pathogens but also several environmental isolates are also subjected to the MLST (Brady *et al*. 2008).

### 3.1.3 MULTILOCUS SEQUENCE TYPING SCHEME FOR *CRONOBACTER* SPP.

Researchers have suggested several different genotyping methods based on the DNA for the

identification, classification and characterization of *Cronobacter* species. The PCR probes for the *rpoB, rpsU* and *dnaG* genes are some of them (Seo and Brackett. 2005; Stoop *et al*. 2009). Still, utilization of these techniques for *Cronobacter* is limited by factors like lack of validation of these techniques against a set of *Cronobacter* strains belonging to 7 species or their discontinued utilization in research studies.

Serogrouping with the help of the PCR seems to be the most advanced non-MLST profiling technique (Jarvis *et al*. 2011; Sun *et al*. 2011). At present, the procedure involving utilization of seven primer pairs screens only 4 out of 7 species and more than 20% (48 out of 231) strains do not yield a PCR product (Jarvis *et al*. 2011; Sun *et al*. 2011) suggesting that some serogroups remain unidentified. Moreover, contradictory findings have been reported indicating existence of single serogroup across multiple species, e.g. *C. muytjensii* O1 and *C. sakazakii* O3 (Jarvis *et al*. 2011). Unlike the above mentioned techniques, MLST utilizing only seven primers pairs has been developed for the entire genus of *Cronobacter* and has proven to be a reliable and efficient technique. This chapter elaborates how the seven loci method has improved our knowledge related to the phylogeny and profiling of *Cronobacter*.

The MLSA and MLST techniques have benefited the *Cronobacter* studies in many ways. They disclosed how diverse the genus *Cronobacter* is and showed how the genus has descended evolutionarily. These techniques not only revealed how closely related are the seven species of *Cronobacter* but also enabled identification of two new species i.e. *C. condimenti* and *C. universalis*. Moreover, clinical studies making use of these techniques indicated that only one clonal lineage *C. sakazakii* ST4 clonal complex is responsible for most of the cases of neonatal meningitis. These findings laid the foundation for future studies for selecting strains in order to study the environmental fitness and virulence of *Cronobacter*. Last but not the least; they enabled development of a curated database available at http://www.pubMLST.org/cronobacter. Prof Stephen Forsythe curates the *Cronobacter* PubMLST database, hosted at University of Oxford, UK. The database contains more than 1000 strains and >350 STs studied through MLST. These strains demonstrate diverse distribution sources, geographically and temporally. The database also contains details of experimental procedures and DNA sequences of 7 alleles for offline evaluation.

One can find the sequence type (ST) of a strain and perform advanced DNA sequence analysis using this database.

Seven housekeeping genes including *atpD, fusA, glnS, gltB, gyrB, infB,* and *ppsA* are subjected to partial sequence analysis while studying *Cronobacter* through MLST technique (Baldwin *et al*. 2009). According to Kucerova *et al*. (2010), these loci are scattered across the genome of *Cronobacter* and are minimally affected by any potential pressures of selective evolution. Moreover, the primers for the seven genes have been developed in a way that the annealing temperatures of these primers are alike. This allows execution of all reactions in a single run thereby making the procedure more rapid. When the loci DNA sequences are compared with the MLST reference database for *Cronbacter*, the sequence type of strains and seven digit allele codes are obtained. Concatenating the 7 allele sequences gives rise to a concern sequence of 3036 nt length for phylogenetic and MLSA analysis.

Initially, the MLST technique was developed for *C. malonaticus* and *C. sakazakii* (Baldwin *et al*. 2009) in order to prove that it is more efficient than the conventional 16S rDNA sequence analysis which was unable to differentiate between these two species. Later, researchers utilized MLST for all of the 7 known *Cronobacter* species (Czerwicka *et al*. 2010; Kucerova *et al*. 2010, 2011; Hamby *et al*. 2011; Joseph and Forsythe, 2011; Hariri *et al*. 2012; Joseph *et al*. 2012a,b). It has also been reported that this method can reliably execute speciation by using *fusA* as single locus (Joseph *et al*. 2012b).

The MLST and MLSA have played a crucial role in improving our knowledge related to *Cronobacter*. However, it is worth mentioning here that the conventional phenotyping (utilization of biotypes) for classification of *Cronobacter* species was defective since certain biotype index strains were classified incorrectly as per the findings made through DNA sequence based MLSA later (Baldwin *et al*. 2009).

The *C. sakazakii* species has been found to be the predominant species in the strains obtained from the PIF and the environmental sampling of the milk powder processing factories. The *C. sakazakii* ST4 strains were found in the powdered formulas, the roof, tanker bays, and the roller driers of the manufacturing plants (Sonbol *et al*. 2013). The desiccation resistant strains of *Cronobacter* spp.

including *C. sakazakii* ST4 could occur in these dry environments. Many species and STs of the *Cronobacter* spp. are found in many food products and ingredients, herbs and spices.

## 3.1.4 AIMS OF THIS CHAPTER

The FAO-WHO (2004) requested that reliable detection and molecular typing methods are developed for the control of *Cronobacter* spp. A multilocus sequence typing scheme (MLST) open access internationally based database (http://www.pubMLST.org/cronobacter/) has been established by our research group. This is supported by international contributors and currently includes the profiles of >1000 strains and 108 whole genomes. The MLST scheme has advanced our understanding of the *Cronobacter* genus, revealing predominant and stable clones associated with neonatal meningitis.

In this chapter, the MLST scheme was applied to three collections of clinical strains that had not previously been profiled as a continuation of previous studies by Susan Joseph (NTU), but with more focussed attention on particular strains. These clinical strains were from USA, Israel and the Czech Republic in which eleven sequence types (STs) were identified spanning the genus of *Cronobacter.*

The key results presented in this chapter have been accepted for publication (Hariri *et al*. 2013; Alsonosi *et al*. 2015).

## 3.2 MATERIALS AND METHODS

The key methods, culture media, culturing conditions and bacterial strain list for this part were described previously in Chapter 2 Materials and Methods

### 3.2.1 GENOMIC DNA EXTRACTION

According to the instructions of the manufacturer, genomic DNA was prepared from 1.5 ml of culture grown overnight in TSB using GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, UK). Nanodrop 2000 (Thermo Scientific, UK) was used to check the concentration and purity of the eluted DNA. For PCR experiments DNA samples with minimum 260/280 nm values of 1.8 and 260/230 nm values of 2 were used, otherwise the DNA was extracted again.

### 3.2.2 PCR AMPLIFICATION OF MLST LOCI

Baldwin *et al.* (2009) described amplification and nested sequencing primers for the MLST loci. Components were used in various concentrations, i.e. ~10 ng chromosomal DNA, 20 pmol reverse and forward primer (Sigma-Aldrich, UK), 1× PCR buffer (Promega, UK) supplemented with 1.5 mM $MgCl_2$, 0.8 mM deoxynucleotide triphosphates and 1.25 U Taq (Promega, UK) were included in each 25 µl amplification reaction mixture. Initial denaturation at 94°C for 2 min; 30 cycles of denaturation at 94°C for 1 min, primer annealing at 58°C for 1 min, extension at 72°C for 2 min; followed by a final extension step of 72°C for 5 min were the reactions conditions for all the primers.

### 3.2.3 AGAROSE GEL ELECTROPHORESIS

1% agarose gel was used to visualize the amplified PCR products. The gel was prepared as follows: 1% (w/v) agarose (Fisher Scientific, UK) was dissolved in 1X Tris-acetate-EDTA (TAE) buffer (Geneflow, UK). To dissolve the agarose completely, the solution was heated in a microwave oven until the solution becomes clear. In the agarose solution, 0.1 µ/ml (v/v) of SYBR® Safe DNA gel stain (Life Technologies – Invitrogen, UK) was added and dissolved well. The gel was then poured into a tray and let it for some time to set. After making wells, 5 µl PCR products were loaded into

each of the well. A 1kb ladder (Promega, UK) was loaded as marker into one well. Gel electrophoresis was performed for 40 minutes at 100V in 1X TAE buffer. The gel was then visualized under ultraviolet (UV) light. After that, the DNA bands can be observed using the InGenius® gel documentation system (Syngene, UK).

### 3.2.4 PCR PRODUCT PURIFICATION

MinElute PCR Purification Kits (Qiagen, UK) were used to purify the amplified products using the manufacturer's protocol. The products were finally eluded in 50 µl of molecular biology grade water (Fisher Scientific, UK). The Nanodrop 2000 (Thermo Scientific, UK) was used to check the concentration and purity of the samples.

### 3.2.5 DNA SEQUENCING

The ABI 3730XL sequencing machines by Eurofins MWG Operon (London, UK) and Source Bioscience (Nottingham, UK) were used for sequencing. The nucleotide sequences were determined on each DNA strand using the nested sequencing primers (Table 3.1).

| Target gene | Locus* | Putative function | | Outer primers (5'-3') | Inner Primers (5'-3') |
|---|---|---|---|---|---|
| *atpD* | ESA_04006 | ATP synthase β chain | Forward | CGACATGAAAGGCGACAT | CGAAATGACCGACTCCAA |
| | | | Reverse | TTAAAGCCACGGATGGTG | GGATGGCGATGATGTCTT |
| *fusA* | ESA_04401 | Elongation factor | Forward | GAAACCGTATGGCGTCAG | GCTGGATGCGGTAATTGA |
| | | | Reverse | AGAACCGAAGTGCAGACG | CCCATACCAGCGATGATG |
| *glnS* | ESA_02658 | Glutaminyl tRNA-synthetase | Forward | GCATCTACCCGATGTACG | GGGTGCTGGATAACATCA |
| | | | Reverse | TTGGCACGCTGAACAGAC | CTTGTTGGCTTCTTCACG |
| *gltB* | ESA_03606 | Glutamate synthase large subunit | Forward | CATCTCGACCATCGCTTC | GCGAATACCACGCCTACA |
| | | | Reverse | CAGCACTTCCACCAGCTC | GCGTATTTCACGGAGGAG |
| *gyrB* | ESA_03973 | DNA gyrase β subunit | Forward | TGCACCACATGGTATTCG | CTCGCGGGTCACTGTAAA |
| | | | Reverse | CACCGGTCACAAACTCGT | ACGCCGATACCGTCTTTT |
| *infB* | ESA_03561 | Translation initiation factor IF-2 | Forward | GAAGAAGCGGTAATGAGC | TGACCACGGTAAAACCTC |
| | | | Reverse | CGATACCACATTCCATGC | GGACCACGACCTTTATCC |
| *ppsA* | ESA_02102 | Phosphoenol pyruvate synthase | Forward | GTCCAACAATGGCTCGTC | ACCCTGACGAATTCTACG |
| | | | Reverse | CAGACTCAGCCAGGTTTG | CAGATCCGGCATGGTATC |

*– Genbank locus of the gene on the *C. sakazakii* BAA-894 genome (Kucerova *et al*. 2010)

**Table 3.1** Details of the seven MLST loci and the primers used for their amplification and sequencing

## 3.2.6 ALLELE AND SEQUENCE TYPE DESIGNATION

For quality control, ChromasLite (Version 2.01, Technelysium Pty Ltd) was used to view the sequence chromatograms. For a given locus, the sequences from both the DNA strands were aligned and cut to the desired allele length with the help of Jalview (Version 2+; Waterhouse *et al*. 2009) in ClustalW (http://www.ebi.ac.uk/clustalw). The newly identified alleles were assigned numbers ( by the database editor; Prof SJ Forsythe) arbitrarily according to the order in the PubMLST database, called allelic profiles. All alleles within the MLST scheme were in frame, to aid the analysis.

## 3.3 CHOICE OF BACTERIAL STRAINS

In this study, the MLST scheme was applied to three collections of clinical strains that had not previously been profiled. These clinical strains were from USA, Israel and the Czech Republic in which eleven sequence types (STs) were identified spanning the genus of *Cronobacter* ; Table 3.2.

| NTU ID | ST | CC | Species | Country | Source | *atpD* | *fusA* | *glnS* | *gltB* | *gyrB* | *infB* | *ppsA* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1579 | 4 | 4 | *C.sakazakii* | USA | CSF (Patient died) | 5 | 1 | 3 | 3 | 5 | 5 | 4 |
| 1566 | 4 | 4 | *C.sakazakii* | USA | CSF | 5 | 1 | 3 | 3 | 5 | 5 | 4 |
| 1570 | 4 | 4 | *C.sakazakii* | USA | CSF (Brain infarction) | 5 | 1 | 3 | 3 | 5 | 5 | 4 |
| 1567 | 4 | 4 | *C.sakazakii* | USA | Feces (Asymptomatic) | 5 | 1 | 3 | 3 | 5 | 5 | 4 |
| 1568 | 4 | 4 | *C.sakazakii* | USA | Opened PIF | 5 | 1 | 3 | 3 | 5 | 5 | 4 |
| 1571 | 4 | 4 | *C.sakazakii* | USA | Opened PIF | 5 | 1 | 3 | 3 | 5 | 5 | 4 |
| 1576 | 4 | 4 | *C.sakazakii* | USA | Tracheal secretion | 5 | 1 | 3 | 3 | 5 | 5 | 4 |
| 1565 | 107 | 4 | *C.sakazakii* | USA | CSF (Brain abscess) | 5 | 50 | 3 | 3 | 5 | 5 | 4 |
| 1572 | 108 | 4 | *C.sakazakii* | USA | Opened PIF | 5 | 8 | 3 | 3 | 5 | 5 | 4 |
| 1577 | 110 | 4 | *C.sakazakii* | USA | CSF | 11 | 1 | 3 | 5 | 8 | 5 | 4 |
| 1578 | 111 | 8 | *C.sakazakii* | USA | PIF reconstitution water | 5 | 8 | 7 | 3 | 5 | 15 | 10 |
| 1573 | 8 | 8 | *C.sakazakii* | USA | Opened PIF | 11 | 8 | 7 | 5 | 8 | 15 | 10 |
| 1574 | 8 | 8 | *C.sakazakii* | USA | Faeces (Diarrheal symptoms) | 11 | 8 | 7 | 5 | 8 | 15 | 10 |
| 1575 | 8 | 8 | *C.sakazakii* | USA | Faeces (Ongoing diarrhoea) | 11 | 8 | 7 | 5 | 8 | 15 | 10 |
| 1569 | 112 | 112 | *C.malonaticus* | USA | Blood (Clinical meningitis (Patient died) | 51 | 7 | 6 | 7 | 62 | 30 | 50 |
| 1580 | 4 | 4 | *C.sakazakii* | Israel | Faeces (Asymptomatic) | 5 | 1 | 3 | 3 | 5 | 5 | 4 |
| 1581 | 4 | 4 | *C.sakazakii* | Israel | Infant Formula | 5 | 1 | 3 | 3 | 5 | 5 | 4 |
| 1582 | 4 | 4 | *C.sakazakii* | Israel | Blender Isolate (Environment) | 5 | 1 | 3 | 3 | 5 | 5 | 4 |
| 1583 | 4 | 4 | *C.sakazakii* | Israel | Faeces (Asymptomatic) | 5 | 1 | 3 | 3 | 5 | 5 | 4 |
| 1584 | 4 | 4 | *C.sakazakii* | Israel | Faeces (Asymptomatic) | 5 | 1 | 3 | 3 | 5 | 5 | 4 |
| 1585 | 4 | 4 | *C.sakazakii* | Israel | Blood (Bacteraemia) | 5 | 1 | 3 | 3 | 5 | 5 | 4 |
| 1587 | 109 | 4 | *C.sakazakii* | Israel | CSF (Brain damage) | 5 | 51 | 3 | 3 | 5 | 5 | 4 |
| 1586 | 83 | 83 | *C.sakazakii* | Israel | Blood | 19 | 16 | 19 | 41 | 19 | 15 | 23 |
| 1826 | 7 | 7 | *C.malonaticus* | Czech Republic | Cannula | 10 | 7 | 6 | 7 | 9 | 14 | 9 |
| 1827 | 7 | 7 | *C.malonaticus* | Czech Republic | Cannula | 10 | 7 | 6 | 7 | 9 | 14 | 9 |
| 1828 | 7 | 7 | *C.malonaticus* | Czech Republic | Nose swab | 10 | 7 | 6 | 7 | 9 | 14 | 9 |
| 1829 | 7 | 7 | *C.malonaticus* | Czech Republic | Throat swab | 10 | 7 | 6 | 7 | 9 | 14 | 9 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1830 | 7 | 7 | *C.malonaticus* | Czech Republic | Throat Swab | 10 | 7 | 6 | 7 | 9 | 14 | 9 |
| 1831 | 7 | 7 | *C.malonaticus* | Czech Republic | Throat Swab | 10 | 7 | 6 | 7 | 9 | 14 | 9 |
| 1832 | 7 | 7 | *C.malonaticus* | Czech Republic | Throat Swab | 10 | 7 | 6 | 7 | 9 | 14 | 9 |
| 1833 | 7 | 7 | *C.malonaticus* | Czech Republic | Stool Dissection | 10 | 7 | 6 | 7 | 9 | 14 | 9 |
| 1834 | 7 | 7 | *C.malonaticus* | Czech Republic | Throat Swab | 10 | 7 | 6 | 7 | 9 | 14 | 9 |
| 1835 | 7 | 7 | *C.malonaticus* | Czech Republic | Throat Swab | 10 | 7 | 6 | 7 | 9 | 14 | 9 |
| 1914 | 7 | 7 | *C.malonaticus* | Czech Republic | Sputum | 10 | 7 | 6 | 7 | 9 | 14 | 9 |
| 1917 | 7 | 7 | *C.malonaticus* | Czech Republic | Sputum | 10 | 7 | 6 | 7 | 9 | 14 | 9 |
| 1836 | 4 | 4 | *C.sakazakii* | Czech Republic | Wound Swab | 5 | 1 | 3 | 3 | 5 | 5 | 4 |
| 1839 | 4 | 4 | *C.sakazakii* | Czech Republic | Smear from of percutaneous endoscopic gastrostomy | 5 | 1 | 3 | 3 | 5 | 5 | 4 |
| 1840 | 4 | 4 | *C.sakazakii* | Czech Republic | Sputum | 5 | 1 | 3 | 3 | 5 | 5 | 4 |
| 1841 | 4 | 4 | *C.sakazakii* | Czech Republic | Sputum | 5 | 1 | 3 | 3 | 5 | 5 | 4 |
| 1842 | 4 | 4 | *C.sakazakii* | Czech Republic | Sputum | 5 | 1 | 3 | 3 | 5 | 5 | 4 |
| 1901 | 4 | 4 | *C.sakazakii* | Czech Republic | Sputum | 5 | 1 | 3 | 3 | 5 | 5 | 4 |
| 1902 | 4 | 4 | *C.sakazakii* | Czech Republic | Sputum | 5 | 1 | 3 | 3 | 5 | 5 | 4 |
| 1903 | 4 | 4 | *C.sakazakii* | Czech Republic | Sputum | 5 | 1 | 3 | 3 | 5 | 5 | 4 |
| 1915 | 4 | 4 | *C.sakazakii* | Czech Republic | Sputum | 5 | 1 | 3 | 3 | 5 | 5 | 4 |
| 1916 | 4 | 4 | *C.sakazakii* | Czech Republic | Sputum | 5 | 1 | 3 | 3 | 5 | 5 | 4 |
| 1838 | 28 | 28 | *Cmuytjensii* | Czech Republic | Sputum | 4 | 24 | 19 | 19 | 4 | 4 | 6 |

**Table 3.2** The bacterial stains used in this study and their MLST profiles. MLST typing technique involving seven loci, each isolate is given a combination of 7 allelic profile numbers. Every distinct combination of these profile numbers denotes a sequence type (ST) for the specific isolate.

## 3.4 RESULTS

### 3.4.1 MLST OF THE *CRONOBACTER* CLINICAL STRAINS.

MLST scheme which was created by Baldwin *et al*. (2009) is based on the seven housekeeping genes: *atpD* (ATP synthase b chain), *fusA* (elongation factor G), *glnS* (glutaminyl t RNA synthetase), *gltB* (glutamate synthase large subunit) *gyrB* (DNA gyrase subunit B), *infB* (translation initiation factor IF-2) and *ppsA* (phosphoenolypyruvate synthase A). Table 3.1.

The alleles of the seven genes of MLST have been effectively amplified and sequenced in this study for 41 strains using the primers defined in (Table 3.1). The *Cronobacter* MLST scheme is hosted with open access at http://www.pubmlst.org/cronobacter. The seven housekeeping genes have been successfully sequenced from all the clinical strains used in this study ;Table 3.2.

### 3.4.2 *CRONOBACTER SAKAZAKII* ST4 STRAINS (UNITED STATES) AND NEONATAL MENINGITIS

This research has analysed 15 *Cronobacter* isolates received from Centre for Disease Control (CDC). Fourteen out of 15 strains were *C. sakazakii* and one strain was *C. malonaticus*. However, there was an uneven distribution according to clinical records: all 5 cerebrospinal fluid (CSF) isolates were either ST4 or within the ST4 complex (clonal group where strains are identical in 4 or more loci). This group included strains from cases during December in Illinois (strain 1577) and in Lebanon, Missouri (strain 1579).

Strain 1577 (ST110), isolated from CSF, is a triple-loci variant of ST4, distinguished by 5/3036 nucleotides: *atpD* (1/390nt),*gltB* (2/507nt), and *gyrB* (2/402nt). Strain 1578 (ST111), isolated from the PIF reconstitution water and associated with the case reported in Illinois, is distinguishable from ST4 in 4/7 loci: *fusA* (5/438nt), *glnS* (1/363), *infB* (4/441), and *ppsA* (19/495). The two Illinois strains, 1577 and 1578 (ST110 and ST111), differed from each other at all loci, with 35/3,036nt differences in total.

Strain 1569 (ST112), isolated from blood, was the first reported meningitis case by *Cronobacter malonaticus* which was from an infant (age <1 month) with severe brain damage which led to their death.

### 3.4.3 *CRONOBACTER SAKAZAKII* ST4 STRAINS (JERUSALEM)

Among the strains collected from Jerusalem, nine were isolated from infants of age ranging from 2 to 36 weeks. The strains were isolated from a hospital belonging to the Hadassah Medical Organisation where 2 infants showed symptoms of bacteraemia and meningitis. These included strains 1585 and 1587 while the other strains were from asymptomatic babies (1580, 1581, 1582, 1583, 1584, and 1586). Seven out of nine strains were previously identified as belonging to the ST4 clonal complex (Block *et al.* 2001; 2002). These strains were unique in that they were all found to be negative for nitrite reduction.

### 3.4.4 *CRONOBACTER SAKAZAKII* ST4 AND *CRONOBACTER MALONATICUS* ST7 STRAINS (CZECH REPUBLIC)

The Czech Republic clinical strains used in this study have been mainly collected from two hospitals Olomouc and Prostejov, and from different departments, sources and patient age. This collection primarily comprised *C. malonaticus* (9/18) were from various age groups belonging to the ST7 (1826-1835) which had been isolated from the two hospitals during a six year period. Other strains (8/18) of *C. sakazakii* were ST4 (1836-1903) which had been isolated from Prostejov hospital, mainly from the department of internal medicine during one year period. Additionally, one strain (1838) was *C.muytjensii* ST28. This strain has been isolated from sputum in 2012 as the only clinical isolate from this species included in this study as shown in Table 3.1. The result revealed the predominance of *C.sakazakii* ST4 strains which have been isolated in 2012 from various sources wound swab and sputum. As well as the *C.malonaticus* ST7 strains which had been also obtained between 2007 and 2012, which included 9 strains. These had been isolated from throat swab, cannula and stool dissection.

Across the three *Cronobacter* species obtained from outbreaks, the majority of STs were identified as *C. sakazakii* CC4 (28/46) compared with (12/46) *C. malonaticus* ST7 and only (1/46) *C. muytijensii* ST. The main *C. sakazakii* STs were CC4 (60 %), and followed by 26% identified as *C. malonaticus* ST7. (Tables 3.3 -3.4)

**Table 3.3** Summary of multilocus sequence typing profile of 41 *Cronobacter* strains obtained between 2002 and 2012.

| Bacterial species | STs (CC)* | Number of isolates | | | Total of strains | Percentage (%) |
|---|---|---|---|---|---|---|
| | | USA | Israel | CR | | |
| ***C. sakazakii*** | **4 (4)** | **7** | **6** | **10** | **28** | **60** |
| | **107 (4)** | **1** | **-** | **-** | | |
| | **108 (4)** | **1** | **-** | **-** | | |
| | **110 (4)** | **1** | **-** | **-** | | |
| | **109 (4)** | **-** | **1** | **-** | | |
| | **111 (8)** | **1** | **-** | **-** | **4** | **8** |
| | **8 (8)** | **3** | **-** | **-** | | |
| | **83** | **-** | **1** | **-** | **1** | **2** |
| ***C. malonaticus*** | **7** | **-** | **-** | **12** | **12** | **26** |
| | **112** | **1** | **-** | **-** | **1** | **2** |
| ***C. muytjensii*** | **28** | **-** | **-** | **1** | **1** | **2** |
| **Total** | | **15** | **8** | **23** | **46** | **100** |

* Clonal complex described as clusters of STs with single locus variants (Joseph *et al*. 2012b).
**%**: Total number of each clonal complex.

**Table 3.4** Breakdown of the *Cronobacter* STs according to species, isolate sources, country of origin and time of isolation.

| Species | ST | No. of strains | Isolation source | Country of isolation | Year of isolation |
|---|---|---|---|---|---|
| *C. sakazakii* | 4 | 21 | Opened PIF, clinical (CSF-tracheal secretion, blood), environment (blender), | Czech Republic, USA, Israel | 2000-2012 |
| *C. malanoticus* | 7 | 9 | Clinical | Czech Republic | 2007-2012 |
| *C. sakazakii* | 8 | 3 | Opened PIF, feces; exposed to PIF | USA | 2011 |
| *C. sakazakii* | 111 | 1 | PIF reconstitution water | USA | 2011 |
| *C. sakazakii* | 108 | 1 | Opened PIF | USA | 2011 |
| *C. sakazakii* | 109 | 1 | Clinical, weaning food | Israel | 2000 |
| *C. sakazakii* | 83 | 1 | Clinical, infant formula, food, herbs, spice | Israel | 2000 |
| *C. malanoticus* | 112 | 1 | Infant formula | Czech Republic | 2007-2012 |
| *C. sakazakii* | 107 | 1 | CSF, exposed to PIF | USA | 2011 |
| *C. muytjensii* | 28 | 1 | Clinical | Czech Republic | 2007-2012 |

## 3.4.5 PHYLOGENETIC RELATIONSHIP OF THE *CRONOBACTE*R CLINICAL STRAINS.

The *Cronobacter* genus has been studied with the help of phylogenetic trees made on the basis of concatenated sequences (comprising of 3036 nucleotides) of the STs of 46 clinical strains (Table 3.2). The interspecific and intraspecific diversity of the genus can be quantified and the strains can be classified on the basis of the source and the virulence groupings.

The clustering of the different species of *Cronobacter* in the genus (Figure 3.2) can be observed by employing the Maximum Likelihood algorithm in MEGA5 (Tamura *et al*. 2011). The Maximum-Likelihood algorithm in MEGA5 was employed for the phylogenetic tree construction. Use of sequences of concatenated seven MLST sequences demonstrated improved resolution and effective clustering of species of *Cronobacter*.

It has been found through phylogenetic studies of *Cronobacter* genus with the help of the MLSA that *C. malonaticus* and *C. sakazakii* have strong genetic relationship as they are close to each other in the cluster. However, they can still be distinguished which is not possible with 16S rDNA sequencing (Baldwin *et al*. 2009).

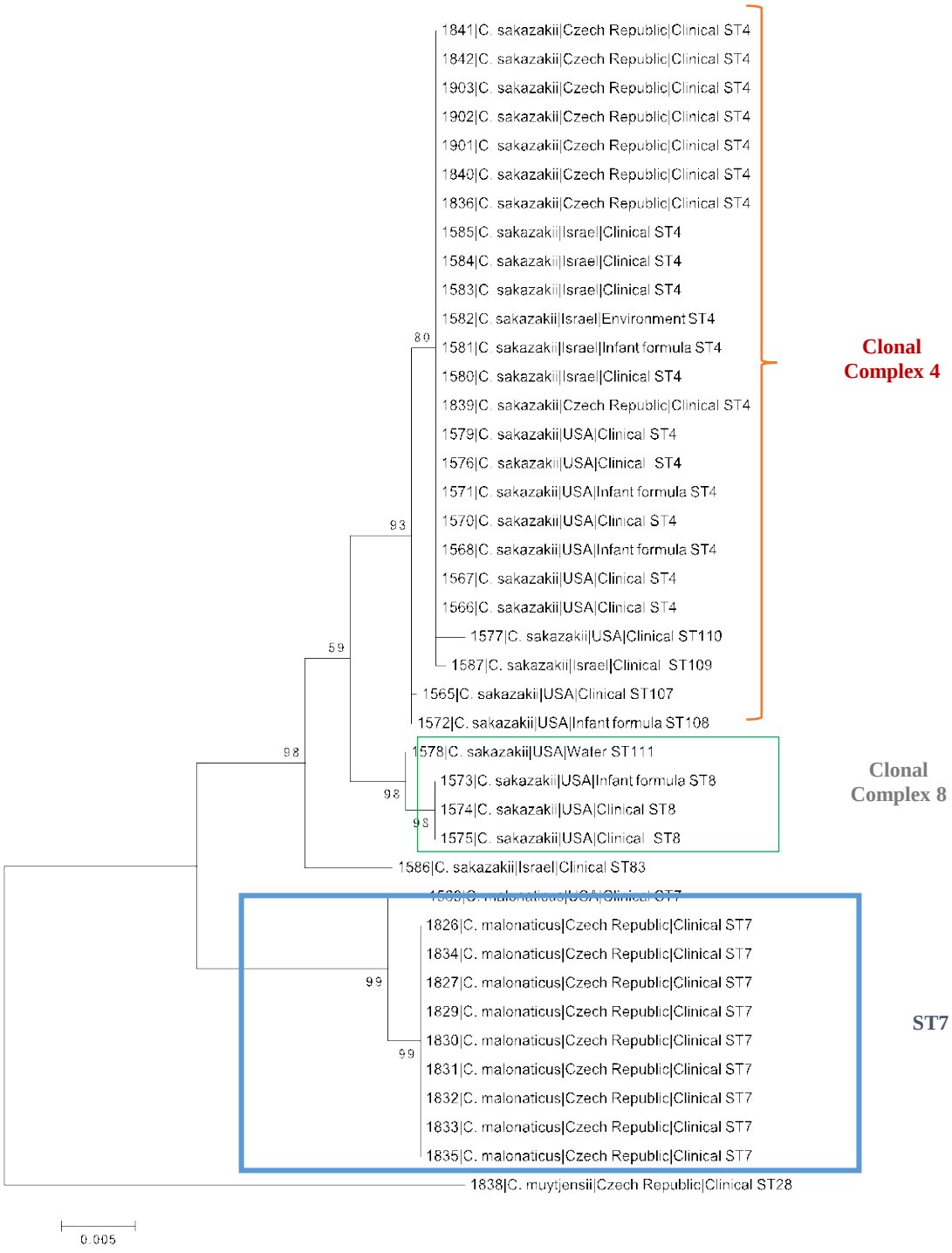


**Figure 3.2** Maximum likelihood tree based on the concatenated sequences (3036 bp) of the 7 MLST loci for the genus *Cronobacter* clinical strains. The STs and clonal complex are indicated at the leaf of each branch. MEGA5 with 1000 bootstrap replicates have been used to assess the quality of the tree produced.

## 3.4.6 GOEBURST ANALYSIS OF DIVERSITY OF *CRONOBACTER* STRAINS DISTRIBUTION IN OUTBREAKS.

The use of the MLST scheme has revealed predominant and key stable clones, within the *Cronobacter* genus which are very important from the point of view of epidemiology of the organism.

The goeBURST analysis has revealed the dominance of certain STs such as ST4 and ST7 in the *Cronobacter* population relationships in the process as shown in Figure 3.3 (A-B-C)

The diversity of sources of isolation based goeBURST analysis of the *Cronobacter* STs has also been done and Figure 3.4 shows that most of the clinical samples contained ST4, ST8, ST7 and other STs related to these strains in different countries. The clinically associated *Cronobacter* strains have been grouped into certain STs (CC) by the *Cronobacter* MLST scheme and stable virulent lineages have been described.

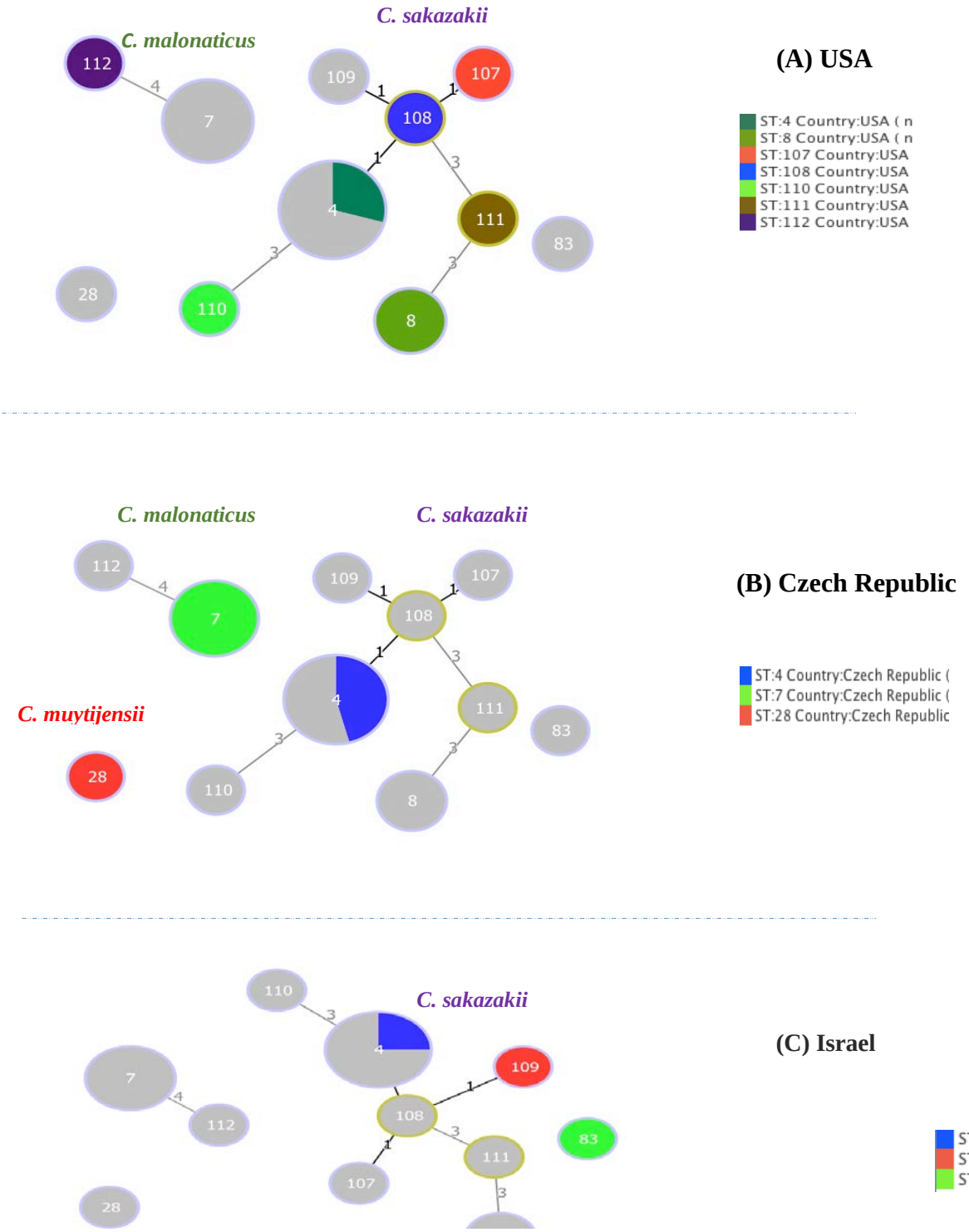


**Figure 3.3** goeBURST analysis of *Cronobacter* STs according to the diversity of the countries of isolation/origin. The threshold for the output was set to triple locus variation. The circles with larger diameters represent the dominant STs. Clusters of linked isolates correspond to clonal complexes. The strain collection was obtained from USA **(A),** Czech Republic **(B)** and Israel **(C)** and compared with the general STs destination (pale shade). The dark shade indicates the stability of certain STs such as the ST4 and ST7 complex isolated from three different countries between 1998-2012.

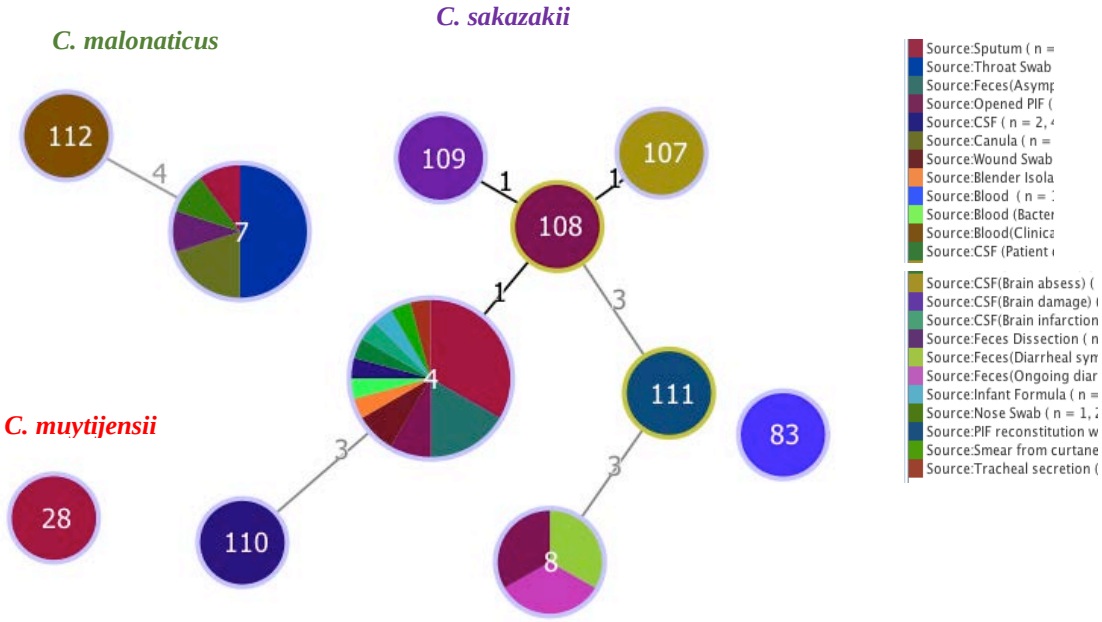


**Figure 3.4** Population snapshot of the *Cronobacter* MLST database generated using the goeBURST algorithm, indicating the clonal complexes and the diversity of the source of the strains. The dominant STs are presented by the circles with large diameters. The *C. sakazakii* STs 4, 110, 109, 107 and 108 are in clonal complex 4 which is a major clonal lineage when one considers the epidemiology of *Cronobacter* species. ST4 has not only proven to be the most common clinical ST, but also the most dominant one.

## 3.5 DISCUSSION

MLST is an efficient molecular technique for typing and has been extensively used for exploring numerous bacteria in the context of epidemiological studies and population genetics (Wirth *et al*. 2006; Martino *et al*. 2011; Merga *et al*. 2011; Urwin and Maiden 2010). In this research, MLST has been used to investigate the evolution and diversity of the genus *Cronobacter*.

This research began as an extension of the study conducted by Baldwin *et al.* (2009) which subjected *C. malonaticus* and *C. sakazakii* to the MLST as these two species were indistinguishable by 16S rDNA sequencing. This research was therefore aimed at subjecting all members of *Cronobacter* genus to the MLST while assuring execution of the technique with precision and accuracy.

The multilocus sequencing of the *Cronobacter* strains of bacteria has helped in defining the *Cronobacter* genus and new detection techniques can be assessed on the basis of this definition. This can be of great use to the regulatory authorities and the food industry in ensuring compliance. The seven housekeeping genes used for the MLST of *Cronobacter* were *atpD, fusA, glnS, gltB, gyrB, infB,* and *ppsA.* Researchers have used some of these genes as target genes for phylogenetic and typing studies of other bacteria belonging to *Enterobacteriaceae* (Dauga 2002; Brady *et al*. 2008; Hedegaard *et al*. 1999; Paradis *et al*. 2005; Young and Park 2007). Housekeeping genes are present at seven sites scattered across the genome. This is the reason why combination of these genes offers better sequence diversity than the conventionally used techniques like 16S rDNA sequencing.

The *Cronobacter* strains have been profiled by the 7 loci MLST scheme. These strains were obtained from the outbreaks occurring in USA, Israel and Czech Republic. The *C. sakazakii* ST4 strain has been found to be the predominant strain in the samples taken from the cerebral spinal fluid (Joseph and Forsythe 2011). The *Cronobacter* strains sent to the CDC in 2011 were also profiled by the MLST and some of these cases had received a lot of public attention. The ST4 and *C. sakazakii* clonal complex 4 constituted the CSF strains and this validated the results obtained from previous research work (Hariri *et al.* 2012).

It has been established through microbiological and epidemiological studies that substitutes of breast milk (PIF product) can act as a source of *Cronobacter* infections, although the source of

infection is not confirmed in many *Cronobacter* outbreaks (Caubilla-Barron *et al.* 2007; FAO-WHO 2004, 2008). However, an important consideration usually ignored in this connection is that fact that a non-infant formula (not intended for neonates) was given to those infants in Tennessee (Himelright *et al*. 2001).

It has been reported by Sonbol *et al*. (2013) that *C. sakazakii* clonal complex ST4 constituted 24% of the strains isolated from the manufacturing plants of milk powder located in Germany and Australia. Moreover, *C. sakazakii* ST4 is reported to be linked with meningitis cases and it must not be overlooked that it has been isolated on numerous occasions from PIF processing plants, milk powder factories and PIF in Australia, Germany, Switzerland and Ireland (Sonbol *et al*. 2013; Power *et al*. 2013).

*Cronobacter* infections which have been found directly connected to the consumption of reconstituted PIF might involve extrinsic or intrinsic contamination of the formula during its preparation or administration. Reports indicate that in many of the cases caused by contaminated infant formula, the reconstituted milk was exposed to an inappropriate temperature that allowed growth of any bacteria present in the formula. Moreover, contamination of devices used for administration of milk has also been reported to be involved in causing neonatal infections. In outbreaks that occurred in the United States and France, young infants were fed with the help of perfusion devices. In particular, prepared feed was supplied gradually into the stomach of the neonate via enteral feeding tube at room temperature (Caubilla-Barron *et al.* 2007; Himelright *et al*. 2001). Similarly, bacteria may also grow inside the syringe and feeding tube unit used for administration of infant feed thereby causing huge quantities of bacterial cells to enter the neonatal body.

It is important to consider that the immune system of neonates is not fully developed and their intestinal microflora is also less dense. Hence, if cells of *Cronobacter* enter the neonatal body in huge numbers, the host intestinal flora is unable to manage the invasion efficiently. Once the intestinal cells of the host are invaded by the pathogen, the immature immune system does not offer sufficient protection against systemic infection. The infectious dose for neonates still needs to be determined.

According to Bowen and Bradden (2008), several neonatal cases of *Cronobacter* infection had no connection with consumption of reconstituted infant formula. Several lines of evidence are in agreement with this report. For instance, colonization by bacteria has been detected in nasogastric enteral feeding tubes. Similarly, *C. sakazakii* has been isolated from ready-to-use formula and feeding tubes from neonates fed breast milk (Hurrell *et al*. 2009a). Furthermore, the bacterium may also be present in the breast milk as isolation of the *C. malonaticus* type strain (LMG 23826$^{T}$) has been reported from breast abscess. In two cases of meningitis, breast milk was found to be a suspected source. Neonates, in some states, are still being fed on breast milk of females having mastitis (Stoll *et al*.2004; Holy & Forsythe 2013). In the United States and Israel, *Cronobacter* infections have been reported among infants which have been fed only on breast milk (Block *et al*. 2002).

The throat and intestine of humans, as well as hospital environment have been reported to contain *Cronobacter* species. Hence, risk of *Cronobacter* infection among neonates cannot be completely eliminated by controlling bacteria in PIF.

Intriguingly, the isolation of *C. sakazakii* has been reported from sources like sputum, trachea, feeding tubes for neonates, ready-to-use infant formula and fed breast milk but no report indicates its isolation from infant formula (Holy & Forsythe 2013). For that reason, it is recommended that broad range of possible sources of *Cronobacter* should be studied during an outbreak, instead of focusing only on PIF. The NICU outbreak that occurred in France in 1994 revealed that multiple strains of *Cronobacter* may colonize a baby and hence all isolates should be genotyped for epidemiological studies so that the sources involved can be traced. (Caubilla-Barron *et al.* 2007).

Adults are the common victims of *Cronobacter* infections which are mainly caused by *C. malonaticus* (Joseph *et al*. 2012b; Kucerova *et al.* 2011). Since the bacterium is frequently present in food, infection is likely to occur through intake of contaminated food. Still, a nasopharyngeal source like that involved in *N. meningitdis* is also possible and this justifies the isolation of bacteria from sputum of pneumonia patients.

### 3.5.1 CLONALITY

Relatedness among different STs of *Cronobacter* has been analysed during this research using the goeBURST algorithm in PHYLOViZ (Francisco *et al*. 2012). It can be seen in the diagram Figure 3.2, that the STs differ in 1, 2 or 3 of the seven loci corresponding to single locus variant (SLV), double (DLV) and triple locus variant (TLV) respectively. ST4 and ST107, for instance, differ only in the *fusA* allele (i.e 5-1-3-3-5-5-4 and 5-50-3-3-5-5-4); therefore they are regarded as single locus variants.

The *C. sakazakii* STs 4, 110, 109, 107 and 108 are in clonal complex 4 which is a major clonal lineage when one considers the epidemiology of *Cronobacter* species. ST4 has not only proven to be the most common clinical ST, but also the most dominant one (see Table 3.2 and Baldwin *et al*. 2009). Still, the term 'clinical' is not specifically descriptive when it comes to the source of strains. These isolates may not necessary reside within the site of infection. For instance, they can be found in conjunctivae swabs in meningitis case patients. Some have been isolated from asymptomatic persons as well. Fortunately, substantial data was at hand for these clinical isolates to disclose what is potentially the most important information brought to light by the MLST analysis related to the trophism and epidemiology of *Cronobacter* infection in neonates. Researchers regard CC4 as the genetic signature for *C. sakazakii* meningitis affecting neonates as most of the isolates have been found to be linked with meningitis patients from six different states during the past 50 years (Joseph and Forsythe. 2011; Hariri *et al*. 2012). Of the sixty-four clinical isolates which have been studied in this research, twenty-nine are *C. sakazakii* ST4 and loci variants (STs 109, 107, 108, and 110). Our knowledge regarding reasons for the predominance of CC4 in patients of neonatal meningitis is still deficient and may depend on the role played by virulence traits and environmental fitness factors. Another possible explanation for the lack of reports of meningitis in adults is because of the blood brain barrier's maturity.

Two ST4 SLVs were found in the fifteen 2011 US strains. Strain 1572 (ST108) demonstrated variation from ST4 profile in the *fusA* loci by 5/438 nt. This strain was isolated from an opened PIF tin. The CSF strain 1565 (ST107) demonstrated variation from the ST4 profile in *fusA* loci by

6/438 nt. Moreover, ST107 and ST108 differ from each other only by 1 nt out of 3036 (concatenated length) in the *fusA* loci position 378 (A: T). The PFGE could not detect this small difference. It is worth mentioning that the bacterial genome is analysed through the MLST and PFGE in different ways. Moreover, the seven MLST loci do not contain any XbaI sites which are the sites for the activity of endonucleases that are employed during the PFGE of *Enterobacteriaceae*.

The *C. malonaticus* ST7 is the dominant sequence type and comprises of several strains isolated during last 30 years from clinical and PIF cases. ST7 includes strains 1826-1835, 1914, 1917 isolated from clinical cases in the Czech Republic. These were detected in sputum, stool dissection, cannula, nose, wound and throat swabs. As per the available data, the majority of these strains were isolated from individuals who were not infants.

The *C. malonaticus* CC112 1569 strain also belongs to the studied 2011 US isolates and was isolated from blood specimen of the infant (less than one month) who died of meningitis. This strain is also of immense importance in this study since until that time *C. malonaticus* was found to mainly cause infections among adults and none of the neonatal meningitis cases were found to be caused by this species (Joseph and Forsythe. 2011). It shows that substantial brain damage can be caused by non-clonal complex 4 strains as well, though rarely.

It can therefore be stated that the MLST technique is an efficient and vigorous typing method for the genus *Cronobacter* as it has demonstrated an increased degree of discrimination between different clinical strains.

Owing to its convenient execution and inexpensive methodology, the MLST has turned into a preferred typing technique as compared to other techniques calling for greater time and effort like the PFGE. Moreover, the enormous amount of data acquired from gene sequencing has facilitated the determination of the substantial diversity demonstrated by the genus *Cronobacter* and also the identification of inter-species evolutionary relationships.

Since control of *C. sakazakii* ST4 can lead to considerable reduction in neonatal fatal infections, identification of different sources of this strain is highly important. Careful evaluation of neonatal

exposure allows in depth study of prevalence of *Cronobacter* species, especially ST4 in healthcare settings, PIF and other sources.

In summary;

The *Cronobacter* MLST scheme is based on the seven genes *atpD, fusA, glnS, gltB, gyrB, infB* and *ppsA*. The expansion of the MLST scheme across the entire genus and subsequent sequence analysis has contributed to a number of key aspects of the genus:

- Revealed the phylogenetic relationships and diversity between the species.
- Revealed the strong clonality of the *C. sakazakii* and *C. malonaticus* species.
- Identified a clonal lineage for a majority of the neonatal meningitic cases – the *C. sakazakii* ST4 clonal complex.

# CHAPTER 4

# SIALIC ACID UTILIZATION AND ITS ROLE IN BACTERIAL PATHOGENICITY

## 4.1 INTRODUCTION

### 4.1.1 SIALIC ACID UTILISATION AND ITS ROLE IN BACTERIAL PATHOGENICITY

Sialic acid or neuraminic acid refers to a family of nine-carbon keto sugars that may be found on mammalian mucosal surfaces such as the intestinal gut lining, brain, secretions of the mouth and lungs as well as milk. These are sites colonized by a wide range of bacteria for which this sialic acid can act as an attachment site and a source of carbon and nitrogen (Wang 2009).

Around fifty different forms of sialic acid have been identified and the 2-keto-3-deoxy-5-acetamido-D-glycero-D-galacto-nonulosonic acid is the most researched form among these. It is mostly known by its abbreviation Neu5Ac. In general, sialic acid exists attached to sugars forming polysaccharides, though it can also be found attached to proteins or lipids forming sialo-glycoconjugates. Except in a small number of eukaryotes, numerous eukaryotic lineages lack conjugates of sialic acid including the majority of protostomes, protists, fungi and plants. According to a proposition, the evolution of sialic acid production took place in animals. Later this process evolved in bacterial commensals and pathogens either through horizontal gene transfer or convergent evolution. Several mechanisms have been evolved in microorganisms which involve utilization of sialic acids as a target for mimicry, degradation and adherence (Severi *et al*. 2007; Almagro-Moreno and Boyd 2009).

*NanH* gene is present in some bacteria enabling them to synthesize sialidase or neuraminidase which brings about the release of sialic acid by cleaving it from glycoconjugate forms. Studies indicate low homology (less than 30%) of *nanH* gene across different groups of bacteria and several organisms have been found to lack this gene (Roggentin *et al*. 1993). Neonatal meningitic *E. coli* K1 is one of those strains which lack sialidase enzyme though it can grow on sialic acid as acarbon source. It is possible that these bacteria exploit the sialidase activity of other bacteria present in the surroundings or that of host cells which produce this enzyme in inflammatory conditions (Severi *et al*. 2009).

A porin present in outer membrane of Gram-negative bacteria namely, NanC, is responsible for the uptake of sialic acid. In the case of inner membrane, three different transporters may be present namely ATP-binding cassette (ABC) transporter, TRAP and NanT. TRAP refers to a tripartite ATP-independent periplasmic transport system and NanT refers to a major facilitator superfamily (MFS) protein. The literature indicates that the single-component NanT transport system is present in all *Enterobacteriaceae* researched so far (Severi *et al*. 2007; Almagro-Moreno and Boyd 2009; Vimr 2012).

As soon as the sialic acid enters the cell, it gets converted into phosphoenolpyruvate (PEP) and N-acetylmannosamine (ManNAc) by the Neu5Ac lyase (NanA). ManNAc is acted upon by NanK which is an ATP-dependent kinase and this activity results in the production of N-acetylmannosamine-6-phosphate (ManNAc-6-P) which in turn is converted into ManNAc-6-P into N-acetylglucosamine-6-phosphate (GlcNAc-6-P) by epimerase (NanE). This is followed by conversion of GlcNAc-6-P by glucosamine-6-P deaminase (NagB) and GlcNAc-6-P deacetylase (NagA) resulting in formation of fructose-6-phosphate which gets consumed in the glycolytic pathway (Figure 4.1). According to Almagro-Moreno and Boyd (2009), the activity of these genes is regulated by NanR which is basically a repressor. Genes encoding the initial 3 enzymes (nanA, nanK and nanE) are mostly present in the form of gene cluster namely *nan* gene cluster. Still, a few exceptional cases have been reported by Vimr (2013). These include *Edwardsiella tarda* and *Citrobacter freundii* and in these bacteria, the *nanE* gene is found to be present in an area separate from the operon. Furthermore, the *nagA* and *nagB* genes are present adjacent to each other though in majority of the bacteria they are not found in close proximity with the *nan* gene cluster.

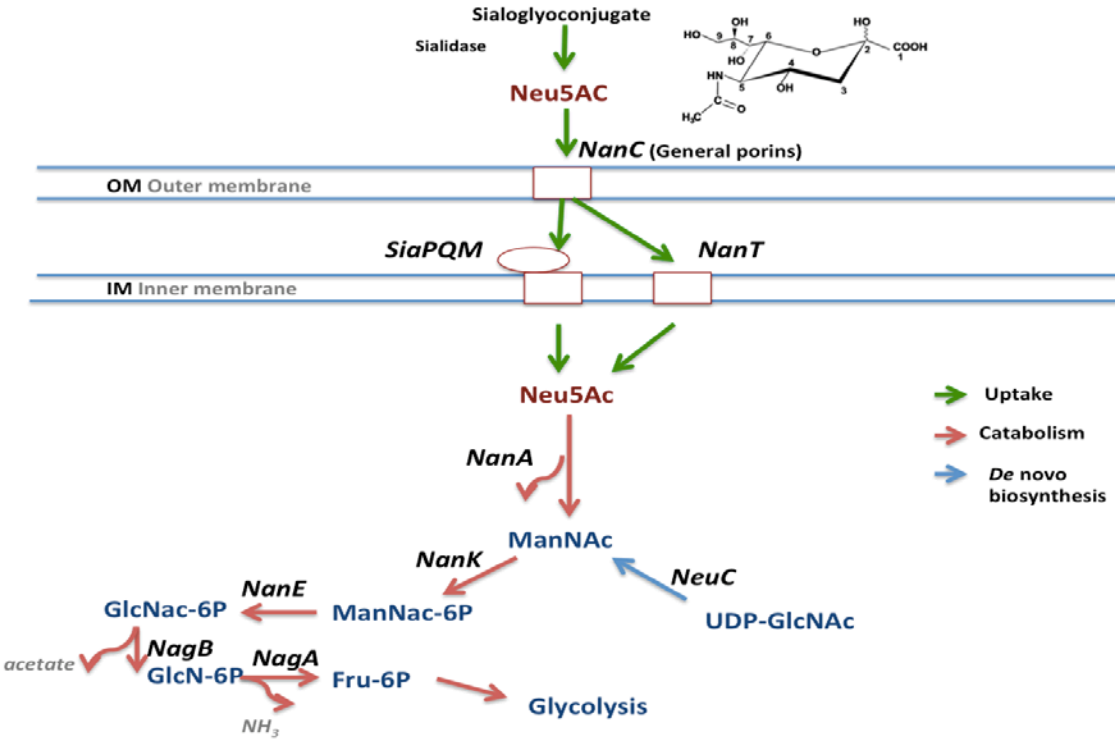


**Figure 4.1** Sialic acid utilization in *Cronobacter sakazakii*.

Vimr (2012) reports an association between uptake of sialic acid and several virulence factors of pathogenic bacteria. For instance, numerous bacteria produce glycolipid capsule in order to evade the host's immunity. Same is the case with neonatal meningitic *E. coli* K1 which utilizes sialic acid to alter its cell surface. Similarly, *Cronobacter* forms a capsule, particularly when cultured on milk agar.

Genes encoding the enzymes for degradation of N-acetylmannosamine and N-acetylneuraminate (*nanKTAR* genes) and the gene cluster responsible for production of a presumed sugar isomerase (*yhcH*) are found to be present at ESA_03609-13 as reported in the past for the *C. sakazakii* BAA-894 (Joseph *et al*. 2012a and 2012b). The rest of the *nan* genes involved in the metabolism of sialic acid still need to be investigated in detail as the bioinformatic studies conducted in past were not successful in identification of any sialidase (*nanH*) gene candidate in *Cronobacter sakazakii*

(Joseph *et al*. 2012b).

A number of infections among low birth weight neonates are caused by *C. sakazakii* and the ability of the organism to grow on sialic acid may be associated with its ability to cause infection. Sialic acid is found in human milk in the form of sialyloligosaccharides and these are present in greatest quantities in colostrums (Wang 2009).

At three of the four lactation stages, mothers of preterm infants produce milk with 13-23% greater quantity of sialic acid as compared to mothers of full-term infants. Sialic acid attached with glycoproteins is also present in infant formulas. Even though the nutritional value of sialic acid still needs to be investigated, it is quite possible that it has a role in the build-up of sialic acid in the brain as an essential part of ganglioside. Wide range of oligosaccharides, lactoferrin, secretory IgA and sialoglycans are also present in breast milk. Oligosaccharides present in breast milk are almost undigestable and hence metabolized by intestinal bacteria thereby encouraging the growth of bacteria in the intestine. Microvilli in the intestine of neonates contain greater quantities of N-acetylglucosamine residues and sialic acid. Conversely, increased quantities of fructose, glucose and mannose residues are present in the case of adults. These microvilli act as site of attachment for bacteria (Sprenger and Duncan 2012; Lewis and Lewis 2012).

According to Wang (2009), the main site of sialic acid in the form of gangliosides (sialylated glycolipids) is the brain. It is therefore possible that the structural and functional establishment of synaptic pathways involve sialic acid. Moreover, *C. sakazakii* might have a developmental reliance on access to the CNS just like the *H. influenzae*, *S. pneumoniae* and *N. meningitidis* that cause meningitis in children younger than five years.

## 4.1.2 AIMS OF THE CHAPTER

The *Cronobacter* genus is composed of seven species and the most severe fatal cases have been reported in infants and neonates (Hariri *et al.* 2013). These bacteria have been known to cause necrotizing enterocolitis and extremely destructive type of meningitis that involves bacteria crossing the blood brain barrier resulting in abscess formation in brain cavity. Multilocus sequence typing has been used to describe the diversity of the genus in previous studies by Joseph *et al*. (2012a and 2012b). Evolutionary analysis suggests that the *C. sakazakii* species separated from the rest of the *Cronobacter* genus 15–23 million years ago (MYA) (Joseph *et al*. 2012). Previous whole genome studies revealed that *C. sakazakii* was the only *Cronobacter* species that has the *nanAKT* gene cluster encoding for sialic acid utilization (Kucerova *et al*. 2010; Joseph *et al*. 2012c). It is plausible that this metabolism may account for the predominance of *C. sakazakii* in neonatal and infant infections. Prior to the study reported here no laboratory studies have been published investigating the growth of *Cronobacter* on sialic acid nor have there been reported of sialidase activity.

This chapter will describe the variation in growth by members of the *Cronobacter* genus on sialic acid, genomic structure and the variation in the gene content of *Cronobacter* associated with sialic acid utilization. Part of results presented in this chapter have been accepted for publication; Joseph *et al*. (2013).

## 4.2 MATERIALS AND METHODS

The key methods, culture media and culturing condition for this section were described previously in Chapter 2 Materials and Methods section.

## 4.3 BACTERIAL STRAINS LIST IN THIS STUDY

| Isolate | Species | Sequence Type [a] | Country | Source | Year |
|---|---|---|---|---|---|
| 658*[b] | *C. sakazakii* | ST1 | USA | Non-infant formula | 2001 |
| 716 | *C. sakazakii* | ST14 | France | Infant formula | 1994 |
| 978 | *C. sakazakii* | ST3 | UK | Clinical | 2007 |
| 984 | *C. sakazakii* | ST3 | UK | Clinical | 2007 |
| 553 | *C. sakazakii* | ST4 | Netherlands | Clinical | 1977 |
| 557 | *C. sakazakii* | ST4 | Netherlands | Clinical | 1979 |
| 558 | *C. sakazakii* | ST4 | Netherlands | Clinical | 1983 |
| 695 | *C. sakazakii* | ST4 | France | Clinical | 1994 |
| 701* | *C. sakazakii* | ST4 | France | Clinical | 1994 |
| 709 | *C. sakazakii* | ST4 | France | Clinical | 1994 |
| 767 | *C. sakazakii* | ST4 | France | Clinical | 1994 |
| 6 | *C. sakazakii* | ST4 | Canada | Clinical | 1990 |
| 20 | *C. sakazakii* | ST4 | Czech Republic | Clinical | 2003 |
| 377 | *C. sakazakii* | ST4 | UK | Milk powder | 1950 |
| 1105 | *C. sakazakii* | ST4 | UK | Weaning food | 2008 |
| 4 | *C. sakazakii* | ST15 | Canada | Clinical | 1990 |
| 12 | *C. sakazakii* | ST1 | Czech Republic | Clinical | 2004 |
| 150 | *C. sakazakii* | ST16 | Korea | Spice | 2005 |
| 680* | *C. sakazakii* | ST8 | USA | Clinical | 1977 |
| 1* | *C. sakazakii* | ST8 | USA | Clinical | 1980 |
| 5* | *C. sakazakii* | ST8 | Canada | Clinical | 1990 |
| 520 | *C. sakazakii* | ST12 | Czech Republic | Clinical | 1983 |
| 690 | *C. sakazakii* | ST12 | France | Clinical | 1994 |
| 696* | *C. sakazakii* | ST12 | France | Clinical | 1994 |
| 693 | *C. sakazakii* | ST13 | France | Clinical | 1994 |
| 681[C] | *C. malonaticus* | ST7 | USA | Clinical | 1977 |
| 510 | *C. malonaticus* | ST7 | Czech Republic | Food | 1985 |
| 507* | *C. malonaticus* | ST11 | Czech Republic | Clinical | 1984 |
| 564* | *C. turicensis* | ST5 | USA | Clinical | 1970 |
| 581* | *C. universalis* | ST54 | UK | Water | 1956 |
| 721 | *C. sakazakii* | ST4 | USA | Clinical | 2003 |
| 92 | *C. turicensis* | ST35 | UK | Herb | 2004 |
| 1218 | *C. sakazakii* | ST1 | USA | Clinical | 2001 |
| 1219 | *C. sakazakii* | ST4 | USA | Clinical | 2009 |
| 1211*[d] | *C. turicensis* | ST19 | Switzerland | Clinical | 2005 |
| 1249 | *C. sakazakii* | ST31 | UK | Clinical | 2010 |

| | | | | | |
|---|---|---|---|---|---|
| 1330*[e] | *C. condimenti* | ST40 | Slovakia | Food | 2010 |
| 1220* | *C. sakazakii* | ST4 | USA | Clinical | 2003 |
| 1221* | *C. sakazakii* | ST4 | USA | Clinical | 2003 |
| 1231* | *C. sakazakii* | ST4 | New Zealand | Clinical | 2005 |
| 1240 | *C. sakazakii* | ST4 | USA | Clinical | 2009 |
| 1225* | *C. sakazakii* | ST4 | USA | Clinical | 2007 |
| 140 | *C. sakazakii* | ST40 | India | Spice | 2005 |
| 582* | *C. dublinensis* | ST36 | UK | Unknown | - |
| 685 | *C. malonaticus* | ST53 | USA | Clinical | 1977 |
| 1210 | *C. dublinensis* | ST106 | Ireland | Environment | 2004 |
| 583 | *C. dublinensis* | ST91 | UK | Environment | 1956 |
| 687 | *C. malonaticus* | ST60 | Czech Republic | Clinical | 2004 |
| 694 | *C. sakazakii* | ST4 | France | Clinical | 1994 |
| 708 | *C. sakazakii* | ST12 | France | Clinical | 1994 |
| 711 | *C. sakazakii* | ST7 | France | Clinical | 1994 |
| 712 | *C. sakazakii* | ST4 | France | Infant formula | 1994 |
| 1545 | *C. malonaticus* | ST84 | Czech Republic | Clinical | - |
| 1553 | *C. turicensis* | ST85 | Slovakia | Unknown | - |
| 1554 | *C. turicensis* | ST87 | Slovakia | Unknown | - |
| 700 | *C. sakazakii* | ST86 | France | Clinical | 1994 |
| 1556 | *C. dublinensis* | ST88 | USA | Clinical | 1979 |
| 1558 | *C. malonaticus* | ST89 | Czech Republic | Clinical | - |
| 1560 | *C. dublinensis* | ST92 | Czech Republic | Food | - |
| 1533 | *C. sakazakii* | ST4 | Germany | Environment | 2006 |
| 1536 | *C. sakazakii* | ST1 | Germany | Environment | 2009 |
| 1537 | *C. sakazakii* | ST4 | Germany | Environment | 2009 |
| 1542 | *C. sakazakii* | ST4 | Germany | Environment | 2009 |
| 691 | *C. sakazakii* | ST4 | France | Clinical | 1994 |
| 692 | *C. sakazakii* | ST4 | France | Clinical | 1994 |
| 698 | *C. sakazakii* | ST4 | France | Clinical | 1994 |
| 699 | *C. sakazakii* | ST1 | France | Clinical | 1994 |
| 702 | *C. sakazakii* | ST4 | France | Clinical | 1994 |
| 703 | *C. sakazakii* | ST12 | France | Clinical | 1994 |
| 705 | *C. sakazakii* | ST4 | France | Clinical | 1994 |
| 706 | *C. sakazakii* | ST4 | France | Clinical | 1994 |
| 707 | *C. sakazakii* | ST4 | France | Clinical | 1994 |
| 713 | *C. sakazakii* | ST13 | France | Infant formula | 1994 |
| 714 | *C. sakazakii* | ST13 | France | Infant formula | 1994 |
| 715 | *C. sakazakii* | ST13 | France | Infant formula | 1994 |
| 730 | *C. sakazakii* | ST4 | France | Clinical | 1994 |
| 1569 | *C. malonaticus* | ST112 | USA | Clinical | 2011 |
| 1587* | *C. sakazakii* | ST4 | Israel | Clinical | 2000 |
| ES15 | *C. sakazakii* | ST125 | Korea | Whole grain | - |
| 1846 | *C. malonaticus* | ST60 | Czech Republic | Ingredient | 2010 |

| | | | | | |
|---|---|---|---|---|---|
| 1880 | *C. turicensis* | ST262 | Czech Republic | Herb | 2011 |
| SP291 | *C. sakazakii* | ST4 | Ireland | Infant formula factory | - |
| Sc-1383$^{T}$*$^{F}$ | *Siccibacter colletis* | ST227 | UK | Ingredients | 2011 |
| Fh-1387* | *Franconibacter helveticus* | ST298 | UK | Spice | 2011 |
| Fh-1392* | *Franconibacter helveticus* | ST229 | UK | Ingredients | 2011 |
| St-1974$^{T}$*$^{G}$ | *Siccibacter turicensis* | ST216 | Switzerland | Fruit powder | 2007 |
| Fh-1975$^{T}$*$^{H}$ | *Franconibacter helveticus* | ST217 | Switzerland | Fruit powder | 2007 |
| Fh-1204* | *Franconibacter helveticus* | ST217 | Jordan | Follow up formula | 2009 |
| Fh-1208* | *Franconibacter helveticus* | ST217 | Portugal | Follow up formula | 2009 |
| Fp-1978* | *Franconibacter pulveris* | ST215 | Switzerland | Infant formula | 2008 |
| Ck-BAA-895 | *Cit.koseri* | ST6 | US | | - |
| LMG23826 | *C. malonaticus* | ST7 | USA | Clinical | 1977 |
| ES713 | *C. sakazakii* | ST218 | USA | Infant formula | - |
| ES35 | *C. sakazakii* | ST8 | Israel | Clinical | - |
| G-2151 | *C. sakazakii* | ST4 | USA | Clinical | - |
| E764 | *C. sakazakii* | ST12 | USA | Clinical | - |
| LMG 23823 | *C. dublinensis* | ST106 | Ireland | Environment | 2004 |
| LMG 23824 | *C. dublinensis* | ST80 | Switzerland | Water | 2004 |
| LMG 23825 | *C. dublinensis* | ST79 | Zimbabwe | Environment | 2003 |
| ATCC 51329 | *C. muytjensii* | ST81 | Unknown | Unknown | - |
| NCTC9529$^{T}$ | *C. universalis* | ST54 | UK | Water | 1956 |
| Fp-G-601/05$^{T}$ | *Franconibacter pulveris* | ST232 | Switzerland | Fruit powder | 2007 |
| Fp-G-1160/04 | *Franconibacter pulveris* | ST231 | Switzerland | Fruit powder | 2007 |
| St-z610 | *Siccibacter turicensis* | ST216 | Unspecified | - | 2007 |
| Fh-1159 (LMG23733) | *Franconibacter helveticus* | - | Switzerland | Fruit powder | 2004 |
| 2089 | *C. sakazakii* | ST1 | France | Clinical | 2004 |
| 2106 | *C. sakazakii* | ST257 | Belgium | Clinical | - |
| 2107 | *C. sakazakii* | ST12 | Belgium | Clinical | - |
| Fp-1991* | *Franconibacter pulveris* | ST232 | UK | Food | 2013 |
| CMCC 45402 | *C. malonaticus* | ST7 | China | Milk | - |
| 2109 | *C. malonaticus* | ST300 | Canada | Unknown | - |
| 530* | *C. muytjensii* | ST49 | Denmark | - | - |
| 2030 | *C. dublinensis* | ST301 | France | - | - |
| 2045 | *C. malonaticus* | ST302 | France | Environmental | - |
| 2046 | *C. malonaticus* | ST302 | France | Environmental | - |
| 2051 | *C. sakazakii* | ST64 | France | Environmental | - |
| HPB5174 | *C. sakazakii* | ST40 | Ireland | Environment | - |
| 2048 | *C. sakazakii* | ST8 | France | Environmental | - |
| 2064 | *C. sakazakii* | ST1 | France | Environmental | - |
| 2087 | *C. sakazakii* | ST100 | France | Environmental | - |
| 2161 | *C. sakazakii* | ST297 | Mexico | Environmental | 2010 |
| NBRC 102416$^{T}$ | *C. sakazakii* | ST8 | Japan | Clinical | 1980 |

| | | | | | |
|---|---|---|---|---|---|
| 48* | *Cit.koseri* | - | Unknown | Clinical | - |
| 1926* | *Ed. Tarda* | - | Unknown | Unknown | - |
| 1927* | *Cit.freundii* | - | Unknown | Unknown | - |

**Table 4.1** List of *Cronobacter* spp. and related *Enterobacteriaceae* isolates included in this study

[a] Sequence type as according to the *Cronobacter* genus multilocus sequence typing scheme database; http://www.pubMLST.org/cronobacter.
*Bacterial strains used for laboratory studies of growth on sialic acid, GM1 and mucin as sole carbon source.
[b]*C. sakazakii* species type strain ATCC29544$^T$.
[c] *C. malonaticus* species type strain LMG 28327$^T$.
[d] *C.turicensis* species type strain LMG 28327$^T$.
[e] *C. condimenti* species type strain.
[F]*Siccibacter colletis* species type strain
[G]*Siccibacter turicensis* species type strain
[H]*Franconibacter helveticus* species type strain.

## 4.4 RESULT

### 4.4.1 GROWTH OF *CRONOBACTER* AND CLOSELY RELATED SPECIES ON SIALIC ACID, GM1 AND MUCIN

The utilization pathway for sialic acid was confirmed through two stages in this study. Firstly 19 *Cronobacter* strains were selected which represented the seven recognized species, and included those from reported clinical cases and species type strains ;1[b],5,658,680,696,701,1220,1221,1225,1231,1587,507,681[c],581,564,1211[d],530,582,1330[e], Table 4.1.

In order to verify whether these strains encoded the *nan* cluster have the capability to grow using sialic acid, ganglioside or mucin as a carbon source, the growth study have been performed by measuring the absorbance of the cultures at 595 nm using a plate reader on minimal media supplemented with A (M9+sialic acid), B (M9+ganglioside), and C (M9+mucin). The data obtained was exported to an excel sheet and the growth curve was made using Graphpad prism.

Eleven strains of *C. sakazakii* grew in minimal medium (M9) with sialic acid**,** monosiaganglioside GM1 and mucin as the only carbon source suggesting that they may have sialidase activity as shown in Figure 4.2. None of other *Cronobacter* species demonstrated growth in these media. *Cronobacter turicensis* (1211[d]) and *Citrobacter. koseri* (48) were used as negative and positive control respectively. This work has been published (Joseph *et al*. 2013).

During the late steps of the current PhD, whole genomes have been studied to advance our understanding of the *Cronobacter* genus (http://www.pubMLST.org/cronobacter). As a result, some of *Cronobacter turicensis* strains appear to encode the nanKTAR cluster. For this reason the next step was to examine the growth of all the *Cronobacter turicensis* strains in our database. Ten out of 24 (42%) have been growing in all the M9 supplemented with sialic acid substrates, showed in the maximum likehood tree of *C. turicensis*; Figure 4.3.

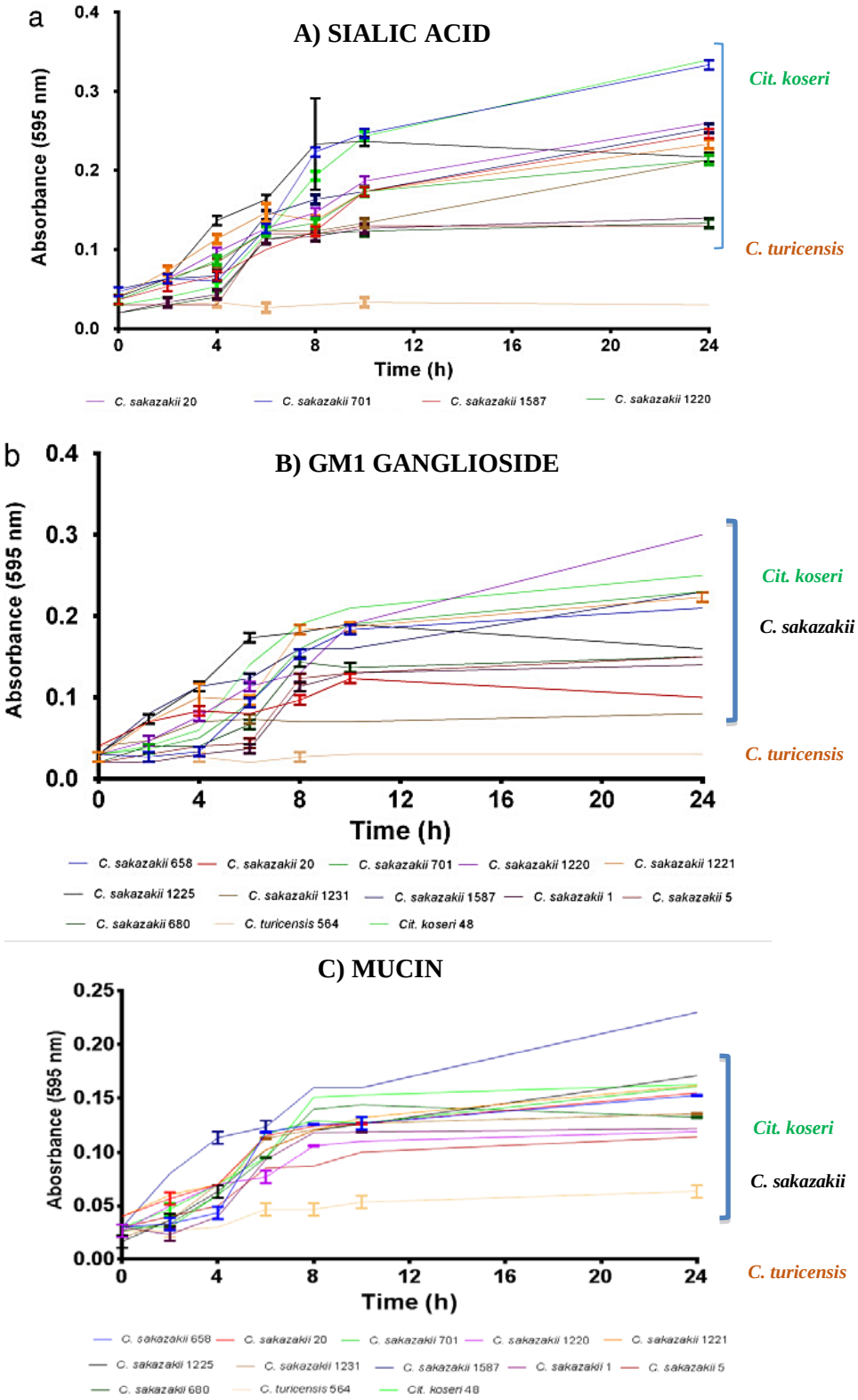


**Figure 4.2** Growth of *Cronobacter* and closely related species in M9 minimal medium supplemented with a) sialic acid, b) GM1 ganglioside and c) Mucin as sole carbon source. Eleven strains of *C. sakazakii* grew in minimal medium (M9) with sialic acid**,** monosiaganglioside GM1 and mucin as the only carbon source suggesting that they may have sialidase activity. None of other *Cronobacter* species demonstrated growth in these media. *Cronobacter turicensis* (1211) and *Citrobacter. koseri* (48) were used as negative and positive control respectively.

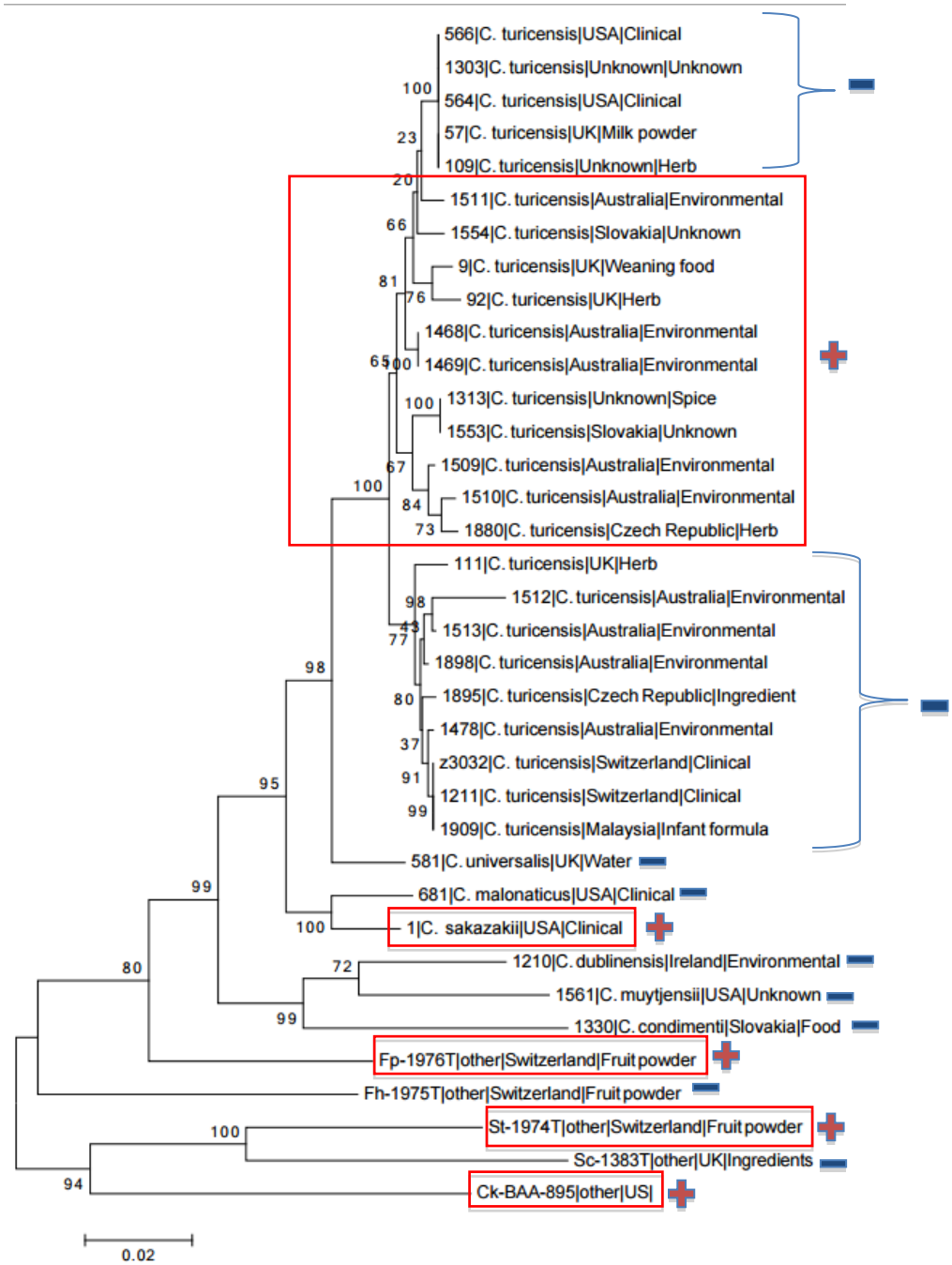


**Figure 4.3** Maximum likehood tree of all *Cronobacter* spp., closely related *Enterobacteriaceae* and *C. turicensis* particularly obtained from http://www.pubMLST.org/cronobacter. Eleven out of 24 (45%) have been growing in all the M9 supplemented with sialic acid substrates. The NTU strains IDs are showed at the top of each branches, the tree is drawn to scale using MEGA5, with 1000 bootstap replicates. This tree based on the concatenated sequences (3,036 bp) of the seven MLST loci.

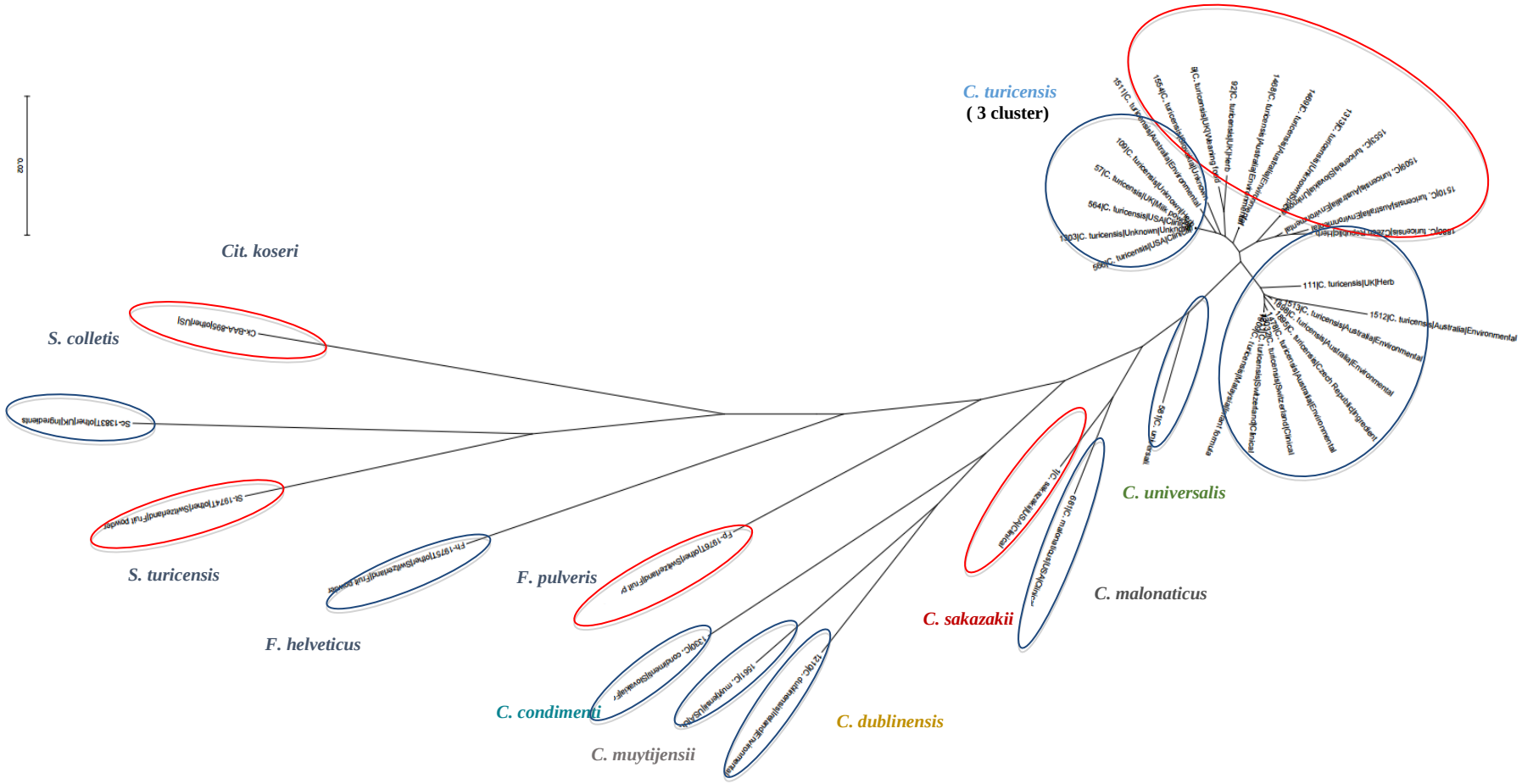


**Figure 4.4** Maximum Likelihood tree all *Cronobacter* spp., closely related *Enterobacteriaceae* and *C. turicensis* particularly based on the concatenated sequences (3,036 bp) of the seven MLST loci. The phylogeny indicated 3 clusters in *C. turicensis*, the red and blue circle indicated the positive and negative species for utilizing sialic acid respectively. The tree is drawn to scale using MEGA, with 1000 bootstrap

### 4.4.2 GENOME STRUCTURE OF POSITIVE SIALIC ACID UTILIZATION GENOMES *CRONOBACTER* SPP. AND CLOSELY RELATED SPECIES OF *ENTEROBACTERIACE*AE.

The Maximum Likelihood tree based on the concatenated sequences (3,036 bp) of the seven MLST loci was used to analyse the variation within *Cronobacter* spp. and closely related *Enterobacteriaceae*. The red and blue circle indicated the positive and negative species utilizing sialic acid respectively (Figure 4.4).

Initially, the genome of *C. sakazakii* 658 was selected since it was the first complete *C. sakazakii* genome available publically (Kucerova *et al*. 2010). The comparison of isolate *C. sakazakii* 658 against *C. turicensis* and closely related *Enterobacteriaceae* (*E. coli, Cit. koseri, F. pulveris* and *S. turicensis*) positive to utilize sialic acid was undertaken using Artemis comparison tool (Carver *et al*. 2005) to consider if the sialic acid utilization cluster genes were acquired or lost in *Cronobacter* as a result of genome evolution.

Figure 4.5 showed the genomic structure of cluster NanKTAR encodings for the proteins involved in the uptake and utilization of exogenous sialic acid to the genomes of *C. sakazakii* BAA-894 (Joseph *et al*. 2013) , *C. turicensis* and closely related *Enterobacteriaceae.* Also the high degree of colinearity of the alignment between different genomes have been shown. Furthermore, the whole cluster is located in a certain location flanked by some conserved housekeeping trait (*gltB*) and starvation gene (*sspA*) thereby suggesting loss from other *Cronobacter* spp. instead of separate acquisition events.

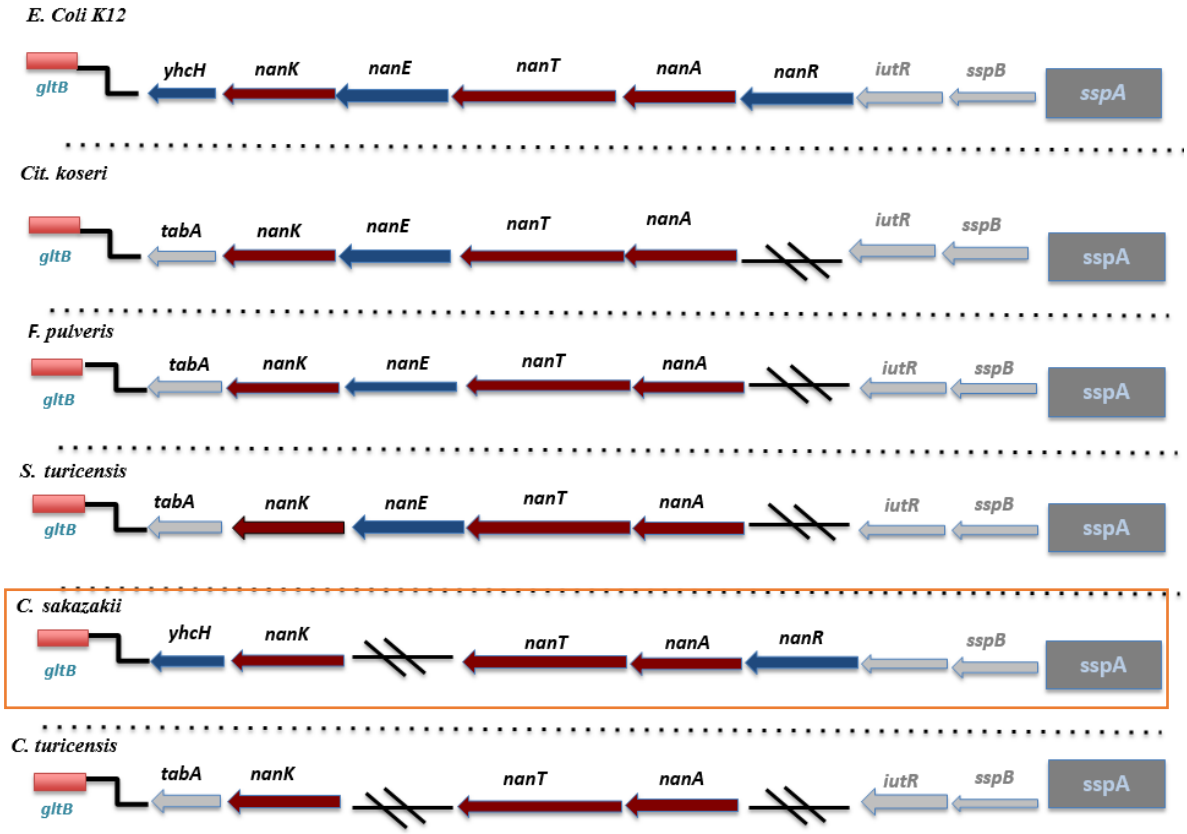


**Figure 4.5** shows the genomic structure of cluster nanKTAR encoding for the proteins involved in the uptake and utilization of exogenous sialic acid to the genomes of *C. sakazakii* BAA-894, *C. turicensis* and closely related *Enterobacteriaceae*. The high degree of colinearity of the alignment between different genomes have been indicated in the red arrow. The whole cluster is located in a certain location flanked by the conserved housekeeping trait (*gltB*) and starvation gene (*sspA*)

### 4.4.3 DISTRIBUTION OF SIALIC ACID UTILIZATION GENES

To provide a platform to confirm the findings of the current study, all of sialic acid metabolism genes in *Cronobacter* and other closely related *Enterobacteriaceae* have been studies (Figure 4.6) by using BLAST facility (http://pubmlst.org/perl/bigsdb/bigsdb.pl?db=pubmlst_cronobacter_isolates&page=plugin&name=BLAST).

One of the interesting gene clusters, unique to the genomes of *C. sakazakii* is the loci cluster ESA_03609–13 *C. sakazakii* BAA-894 loci number. This cluster encodes for the proteins involved in the uptake and utilization of exogenous sialic acid.

- A putative sugar isomerase (YhcH) ESA_03609
- *NanKTAR* genes involved in the N-acetylneuraminate and N-acetylmannosamine degradation pathway ESA_03610-13.
- N-acetylmannosamine kinase *nanK* gene (ESA_03610)
- Sialic acid permease transporter *nanT* (ESA_03611)
- N-acetylneuraminate lyase *nanA* (ESA_03612)
- Transcriptional regulator from the GntR family *nanR* (ESA_03613).

The *nanE* locus encoding the enzyme N-acetylmannosamine-6-phosphate-2epimerase was located separate from this cluster at ESA_00529, and unlike the rest was found conserved across the genomes of the *Cronobacter* genus. For that reason an additional *nanE* locus from *Cit. koseri* (CKO_04626), *Escherichia coli* str. K-12 (Y75_P3143) and *Edwardella tarda* (ETAF_0472) have been used for comparative purposes.

ESA_03302, encoding NanC was another locus involved in this metabolic cycle – the N-acetylneuraminic acid outer membrane channel protein. This was found to be unique to the genomes of *C. sakazakii*. ESA_02661 (nagB), ESA_02662 (nagA) and ESA_03772 (neuC) were also essential to the sialic acid metabolism cycle. They were found preserved across the genomes of the *Cronobacter* genus and are located neighboring to each other in all the genomes.

Essentially *nanAKRT* and *yhcH* were only found in the genomes of 100 % (n= 73) *C. sakazakii*, 66 % (n=6) of *C.turicensis* and none in the other *Cronobacter* species. These genes were also found in *Franconibacter pulveris* (n=4) and *Siccibacter turicensis* (n=3), and were not found in the related *Enterobacteriaceae* members *Franconibacter helveticus* or *Siccibacter colletis*. *NeuC* encoding for UDP-N-acetylglucosamine 2-epimerase was found in all *Cronobacter* and related species.

All *C. sakazakii* strains can hypothetically transport the exogenous sialic acid into the cytoplasm of their cells. Interestingly, all the *Cronobacter* species genomes also showed the presence of the genes (*siaPQM*) encoding for the TRAP transporter.

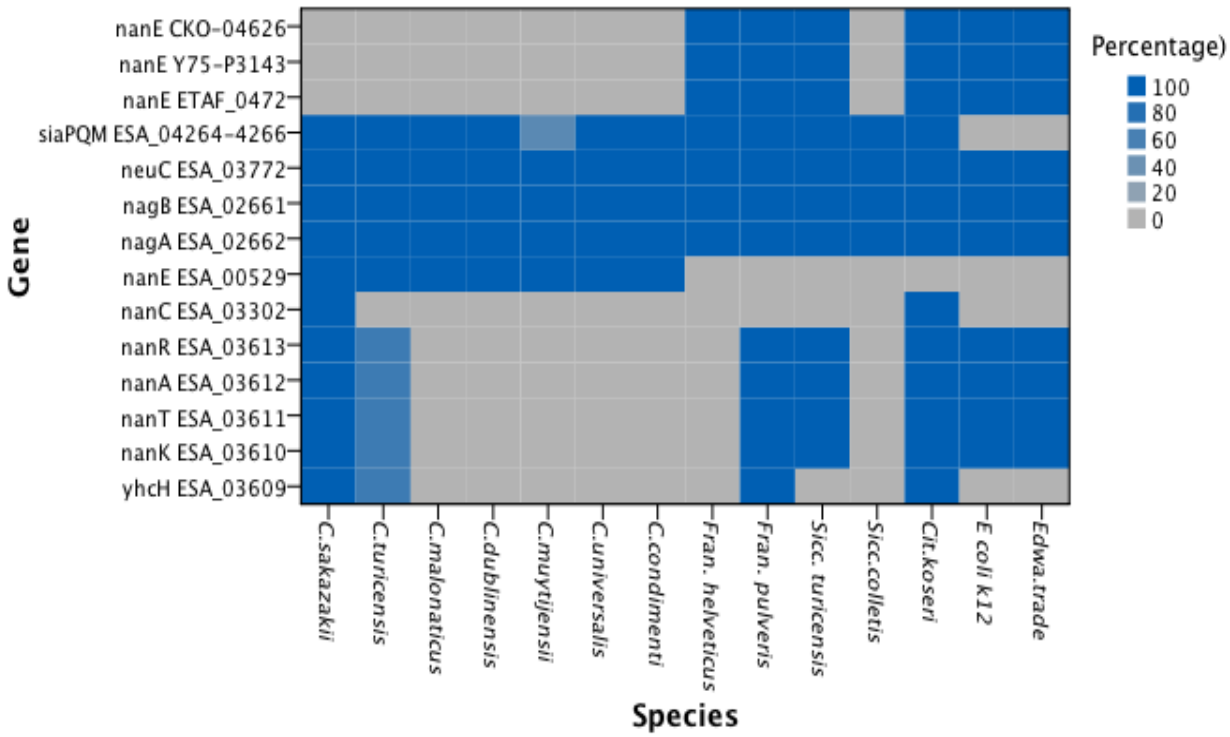


**Figure 4.6** Distribution of the sialic acid utilisation and other related genes across the sequenced genomes of the *Cronobacter* genus and closely related species.

### 4.4.4 % GC CONTENT OF SIALIC ACID UTILIZATION GENES

The GC % content analysis of each sialic acid utilization genes was carried out to indicate possible horizontal gene transfer via calculated using WWW.endmemo.com software. The *nanT*, *nanA* and *nanR* genes of the *C.sakazakii* genomes revealed average GC% values of 57.14%, 57.22% and 56.32% respectively which is comparable to the average 56% GC content of the *C. sakazakii* genome BAA-894. Whereas *nanK* and *nanE* revealed slightly higher values of 62.21% and 63.18% respectively. In contrast *nanC* gene had a much lower 47.44 GC% content, which is closely related with *Cit. koseri* 48% organism.

The *nagA*, *nagB*, *neuC* and *siaPQM* genes in *Cronobacter sakazakii* revealed GC% content values of 56.39%, 53.05%, 59.20% and 55.28% respectively.

However, the *nanT*, *nanA* and *nanR* genes of the *C. turicensis* genomes revealed average GC% values of 57.07%, 57.45 % and 53.76% respectively which is comparable to the average 58.4% GC content of the *Cronobacter turicensis* z3032. In contrast, *nanK* and *nanE* revealed slightly higher values of 61.64% and 62.60% respectively.

The *nagA*, *nagB*, *neuC* and *siaPQM* genes of the *C. turicensis* genomes as well revealed GC% content values of 57.52%, 53.8, 59.85 % and 55.18 % respectively. Table 4.2 shown the GC% content value of *C. sakazakii* and *C. turicensis* and other closely related species.

| **Genes** | **Species** | | | | |
|---|---|---|---|---|---|
| | ***C. sakazakii*** | ***C. turicensis*** | ***Franconibacter pulveris*** | ***Siccibacter turicensis*** | ***Cit. koseri*** |
| **Average (%)** | **56** | **57.21** | **56.60** | **57.80** | **53.80** |
| ***yhcH*** | 54.62 | 55.05 | 52.96 | - | 55.13 |
| ***nanK*** | 62.21 | 61.64 | 62.67 | 70.58 | 62.44 |
| ***nanT*** | 57.14 | 57.07 | 58.69 | 61.46 | 57.44 |
| ***nanA*** | 57.22 | 57.45 | 56.38 | 58.88 | 55.65 |
| ***nanR*** | 56.32 | 53.76 | 56.56 | 60 | 56.34 |
| ***nanC*** | 47.44 | - | - | - | 48 |
| ***nanE*** | 63.18 | 62.6 | - | - | - |
| ***nagA*** | 56.39 | 57.52 | 56.22 | 56.3 | 53.35 |
| ***nagB*** | 53.05 | 53.8 | 53.05 | 54.55 | 52.18 |
| ***neuC*** | 59.2 | 59.85 | 52.76 | 53.22 | 55.15 |
| ***siaPQM*** | 55.28 | 55.18 | 57.29 | 57.2 | 54.23 |

**Table 4.2** GC % content values of *C. sakazakii*, *C. turicensis* and other closely related species.

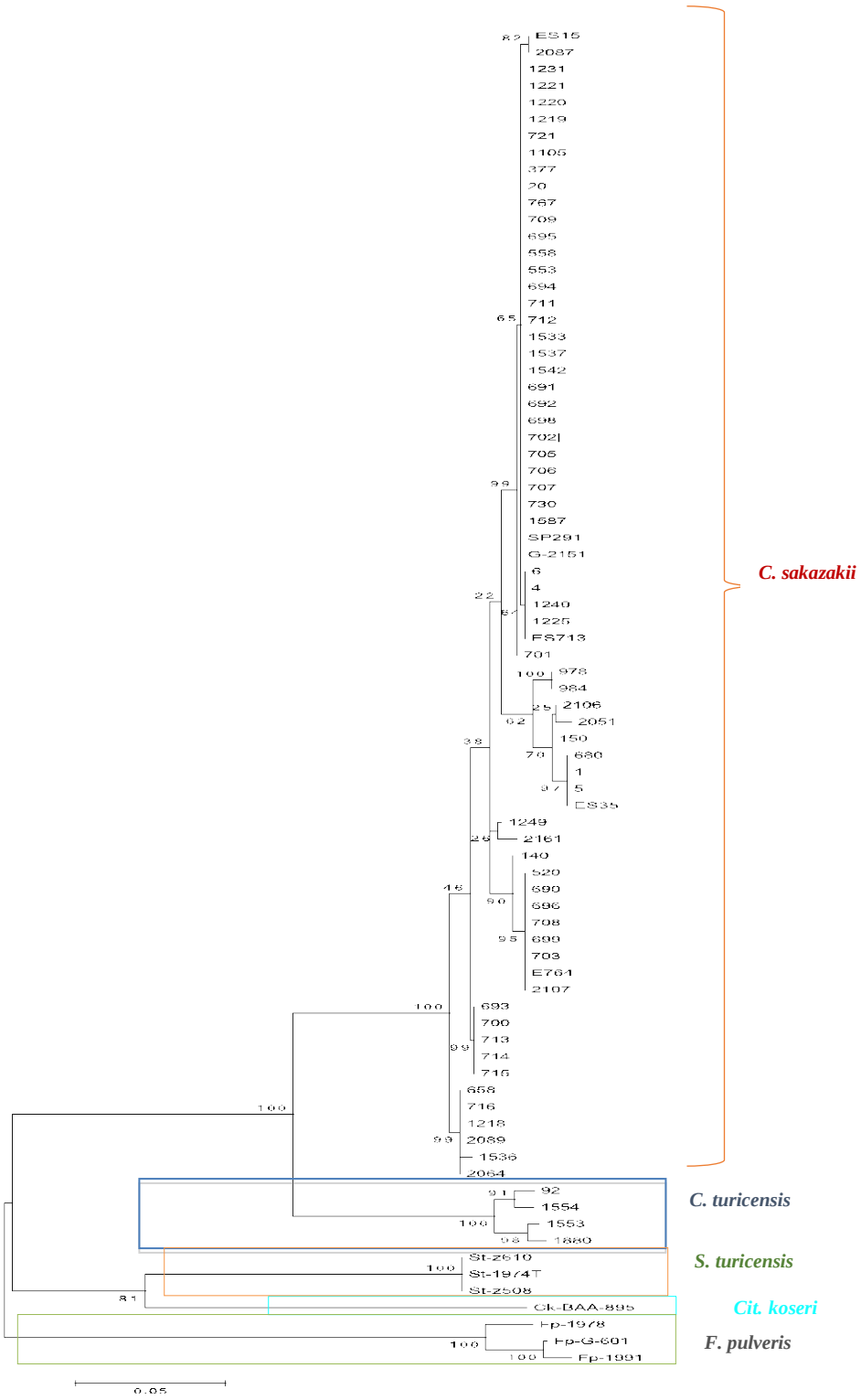


**Figure 4.7** Maximum likehood tree of protein NanA of *C.sakazakii* and *C.turicensis* and related *Enterobacteriaceae* species. The NTU strains IDs are showed at the top of each branches, the tree is drawn to scale using MEGA5, with 1000 bootstap replicates.

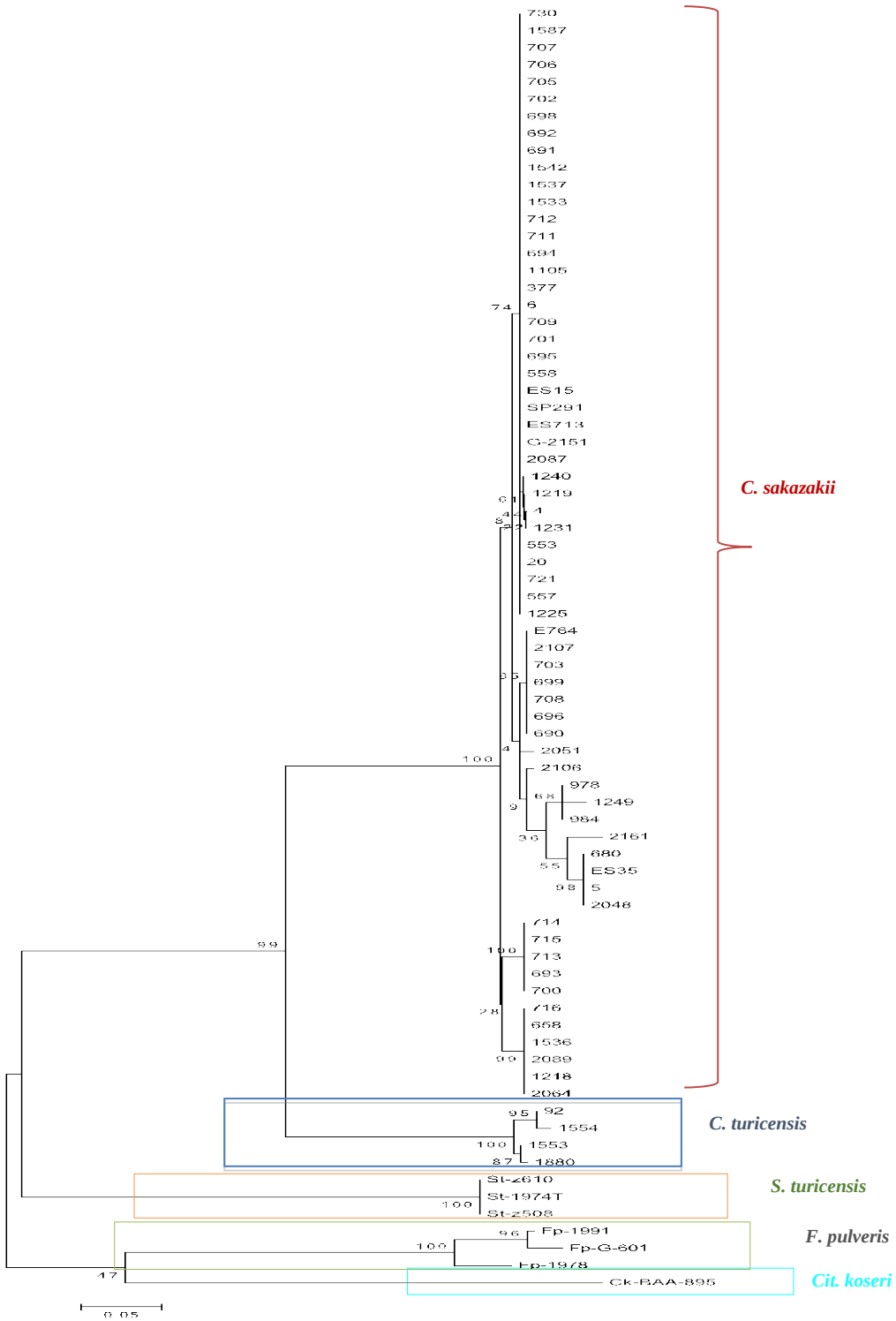


**Figure 4.8** Maximum likehood tree of protein NanR of *C. sakazakii*, *C.turicensis* and related *Enterobacteriaceae* species. The NTU strains IDs are showed at the top of each branches, the tree is drawn to scale using MEGA5, with 1000 bootstap replicates.

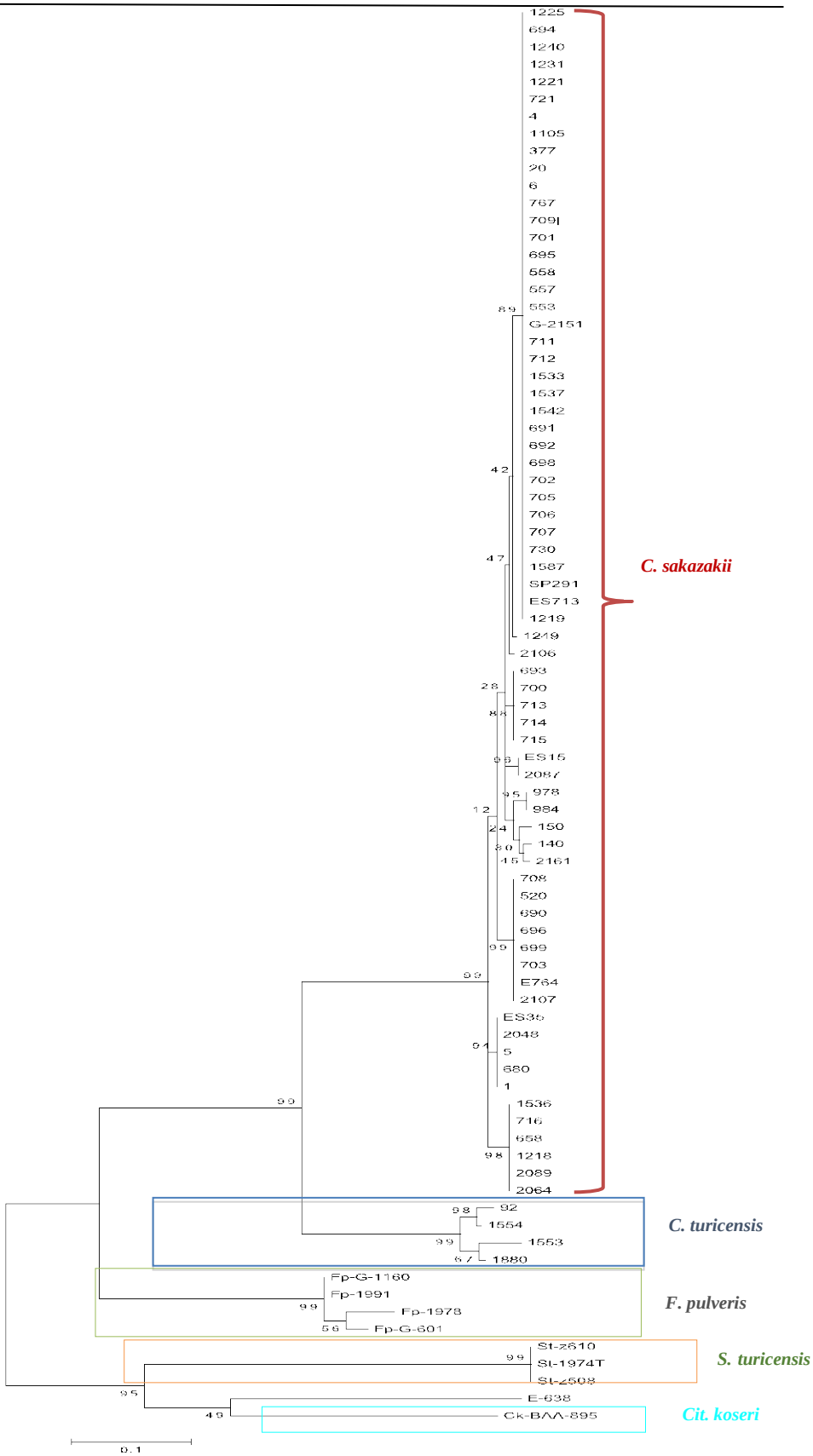


**Figure 4.9** Maximum likehood tree of protein NanK of *C.sakazakii*, *C.turicensis* and related *Enterobacteriaceae* species. The NTU strains IDs are showed at the top of each branches, the tree is drawn to scale using MEGA5, with 1000 bootstap replicates.

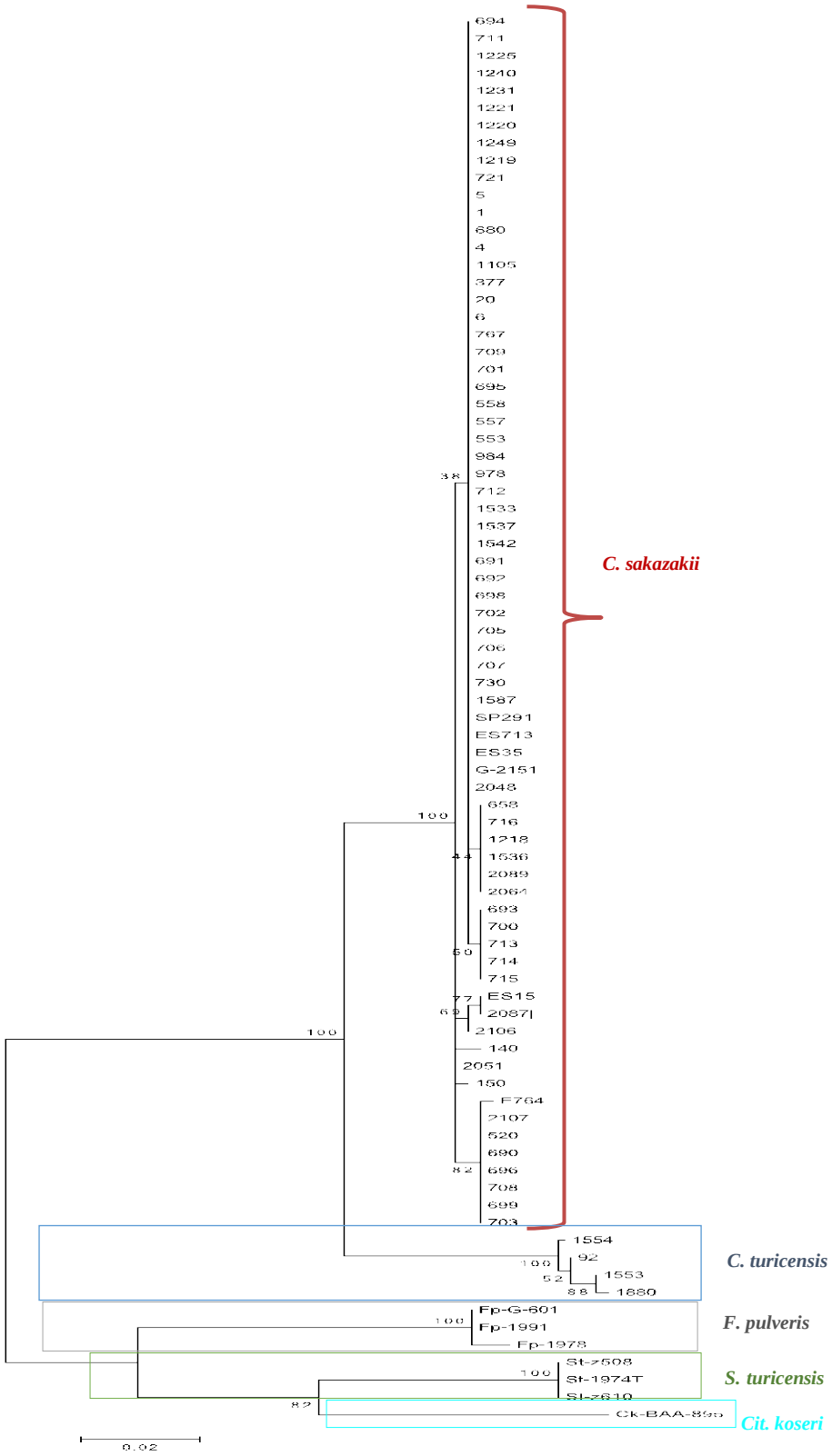


**Figure 4.10** Maximum likehood tree of protein NanT of *C.sakazakii*, *C.turicensis* and related *Enterobacteriaceae* species. The NTU strains IDs are showed at the top of each branches, the tree is drawn to scale using MEGA5, with 1000 bootstap replicates.

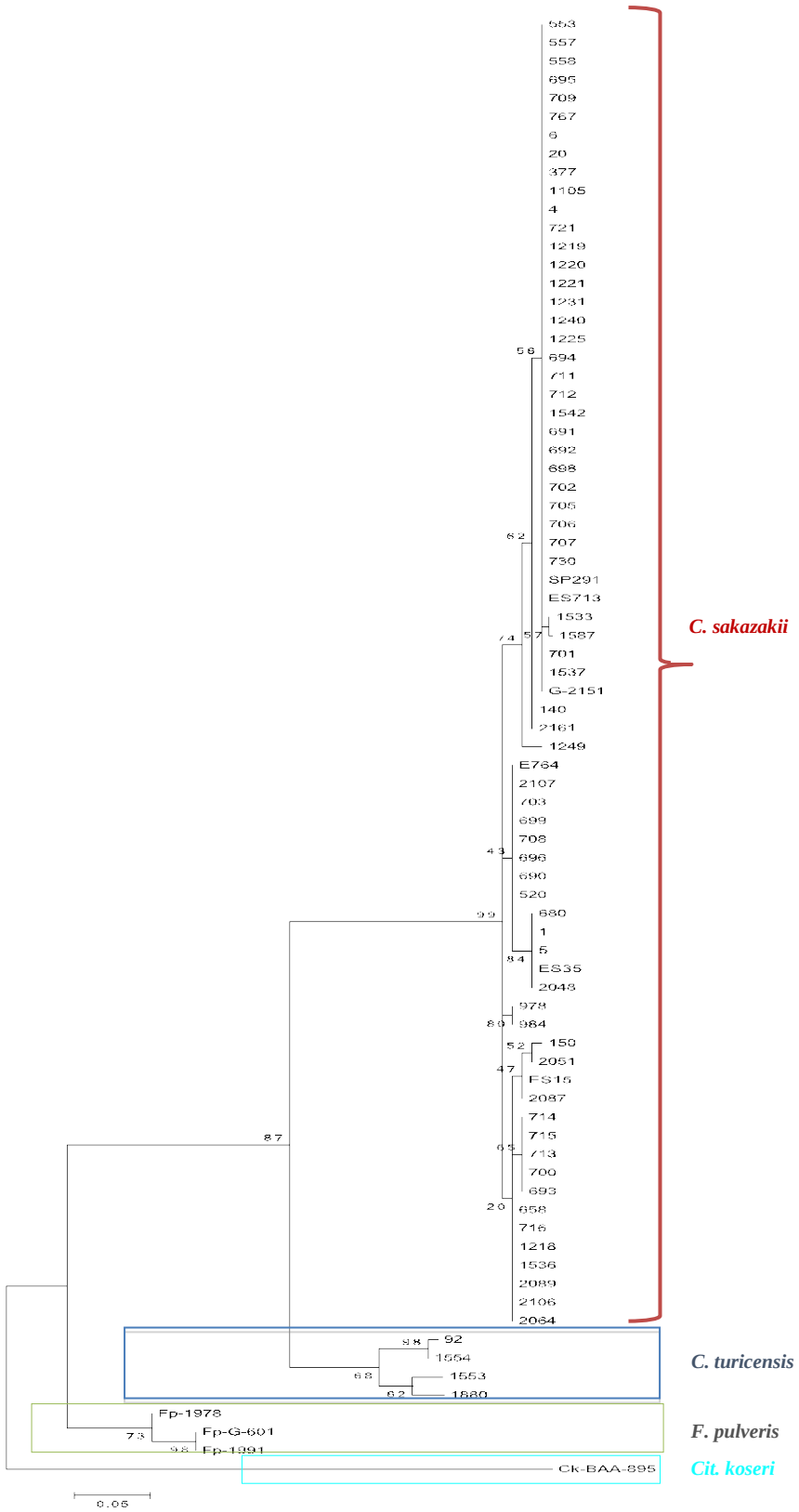


**Figure 4.11** Maximum likehood tree of protein yhcH of *C. sakazakii*, *C. turicensis* and related *Enterobacteriaceae* species. The NTU strains IDs are showed at the top of each branches, the tree is drawn to scale using MEGA5, with 1000 bootstap replicates.

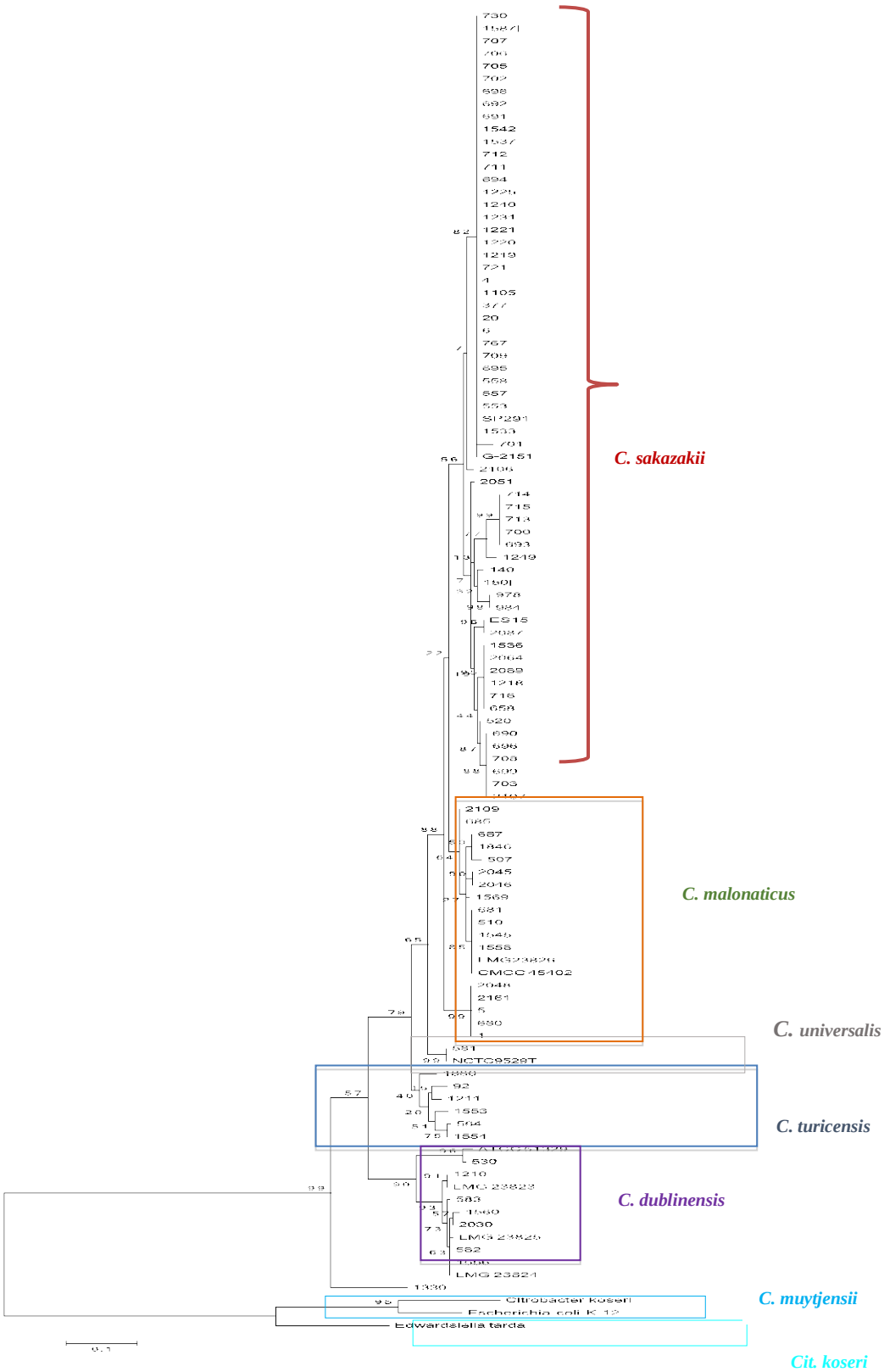


**Figure 4.12** Maximum likehood tree of protein nanE of *Cronobacter* and related *Enterobacteriaceae* species. The NTU strains IDs are showed at the top of each branches, the tree is drawn to scale using MEGA5, with 1000 bootstap replicates.

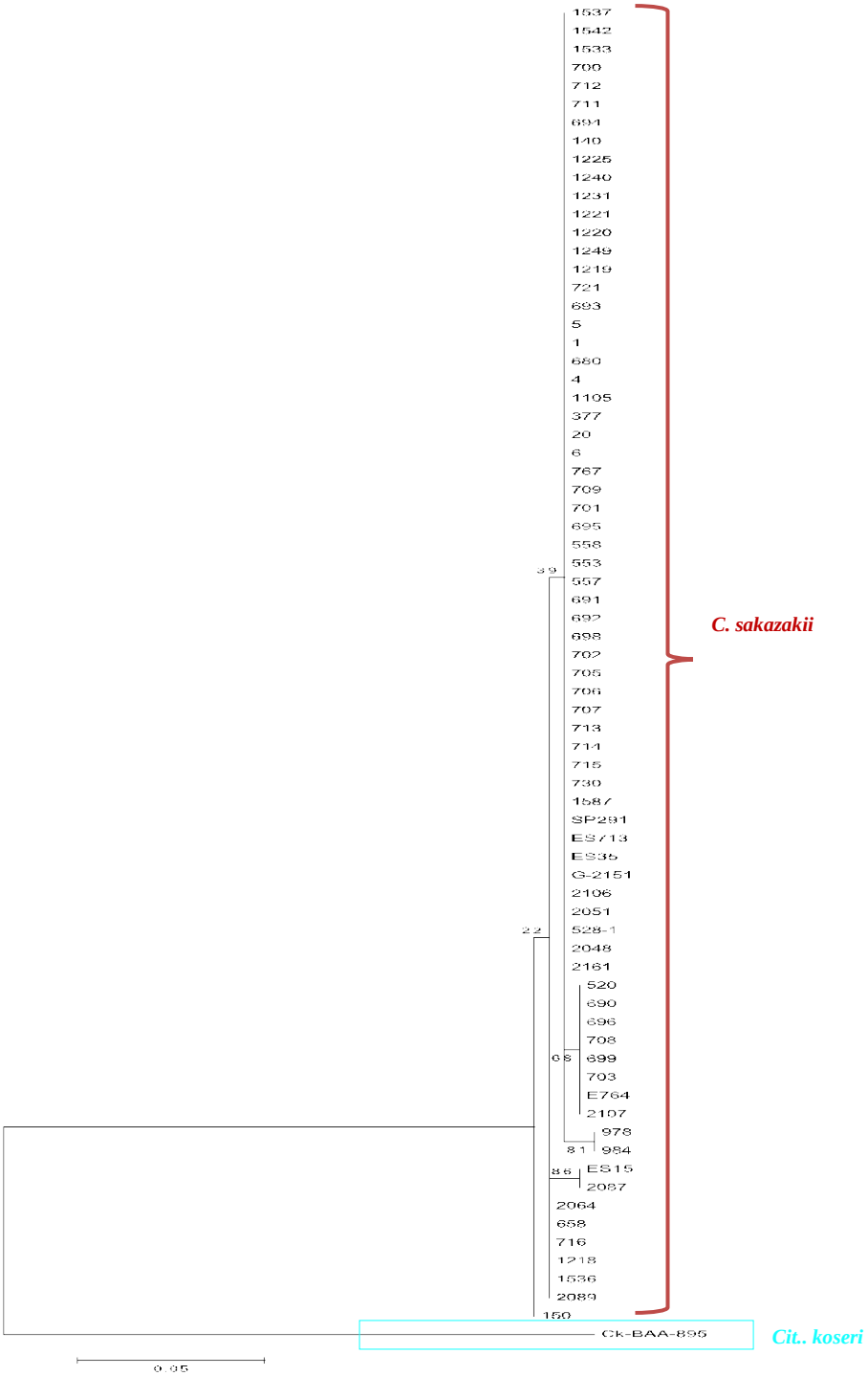


**Figure 4.13** Maximum likehood tree of protein NanC of *C. sakazakii* and related *Enterobacteriaceae* species. The NTU strains IDs are showed at the top of each branches, the tree is drawn to scale using MEGA5, with 1000 bootstap replicates.

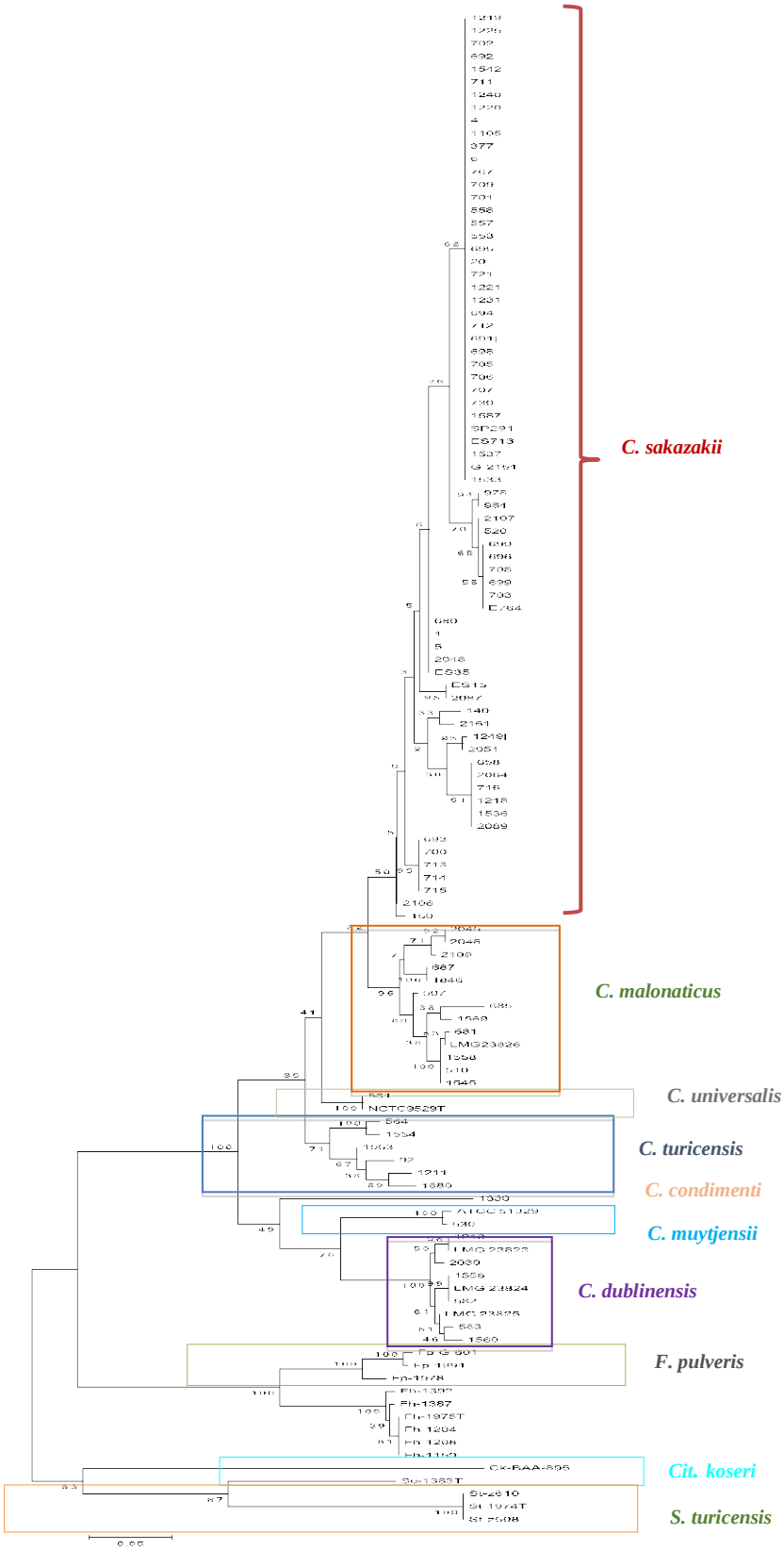


**Figure 4.14** Maximum likehood tree of protein NagA of *Cronobacter* and related *Enterobacteriaceae* species. The NTU strains IDs are showed at the top of each branches, the tree is drawn to scale using MEGA5, with 1000 bootstap replicates.

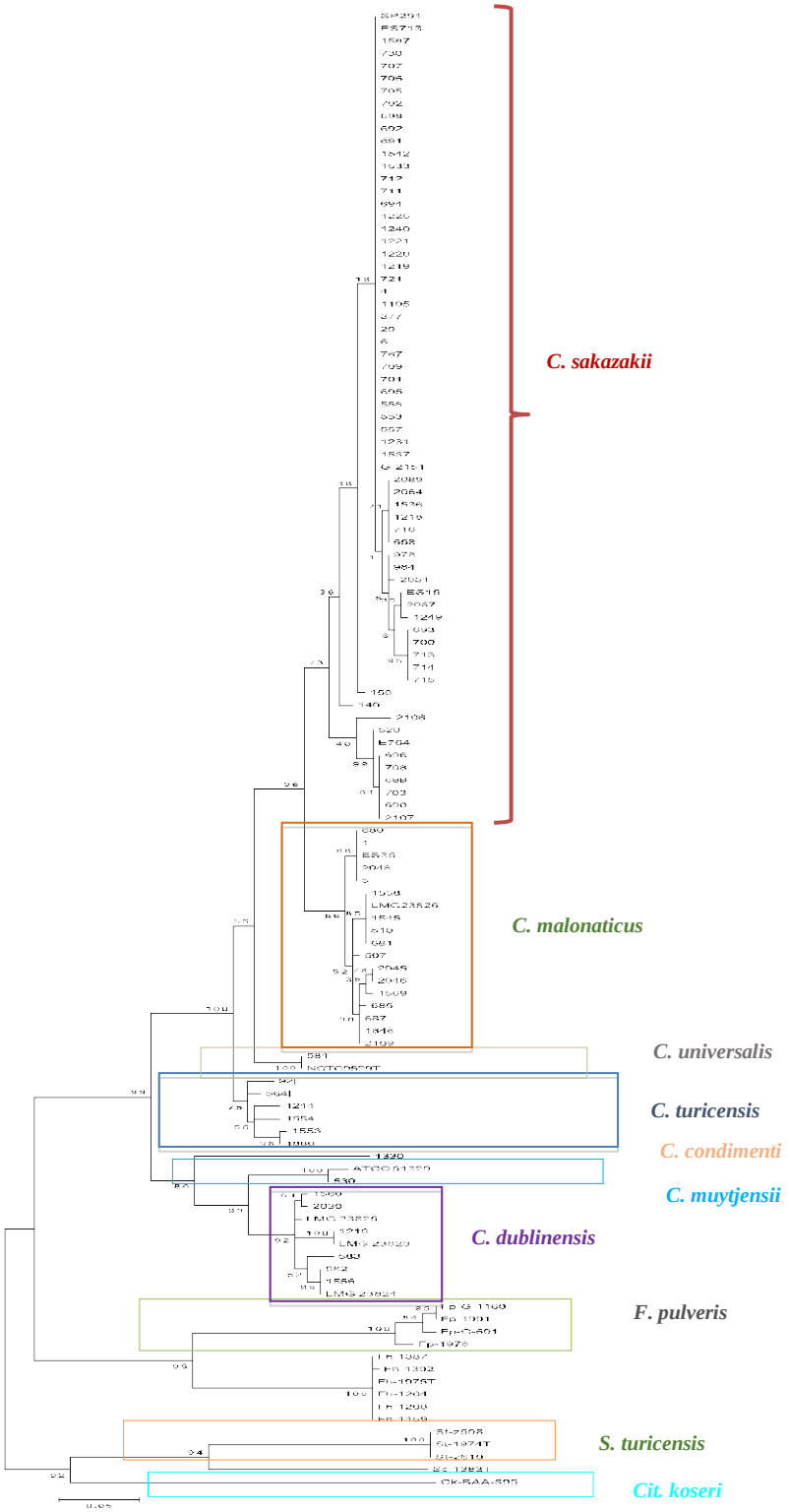


**Figure 4.15** Maximum likehood tree of protein NagB of *Cronobacter* and related *Enterobacteriaceae* species. The NTU strains IDs are showed at the top of each branches, the tree is drawn to scale using MEGA5, with 1000 bootstap replicates.

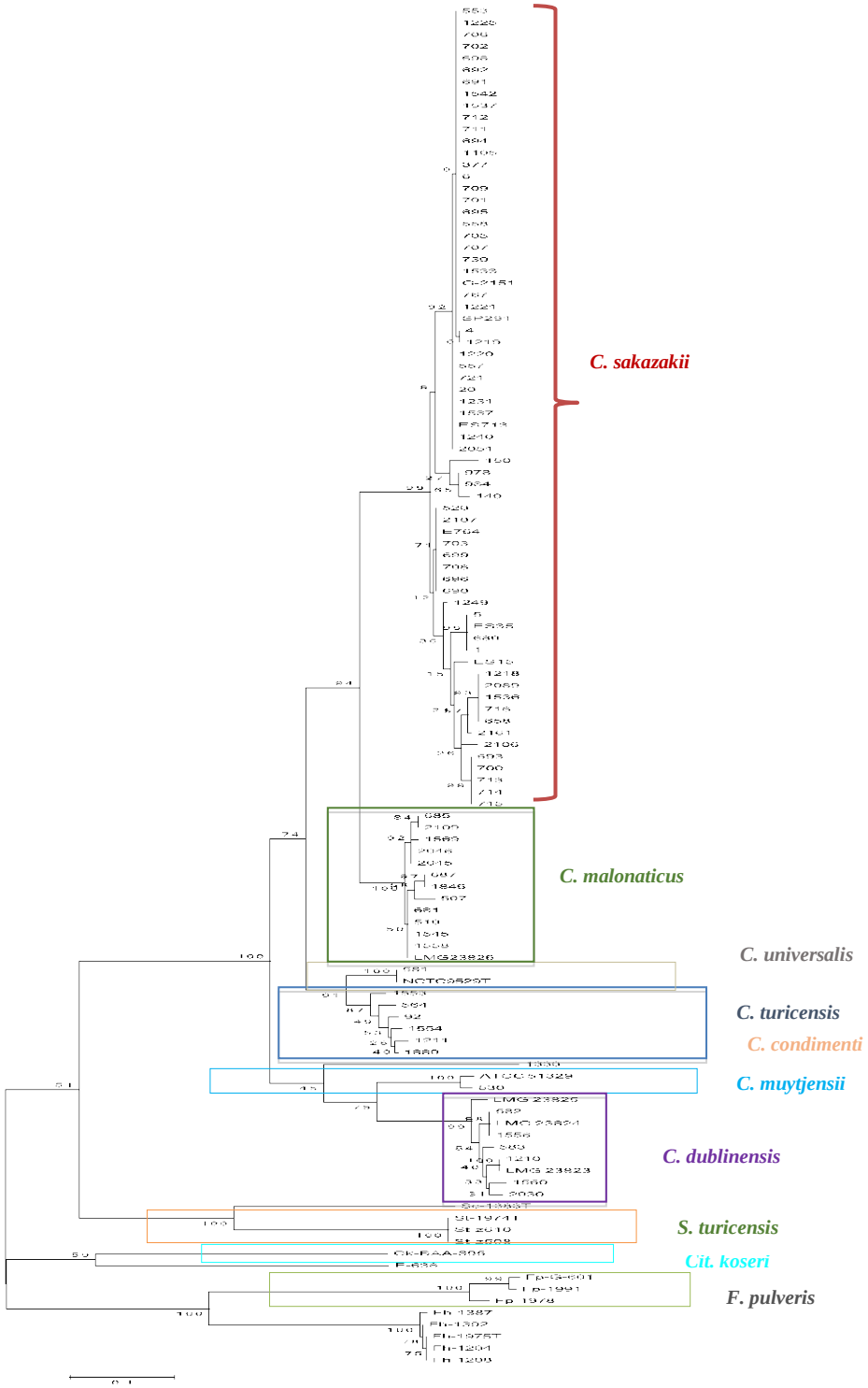


**Figure 4.16** Maximum likehood tree of protein *NeuC* of *Cronobacter* and related *Enterobacteriaceae* species. The NTU strains IDs are showed at the top of each branches, the tree is drawn to scale using MEGA5, with 1000 bootstap replicates.

### 4.4.5 PHYLOGENETIC ANALYSIS

The uniqueness of the core sialic acid-related gene cluster to the *C. sakazakii* and some of *C.turicensis* genomes hints at a role in the evolution of the virulence of the organism. The predicted amino acid sequences of the proteins encoded by these nan cluster genes were individually analysed and their phylogenetic relationships observed with closely related Gram-negative bacteria have been indicated in Fig. 4.7 to 4.16. In the case of each of the genes, the *C. sakazakii* sequences formed an independent cluster of their own, with the other *Enterobacteriaceae Cit. koseri*, *Franconibacter pulveris* and *Siccibacter turicensis* members clustering on the neighbouring branches.

In the *nanA* (Fig.4.7) and *nanR* (Fig.4.8) phylogenetic trees of predicted amino acid, the *C. sakazakii* cluster appeared to evolve on the same branch as *C. turicensis*. With the others forming a separate clade *Cit. koseri*, *Franconibacter pulveris* and *Siccibacter turicensis*.

As well as, the *nanK* (Fig.4.9) and *nanT* (Fig.4.10) *C. sakazakii* and *C. turicensis* clusters appears to have greater phylogenetic distance from the other closely species, with a clear split of the population into two clades, one of them being that of the *C. sakazakii* and *C. turicensis* cluster.

The *nanE* gene was found across the *Cronobacter* genus, and the phylogenetic analysis of the nanE protein sequences (Fig 4.12) revealed the *Cronobacter* cluster to have a common and closely related evolutionary clade with *Cit. koseri*, *Escherichia coli*. K-12 and *Edwardsiella trade*.

*C. sakazakii nanC* demonstrated more than 50% homology with *Cit.koseri* (Figure 4.13) Moreover, all *Cronobacter* species were found to have *nagA*, *nagB*, and *neuC* genes as shown in Figures 4.14-4.16. When these protein sequences were subjected to phylogenetic analysis, it was found that the evolutionary clade of *Cronobacter* spp. sequences were quite distinct from other closely related members of *Enterobacteriaceae* which constitute an adjacent clade, through both share the evolutionary lineage. A dissimilar branching pattern has been demonstrated by *nan* genes sequence from the *Cit. koseri* when it was subjected to phylogenetic analysis. It implies that there can be distinct evolutionary paths adopted by *nan* genes in case of the genus *Cronobacter*.

## 4.5 DISCUSSION

It has been reported by Joseph *et al*. (2013) that the region ESA_03609–13 on the genome of *C. sakazakii* BAA-894 encodes for the uptake and consumption of exogenous sialic acid. Moreover, this region was unique to the genome of *C. sakazakii* based on RAST analysis. This exclusive characteristic is quite intriguing in terms of virulence and epidemiology of *Cronobacter* species. Sialic acid metabolism might have a role in high prevalence of *C. sakazakii* infections among infants and neonates. In contrast, the *nanE* gene have been found located in a distinct site (ESA_00529) separate from the nan cluster site (ESA_03610-12). The same have been observed with other Gram-negative bacteria such as *Cit. freundii* and *Ed. tarda* (Vimr 2012). It potentially points towards a distinct evolutionary lineage for the gene. Moreover, genes for NanT inner membrane transporter protein and NanC outer membrane porin are present in all *C. sakazakii* strains. For that reason, all of them are able to uptake sialic acid from the environment into the cytoplasm of the cell. It is surprising to note that all *Cronobacter* possess genes for TRAP transporter i.e. *siaPQM* (Figure 4.4).

These laboratories experiments have confirmed that *C. sakazakii* is not the only member of *Cronobacter* genus which can utilize sialic acid as a source of carbon as some of *C. turicensis* have this ability. Colonization by *C. sakazakii* in the intestinal tract of humans and consumption of sialic acid from the infant formula, breast milk and brain cell might be due to acquisition of genes responsible for utilization of sialic acid (Almagro-Moreno and Boyed 2009).

Growth of *C. sakazakii* on ganglioside GM1, as shown in Figure 5 b, shows that the bacterium can produce the sialidase enzyme. This finding has not been reported earlier though researchers had carried out gene sequencing in order to detect the *nanH* gene in the genome of these bacteria. Researchers conducted an extensive research for Asp-box motifs and sialidase RIP as the homology between the genes coding for sialidases is found to be less than 30% (Kim *et al*. 2011). Growth of *C. sakazakii* on GM1 also proves that the organism is capable of degrading the ganglioside. Sialic acid residues, glucose, N-acetyl-galactose, and galactose constitute to form GM1. These building blocks are linked through β 1–3 and β 1–4 linkages and are attached to

steroid. Hence, it has been postulated that degradation of GM1 by different lipases (ESA_02127 & ESA_02202), esterases (ESA_00377 & ESA_00776), β-acetyl-hexosaminidases (ESA_02237 and ESA_02655) and β-galactosidases (ESA_01827, ESA_02977 & ESA_03417) results in formation of metabolisable sugar residues thereby enabling the organism to grow on GM1.

An interesting observation made was that *nanE* and TRAP transporter (*siaPQM)* were found conserved across the genomes of the *Cronobacter* genus in contrast with *nanA*, *nanT* and *nanK* (Figure 4.5). GC content of the entire genome of *C. sakazakii* is 56% which is significantly greater than the GC content of *nanC* gene i.e. 47.44%. Slight aberration in the GC content values of the *nanE* and *nanK* genes have also been found (63.18%-62.21% respectively) Table 4.2. This is in agreement with a past observation recorded during an evolutionary investigation of the nan clusters present in members of *Enterobacteriaceae* such as *Yersinia* species, *E. coli* and *Salmonella enterica* (Almagro-Moreno and Boyed. 2009). For this reason, it can be stated that *nan* clusters might have evolved in these bacteria in a mosaic fashion. These findings point towards the fact that it is highly likely that acquisition or lost of *nanC* and *nanE* clusters by the members of *Cronobacter* and *nanAKT* cluster by *C. sakazakii* could have been the result of the horizontal transfer events. This research also analysed the *nanAKT* cluster genes acquisition or loss based on gene location, the high degree of colinearity of the *nanAKT* cluster alignment between different genomes have been shown in figure 4.5. Furthermore, the whole cluster is located in a certain location flanked by some conserved housekeeping trait (*gltB*) and starvation gene (*sspA*) suggesting loss from other *Cronobacter* spp. instead of separate acquisition events. Moreover, because of close adaptation of intracellular microorganism to the physiologically stable environments of their host cells, a reductive genome evolution happened that led to the loss of some genes not crucial for life within the host. This is called evolution by reduction (Dobrindt and Hacker 2001). Sequence of proteins coded for utilization of sialic acid in *C. turensis* and *C. sakazakii* have also been determined during the phylogenetic analysis (Figures 4.5-4.11). This finding indicated that the evolution of the *nanATKR* genes as a lineage in *C. sakazakii* and certain strains of *C. turicensis* was independent of closely related *Enterobacteriaceae* family.

Expression of sialic acid utilisation gene cluster can be affected by levels of nutrients in the

environment since the *nanATK* gene cluster is found proximate to the stringent starvation gene homologue (*sspA*, ESA_03615) in *C. sakazakii*. *C. sakazakii* also possesses other related genes like *nagA* and *nagB* responsible for the formation of fructose-6-phosphate which is also indicative of the fact that the organism can utilize sialic acid as a source of carbon or nitrogen. Human milk, brain and GIT serve to be the three main sources of sialic acid in mammals for commensal as well as pathogenic bacteria.

Researchers have found that human milk is a rich source of sialic acid and highest concentrations of sialic acid have been detected in colostrum up to three months after child birth. For that reason, human beings are exposed to sialic acid right from their infancy. It has been proposed that this exposure affects the concentration of sialic acid in brain (Wang *et al*. 2001). Concentration of sialic acid in cell membranes inside the brain is 20 times higher than its concentration in other mucosal membranes. Especially, the gangliosides of brain contains high concentration of Neu5Ac giving rise to sialyated glycolipids. Similarly, epithelium of human intestine contains high concentrations of sialic acid. Moreover, levels of sialic acid and N-acetylglucosamine that residue in intestinal mucosa of an infant are considerably greater than that of adults (Wang. 2009). There is an intriguing clinical association between the above mentioned sites of sialic acid build up and epidemiology of *Cronobacter* species. During the course of neonatal meningitis, this bacterium causes NEC and intensive brain damage. High concentration of sialic acid in the above mentioned sites relates with the *C. sakazakii* infections as majority of neonatal infections with *C. sakazakii* have been found to occur during infancy. In particular, half of the cases were reported in the first week of birth and three quarter cases were reported within one month (Lai 2001). In addition the sources of sialic acid, PIF products contain sialic acid, but in lesser quantity (<25%) than human milk. In contrast to the form of sialic acid in human milk i.e. oligosaccharide-bound form, infant formula usually contains sialic acid in glycoprotein bound form (Wang *et al*. 2001).

In summary;

- A key finding from the comparative genomic study was the unique cluster of genes in *C. sakazakii* and some of *C. turicensis* encoding for the utilization of exogenous sialic acid.

- Since this is also the species most associated with the neonatal meningitic infections, this association could prove to be a crucial link to the pathogenicity of the organism.

# CHAPTER 5

# DETECTION OF VIRULENCE ASSOCIATED GENES OF *C.SAKAZAKII* CLINICAL STRAINS USING PCR AND COMPARATIVE GENOMIC ANALYSIS VIA THE PubMLST DATABASE

## 5.1 INTRODUCTION

Despite *Cronobacter* spp. infections being infrequent, they are still of high concern due to the severity of the infection that the organism causes, as well as the sensitive age group of the neonates that are affected by them. Ever since the *C. sakazakii* BAA-894 and *C. turicensis* z3032 genomes became available, examination of the virulence of the organism can be examined at the genomic level (Kucerova *et al*. 2010; Stephan *et al*. 2010). Type VI secretion system, iron acquisition, enterobactin and aerobactin synthesis are acknowledged as potential virulence factors in *Cronobacter* spp. (Kucerova *et al*. 2011; Hartmann *et al*. 2010; Franco *et al*. 2011).

### 5.1.1 IRON UPTAKE

Iron acts as an essential mineral for many cellular functions including electron transport, ATP production via oxidative phosphorylation, DNA metabolism, protection against oxidative stress and regulation of gene expression, but it is toxic and poorly soluble in its free ferric form (Crosa *et al*. 2004). Pathogenic bacteria must be able to compete for this very limited supply of iron with their host organism in order to survive and propagate in the host. Furthermore, the decreased iron availability in host organisms can serve as a stimulus triggering expression of virulence-related genes. It has been shown that increased iron availability correlates with increased virulence of *Escherichia, Klebsiella, Listeria, Neisseria, Pasteurella, Shigella, Salmonella, Vibrio,* and *Yersinia,* as reviewed in Raymond *et al*. (2003). The efficiency of iron assimilation via expression of iron uptake mechanisms is hence an important aspect of bacterial virulence.

The ability of iron within a host varies with its tendency to form complexes with iron binding proteins such as haemoglobin, transferrin, lactoferrin and ferritin. However, bacteria can not directly utilise these sources (Lin *et al*. 2012). Therefore, pathogenic bacteria require various iron acquisition systems to obtain iron from the host environment. Many bacteria do this by releasing siderophores, which are compounds with a high affinity to chelate iron from iron binding proteins.

Banin *et al.* (2005) found that iron starvation can prevent bacterial growth and formation of a biofilm.

Bacteria have evolved several mechanisms to cope with iron scarcity and scavenge iron from the host environment: use of proteases that cleave the iron-binding proteins to obtain free iron, reducing $Fe^{3+}$ to $Fe^{2+}$ followed by the release of iron from a protein complex, and siderophore synthesis (Henderson & Payne 1994). Siderophore production is probably the best studied mechanism of iron acquisition in pathogenic bacteria. Siderophores are high-affinity iron-binding compounds produced by various bacterial species as a response to iron depletion. Firstly, the ferric siderophore complex binds to the receptor protein on the microbial cell surface, then the complex is translocated across the outer and inner membrane, and finally, iron is released for metabolism inside the cell (Crosa *et al*. 2004). Iron uptake via the catechol siderophore enterobactin (or entrochelin) is the best described among the siderophore-mediated iron uptake systems in prokaryotes. The biosynthesis of aerobactin requires the expression of the operon *entABCDEF* and the transport system for enterobactin is encoded by genes *fepABCD* and *fepG*. In addition, some *Enterobacteriaceae* are able to synthesize a hydroxamate siderophore aerobactin, which has also been linked to increased virulence in members of *Enterobacteriaceae* (Lafont *et al*. 1987, Martinez *et al*. 1994). Aerobactin system was first described on the large *E. coli* plasmid pColIV-K30 by Warner *et al*. (1981) and the products of the biosynthetic pathway were identified by de Lorenzo *et al*. (1986). Aerobactin synthesis requires the expression of four genes *iucABCD*. Aerobactin is then secreted into the extracellular environment and the iron-aerobactin complex binds to a specific outer membrane TonB-dependent receptor IutA. Interestingly, the aerobactin synthesis genes *iucABCD* and the outer membrane receptor gene *iutA* are located in the same operon, whereas the genes required for trans-membrane transport which encode the periplasmic binding protein FhuB and the inner membrane permease FhuCD form a different genetic cluster that is not related to aerobactin synthesis (Crosa *et al*. 2004).

### 5.1.2 *CPA* (PLASMINOGEN ACTIVATOR)

It was discovered through *in silico* analysis of pESA3 that there was an omptin superfamily homologue, pESA3p05434, present in it, recently named cpa by Franco *et al*. (2011). Cpa has a common identity with the plasminogen activators Pla of *Yersinia pestis* and PgtE of *Salmonella enterica.*

After the systematic invasion by *Cronobacter*, excessive presence of outer-membrane protease Cpa (plasminogen activator) was observed by Franco *et al*. (2011). Not only does cpa provide protection from the bacterial activity of the serum by cleaving the accompanying components C3 and C4b, it also stimulates the plasminogen and inactivates a2-AP (plasmin inhibitor) (Franco *et al*. 2011a; Schwizer *et al*. 2013). Interestingly, it has been stated that, of all the diverse *Cronobacter* spp., *C. sakazakii* had the greatest resistance against the terminating impact of the serum – a statement which may help understand its powerful pathogenic potential and the presence of pESA3-borne cpa in *C. sakazakii* strains. The function of alternative surface structures such as LPS, OmpA and exopolysaccharide (capsule), which allow the pathogen to tolerate the bactericidal activity of serum and avert the immune system is yet to be discovered (Schwizer *et al*. 2013).

### 5.1.3 TYPE VI SECRETION SYSTEMS

T6SS is one of the bacterial secretory machinery that help in the transportation of proteins over the bacterial cell membranes. There are different types of T6SS in organisms, with the various systems being formed of a variety of genes, from 12 to over 20. There may be various clusters of T6SS genes, which are not essentially the same as each other, and it is highly likely that not all of them are functional. In several organisms like *P. aeruginosa* and *E. coli*, these areas have been categorized as pathogenicity islands.

These secretion systems have facilitated the transportation of molecules over the membranes so that they can be discharged into the adjacent medium or the eukaryotic host cell. The translocation process in Gram-negative bacteria may either carry on as a single step process through the inner and outer membranes (as can be seen in types 1, 3, 4 and 6 secretion systems), or as a step-by-step

procedure that includes transport into the periplasmic space, followed by secretion out of the cell across the outer membrane (as can be seen in types 2 and 5 secretion systems).(Tseng *et al*. 2009).

It was as early as 1996 that there were reports of a protein transport system in studies that were carried out on the secretion of the haemolysin co-regulated protein Hcp in *Vibrio cholerae* (Williams *et al*. 1996), and consequently, in other groups of micro-organisms. In 2003, there were further investigations of such a region using an *in silico* study of the *V. cholerae* genome, which was referred to as IAHP (IcmF-associated homologous protein) clusters at that time due to the similarity of a particular protein to IcmF proteins seen in certain T4SSs (Das *et al*. 2003). This protein secretion system was categorized as "Type VI" in 2006 in a subsequent study on *V. cholera*e, which explained the export process of the Hcp and VgrG proteins, and their contribution towards virulence of the organism (Pukatzki *et al*. 2006).

A classic T6SS mainly consists of the IcmF and IcmH-like proteins, CIpV ATPase, a putative lipoprotein and the proteins Hcp and VgrG (valine glycine repeats), in which the last two are also the secreted factors. This secretory system may be managed either through transcriptional activator of the AraC family or σ-54, or with the help of a threonine phosphorylation signalling cascade. There are reports of a relationship between T6SSs and virulence functions in several pathogens, like *E. coli, P. aeruginosa, V. cholerae* and several others. These carryout activities like host cell adhesion and invasion, macrophage survival and cytotoxicity. However, in addition to pathogenicity, T6SS has also been linked to physiological functions of certain organisms like root colonization by the nitrogen-fixing *Rhizobium* spp., in addition to other activities like quorum sensing and biofilm creation (Bingle *et al*. 2008; Cascales. 2008; Leung *et al*. 2011).

### 5.1.4 AIMS OF THIS CHAPTER

It is not proven that all *Cronobacter* species are infective in infants. Until now *C. sakazakii* has been the most prevailing among clinical cases. It has been found in sequence analysis that there are several plausible reasons for virulence; however, most of them need to be tested further in laboratories for affirmation (Kucerova *et al*. 2010; Joseph *et al*. 2012). Also, there are now (April, 2015) genomes of 107 *Cronobacter* strains, including 37 *C. sakazakii* ST4.

The aim of this chapter was to investigate the pathogenesis of *Cronobacter sakazakii* by determining the presence of a number of key virulence associated genes which included: two iron acquisition system gene clusters (*eitA* and *iucC*), *Cronobacter* plasminogen activator (*Cpa*), and type IV secretion (T6SS) gene cluster using laboratory studies in *Cronobacter sakazakii* strains, particularly with ST4 strains regarding the location on plasmid and total DNA.

In addition, *C. sakazakii* clinical strains have been examined furthermore using BLAST analysis from PubMLST *Cronobacter* database with a particular interest in the iron acquisition system. Plasmid profiling experiments were carried out in this study as well.

## 5.2 MATERIALS AND METHODS

The key methods, culture media and culturing conditions for this part were described previously in Chapter 2 (Section 2.6) Materials and Methods.

## 5.3 BACTERIAL STRAINS USED IN PCR SCREENING STUDY

| Isolate | Species | Sequence Type [a] | Country | Source | Year |
|---|---|---|---|---|---|
| 658*[b] | *C. sakazakii* | ST1 | USA | Non-infant formula | 2001 |
| 555 | *C. sakazakii* | ST1 | Netherlands | Clinical | 1979 |
| 12 | *C. sakazakii* | ST1 | - | Faecal- Clinical | 2003 |
| 680* | *C. sakazakii* | ST8 | USA | CSF -Clinical | 1977 |
| 1* | *C. sakazakii* | ST8 | USA | Throat - Clinical | 1980 |
| 5* | *C. sakazakii* | ST8 | Canada | Clinical | 1990 |
| 520* | *C. sakazakii* | ST12 | Czech Republic | Clinical | 1983 |
| 696* | *C. sakazakii* | ST12 | France | Faecal-Clinical (NECII) | 1994 |
| 553* | *C. sakazakii* | ST4 | Netherlands | Clinical | 1977 |
| 557* | *C. sakazakii* | ST4 | Netherlands | Clinical | 1979 |
| 558* | *C. sakazakii* | ST4 | Netherlands | Clinical | 1983 |
| 695* | *C. sakazakii* | ST4 | France | Trachea-Clinical(Fatal NECII) | 1994 |
| 701* | *C. sakazakii* | ST4 | France | Peritoneal- Clinical(Fatal NECII) | 1994 |
| 767* | *C. sakazakii* | ST4 | France | Trachea-Clinical(Fatal meningitis) | 1994 |
| 6* | *C. sakazakii* | ST4 | Canada | Clinical | 1990 |
| 20* | *C. sakazakii* | ST4 | Czech Republic | Faecal-Clinical | 2003 |
| 721* | *C. sakazakii* | ST4 | USA | CSF –Clinical | 2003 |
| 1219* | *C. sakazakii* | ST4 | USA | CSF-Clinical (Fatal meningitis) | 2009 |
| 1220* | *C. sakazakii* | ST4 | USA | CSF –Clinical (Brain abscess) | 2003 |
| 1221* | *C. sakazakii* | ST4 | USA | CSF –Clinical (Meningitis) | 2003 |
| 1222 | *C. sakazakii* | ST4 | USA | Blood- Clinical | 2003 |
| 1223 | *C. sakazakii* | ST4 | USA | Blood- Clinical | 2004 |
| 1231* | *C. sakazakii* | ST4 | New Zealand | Faecal-Clinical (Meningitis) | 2005 |
| 1240* | *C. sakazakii* | ST4 | USA | CSF –Clinical | 2009 |
| 1241 | *C. sakazakii* | ST4 | USA | Blood- Clinical | 2009 |
| 1242 | *C. sakazakii* | ST4 | USA | Brain- Clinical | 2009 |
| 1225* | *C. sakazakii* | ST4 | USA | Blood -Clinical(Fatal meningitis) | 2007 |
| 730* | *C. sakazakii* | ST4 | France | Clinical(NECI) | 1994 |
| 1585 | *C. sakazakii* | ST4 | Israel | Blood –Clinical (Bacteraemia) | 1999 |
| 1587* | *C. sakazakii* | ST4 | Israel | CSF –Clinical (Sever anatomical damage brain) | 2000 |
| 4* | *C. sakazakii* | ST15 | Canada | Clinical | 1990 |
| 1588 | *C. sakazakii* | ST14 | Israel | Blood- Clinical | 2012 |
| 1586 | *C. sakazakii* | ST9 | Israel | Blood –Clinical | 1998 |
| 150* | *C. sakazakii* | ST16 | Korea | Spice | 2005 |

| | | | | | |
|---|---|---|---|---|---|
| 140* | *C. sakazakii* | ST40 | India | Spice | 2005 |
| 1249* | *C. sakazakii* | ST31 | UK | Clinical | 2010 |

*Strain have been genome sequenced in the *Cronobacter* PubMLST *Cronobacter* database

**Table 5.1** List of *Cronobacter. sakazakii* isolates included in this study.

## 5.4 RESULTS

### 5.4.1 SCREENING OF VIRULENCE ASSOCIATED GENES CARRIAGE IN PLASMID AND TOTAL DNA (CHROMOSOME AND PLASMID)

Genes implicated in virulence and associated with RepFIB plasmids were studied as designated by Franco *et al*. (2011b) with some modification. Plasmids and total DNA were used because some genes such as T6SS are located on both chromosomal and plasmid DNA. Plasmid extraction was prepared as described in Chapter 2 (Section 2.5). Thirty-six strains were used to compare their virulence traits, according to their sequence type. Heat mapping was generated to show the presence/absence or location of these genes. Of these 36 strains, 23 were ST4 and 13 strains were non-ST4 strains of which three strains were ST1, three strains were ST8 and two strains were ST12.

#### 5.4.1.1 *CRONOBACTER* PLASMINOGEN ACTIVATOR *(CPA)* GENE

The presence of *cpa* gene was investigated in the plasmids and results are shown in Figure 5.1-5.2. To confirm the presence of the *cpa* locus, the conserved flanking regions upstream and downstream of the *cpa* locus on pESA3 plasmid were amplified as a positive control. Most of the strains produced 306bp amplicons, negative strains were unable to show any PCR products. The plasmid PCR screening indicated that 87% of *C. sakazakii* ST4 strains (n=23) were harbouring the *cpa* gene. The *cpa* gene was present in all ST1 strains (n=3) while none of the ST8 (n=3) or ST12 (n=2) strains contained this gene. Similarly, the presence of the *cpa* gene was investigated in total DNA and the results have been shown in Figure 5.1-5.2. Ninety-six percent of *C. sakazakii* ST4 strains (n=23), 100 % of ST1, 50% of ST12 and 11% of ST8 strains were harbouring the *cpa* gene.

### 5.4.1.2 TYPE VI SECRETION SYSTEM (T6SS) LOCUS

A previous investigation by Franco and colleagues (2011) revealed that the T6SS cluster in the pESA3 plasmid is large, 16,937bp in size. Due to the large size of the T6SS cluster, four separate PCR assays have been designed based on the conserved flanking regions of the type T6SS present in pESA3. Primers specific to the *vgrG* gene as well as the regions IntT6SS left, T6SS R end and IntT6SS Right have been used in this work as previously designed by Franco *et al*. (2011). Figure (5.1): T6SS cluster of pESA3 consist of 16 ORF (16.937 bp) long (ESA_pESA3p05491 to -5506)* Table (2.3) Section 2.6. Primers used for virulence genes investigations.

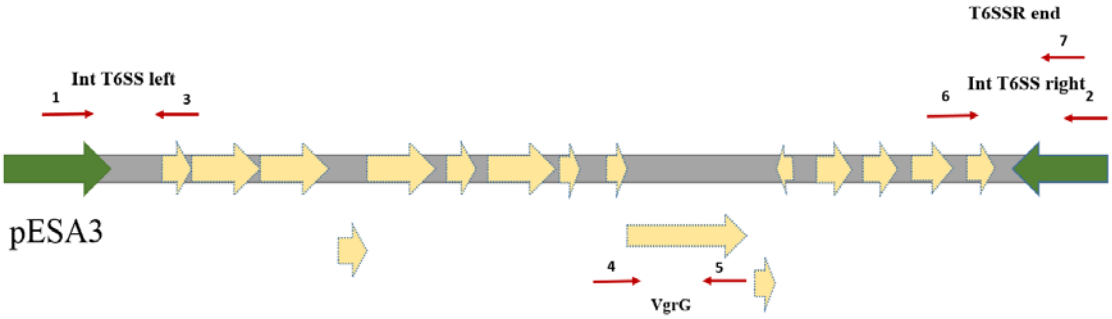


**Figure 5.1** T6SS cluster of pESA3 consist of 16 ORF (16.937 bp) long (ESA_pESA3p05491 to -5506*) PCR primer showed in arrow with number; Primer 1, Δt6ssfw; primer 2, Δt6ssrv; primer 3, t6ssrv; primer 4, vgrGfw; primer 5, vgrGrv; primer 6, t6ssfw; primer 7, t6ssrv3.
*– Genbank locus of the gene on the C. sakazakii BAA-894 genome (Kucerova et al. 2010)

All investigated strains were harbouring at least one of the investigated sites in the T6SS loci in plasmid DNA. Based on the plasmid and total DNA, 71% of *C. sakazakii* ST4 strains (n=23) harboured the *vgrG* effector protein, while all of ST1 and ST8 were positive. However, 50% of ST12 strains were harboured for this effector. Figure 5.2.

Out of the 23 strains tested, based on plasmid PCR screening one ST4 strain 553 (4 %) was harbouring the 5' region of the T6SS loci (IntT6SS Left), (100%) ST1, 1 of 3 (33 %) ST8 strains;

and 50% ST12 strains possessing the region of this cluster IntT6SS Left. These amplicons could have a similar DNA sequence to the primers or there are other T6SS regions. Similarly, the presence of the IntT6SS Left, the cluster was investigated in total DNA and the results shown in Figures 5.1-5.2. Ten out of 23 (43 %) of *C. sakazakii* ST4 strains, 100% of ST1(n=3), 50% of ST12 (n=2) and 33% (n=3) of ST8 strains were harbouring this region.

Moreover, based on plasmid PCR screening 3% of ST4 strain (n=23) was harbouring another right side region of T6SS loci which have been amplified (T6SS R end and Int T6SS Right), While all of ST1(n=3), 33% of ST8 strains (n=3); and 50% ST12 strains (n=2) were positive. These amplicons could have a similar DNA sequence to the primers or there are other T6SS regions. Similarly, the presence of the T6SS R end and Int T6SS Right, for right side of the T6SS cluster was also investigated in total DNA and the results have been shown in Figures 5.1-5.2. Nine out of 23 (39 %) of *C. sakazakii* ST4 strains, 50% of ST12 and 33% of ST8 strains were harbouring both of T6SS R end and Int T6SS Right region. While all strains of ST1 were positive for this regions.

In order to verify the presence of T6SS patterns in the genomes, BLAST analysis have been used to confirm the presence of this region (ESA_pESA3p05491-5506) (Franco *et al*. 2011). As a result, they were a variation observed within all the *C. sakazakii* clinical strains. However, T6SS patterns have been detected in the most clinical cases such as NECII and severe meningitis strains 696 (ST12), 5 (ST8), 553, 721, 1219, 1220, 1221, 1225, 1231, 1240, 1251 (ST4) and 140 (ST40). Table 5.2. Some of these strain lacking plasmid pESA3 such as 696 (ST12) and 1220 and 1241 (ST4). Therefore is not necessarily plasmid borne, but could be inserted in the chromosome.

| | ID | ST | Source | Plasmid PCR | | | Total DNA PCR | | | % of present T6SS patterns in the *Cronobacter* PubMLST genomic database* |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | IntT6SS left | T6SS R end | IntT6SS Right | IntT6SS left | T6SS R end | IntT6SS Right | |
| *C. sakazakii* | 520 | 12 | Clinical | - | - | - | - | - | - | 6 |
| *C. sakazakii* | 696 | 12 | Clinical | + | + | + | + | + | + | 78 |
| *C. sakazakii* | 1 | 8 | Clinical | - | - | - | - | - | - | 33 |
| *C. sakazakii* | 5 | 8 | Unk | + | + | + | + | + | + | 63 |
| *C. sakazakii* | 680 | 8 | Clinical | - | - | - | - | - | - | 33 |
| *C. sakazakii* | 555 | 1 | Clinical | + | + | + | + | + | + | N |
| *C. sakazakii* | 658 | 1 | Non-infant formula | + | + | + | + | + | + | 100 |
| *C. sakazakii* | 12 | 1 | Clinical | + | + | + | + | + | + | N |
| *C. sakazakii* | 558 | 4 | Unk | - | - | - | - | - | - | 15 |
| *C. sakazakii* | 553 | 4 | Unk | + | + | + | + | + | + | 87 |
| *C. sakazakii* | 767 | 4 | Clinical | - | - | - | - | - | - | 27 |
| *C. sakazakii* | 695 | 4 | Clinical | - | - | - | - | - | - | 22 |
| *C. sakazakii* | 20 | 4 | Clinical | - | - | - | - | - | - | 34 |
| *C. sakazakii* | 557 | 4 | Unk | - | - | - | - | - | - | 33 |
| *C. sakazakii* | 6 | 4 | N | - | - | - | - | - | - | 8 |
| *C. sakazakii* | 730 | 4 | Clinical | - | - | - | - | - | - | 22 |
| *C. sakazakii* | 1242 | 4 | Clinical | - | - | - | - | - | - | N |
| *C. sakazakii* | 1223 | 4 | Clinical | - | - | - | - | - | - | N |
| *C. sakazakii* | 1222 | 4 | Clinical | - | - | - | - | - | - | N |
| *C. sakazakii* | 1585 | 4 | Clinical | - | - | - | - | - | - | N |
| *C. sakazakii* | 1587 | 4 | Clinical | - | - | - | - | - | - | 34 |
| *C. sakazakii* | 701 | 4 | Clinical | - | - | - | - | - | - | 27 |
| *C. sakazakii* | 721 | 4 | Clinical | - | - | - | + | + | + | 50 |
| *C. sakazakii* | 1219 | 4 | Clinical | - | - | - | + | + | + | 50 |
| *C. sakazakii* | 1220 | 4 | Clinical | - | - | - | + | + | + | 50 |
| *C. sakazakii* | 1221 | 4 | Clinical | - | - | - | + | + | + | 50 |
| *C. sakazakii* | 1225 | 4 | Clinical | - | - | - | + | + | + | 50 |
| *C. sakazakii* | 1231 | 4 | Clinical | - | - | - | + | + | + | 50 |
| *C. sakazakii* | 1240 | 4 | Clinical | - | - | - | + | + | + | 50 |
| *C. sakazakii* | 1241 | 4 | Clinical | - | - | - | + | + | + | N |
| *C. sakazakii* | 4 | 15 CC4 | Clinical | - | - | - | - | - | - | 34 |
| *C. sakazakii* | 140 | 40 | Spice | - | - | - | + | - | - | 50 |
| *C. sakazakii* | 150 | 16 | Spice | - | - | - | - | - | - | 13 |
| *C. sakazakii* | 1586 | 9 | Clinical | - | - | - | - | - | - | N |
| *C. sakazakii* | 1588 | 14 | Clinical | - | - | - | - | - | - | N |
| *C. sakazakii* | 1249 | 31 | Clinical | - | - | - | - | - | - | 32 |

**Table 5.2** Type VI Secretion System (T6SS) patterns, of screening of clinical *C. sakazakii* strains in plasmid and total DNA (chromosome and plasmid) including PCR results and genome BLAST analysis.
* BLAST analysis from PubMLST *Cronobacter* database **N**: was not investigated.

### 5.4.1.3 IRON ACQUISITION GENES *eitA* AND *iucC*

The presence of the iron acquisition gene clusters *eitA* and *iucC* was investigated using designed PCR probes of *eitA* and *iucC* genes (Franco *et al*. 2011b). The results are shown in Figure 5.2. The presence of the gene *eitA* was confirmed in 96% of *C. sakazakii* ST4(n= 23) while all of ST1 and ST8 (n=3), and 50% of ST12 strains were harbouring the *eitA* gene in both plasmid and total genomic DNA. The presence of iron acquisition *iucC* gene was also investigated. Ninety-six percent of ST4 strains (n=23), 50% of ST12 strains (n=2).While all of ST1 and ST8 strains were found to be positive for the *iucC* gene in both plasmid and total genomic DNA.

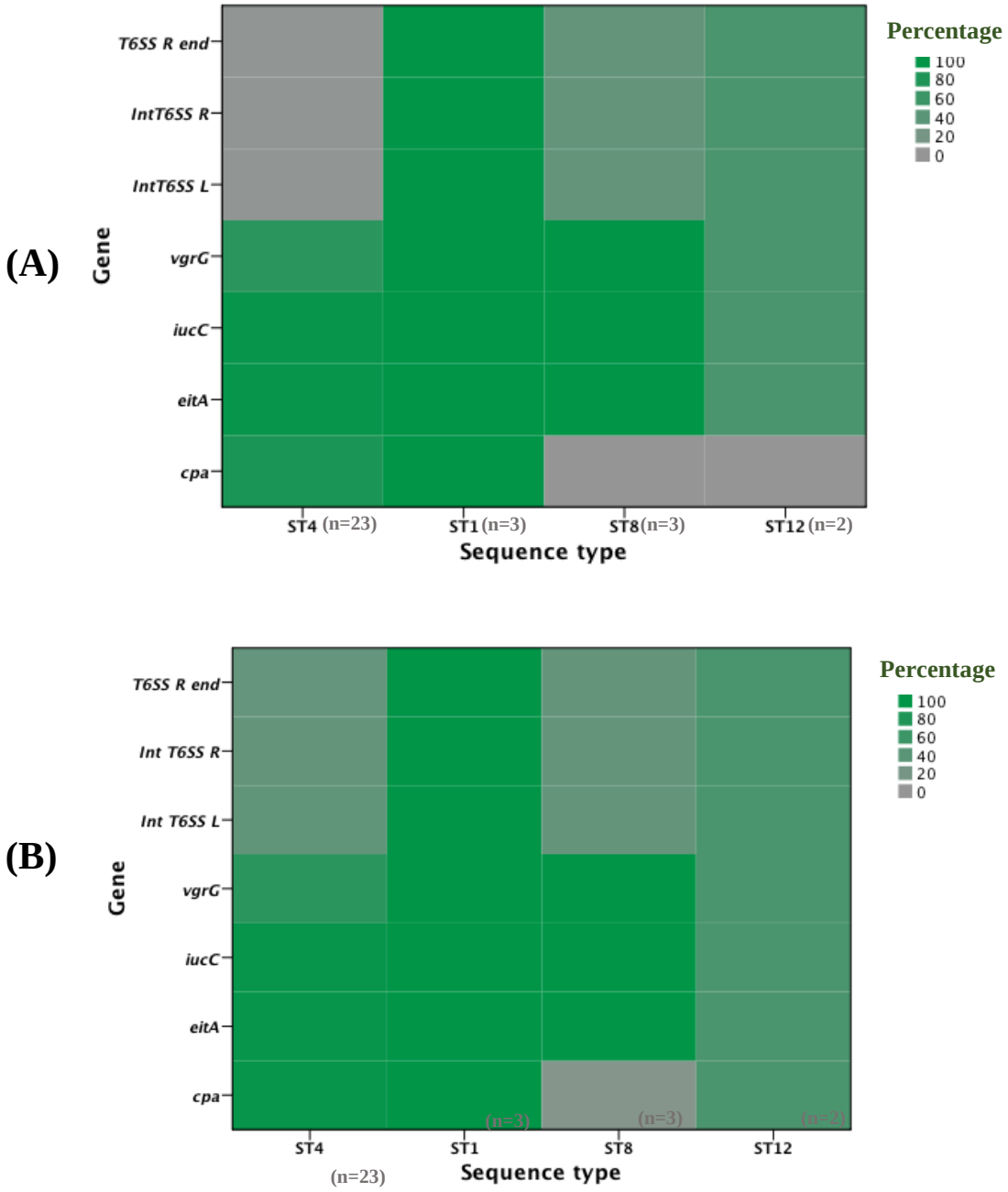


**Figure 5.2** The heat map showing the presence/absence of a number of key virulence associated genes which included: two iron acquisition system gene clusters (*eitA* and *iucC*), *Cronobacter* plasminogen activator (*Cpa*), and type IV secretion (T6SS) gene cluster using laboratory studies in *Cronobacter sakazakii* clinical strains **(A)** on plasmid DNA **(B)** on total DNA in different sequence types of *C. sakazakii.* The difference in colour indicates the presence/absence or percentage of a gene present on plasmid. The heat map was generated using SPSS (version 21).

## 5.4.2 CORRELATION OF *CRONOBACTER* PLASMINOGEN ACTIVATOR (*CPA*) GENE LOCUS, SERUM RESISTANCE AND GENOME STUDY.

This section was to determine whether *cpa* is required for serum resistance and virulence of *C. sakazakii*. Serum resistance assay was carried out on a selection of the clinical *C. sakazakii* strains (n = 36) from the MLST database and PCR screening of *cpa* gene. Most of ST4, ST1, ST8 and ST12 clinical strains were tested (Table 5.3). The presence of *Cpa* in the plasmid could be essential for survival in human serum in all ST4 except strain 6.

In order to verify the present of this gene in the genome, BLAST facility analysis have been used to confirm the presence of *cpa* gene (ESA_pESA3p05434) in *C.sakazakii*. Moreover, the present of Pla plasminogen activator of *Yersinia pestis* (NC_019235) have been undertaken in this research also because its significant homology share with the *cpa*.

As a result, there was no variation observed within the ST4 lineage. In contrast, a considerable degree of variation was observed within non ST4 the ST1 strain 658 with ST8, ST12, ST16, ST40 and ST31 that could some extent explain the variation the host susceptibility. The sequences of *cpa* and *Pla* plasminogen activator were trimmed, aligned using the online tool of Cluster W and MEGA 5 and the phylogeny tree was constructed as shown in figure 5.3.

| Species | Strains | ST | Source | Laboratory PCR screening | | Laboratory | |
|---|---|---|---|---|---|---|---|
| | | | | Plasmid | Total DNA | Serum resistance assay | *Cpa* gene in the *Cronobacter* PubMLST genomic database* |
| *C. sakazakii* | 520 | 12 | Clinical | - | - | S | Absent |
| *C. sakazakii* | 696 | 12 | Clinical | - | + | R | Present |
| *C. sakazakii* | 1 | 8 | Clinical | - | - | S | Absent |
| *C. sakazakii* | 5 | 8 | Unk | - | + | R | Present |
| *C. sakazakii* | 680 | 8 | Clinical | - | - | S | Absent |
| *C. sakazakii* | 555 | 1 | Clinical | + | + | R | N |
| *C. sakazakii* | 658 | 1 | Non-infant formula | + | + | R | Present |
| *C. sakazakii* | 12 | 1 | Clinical | + | + | R | N |
| *C. sakazakii* | 558 | 4 | Unk | + | + | R | Present |
| *C. sakazakii* | 553 | 4 | Unk | + | + | R | Present |
| *C. sakazakii* | 767 | 4 | Clinical | + | + | R | Present |
| *C. sakazakii* | 695 | 4 | Clinical | + | + | R | Present |
| *C. sakazakii* | 20 | 4 | Clinical | + | + | R | Present |
| *C. sakazakii* | 557 | 4 | Unk | + | + | R | Present |
| *C. sakazakii* | 6 | 4 | | - | - | S | Absent |
| *C. sakazakii* | 730 | 4 | Clinical | + | + | R | Present |
| *C. sakazakii* | 1242 | 4 | Clinical | + | + | R | N |
| *C. sakazakii* | 1223 | 4 | Clinical | + | + | R | N |
| *C. sakazakii* | 1222 | 4 | Clinical | + | + | R | N |
| *C. sakazakii* | 1585 | 4 | Clinical | + | + | R | Absent |
| *C. sakazakii* | 1587 | 4 | Clinical | + | + | R | Present |
| *C. sakazakii* | 701 | 4 | Clinical | + | + | R | Present |
| *C. sakazakii* | 721 | 4 | Clinical | + | + | R | Present |
| *C. sakazakii* | 1219 | 4 | Clinical | - | + | R | Present |
| *C. sakazakii* | 1220 | 4 | Clinical | + | + | R | Present |
| *C. sakazakii* | 1221 | 4 | Clinical | + | + | R | Present |
| *C. sakazakii* | 1225 | 4 | Clinical | + | + | R | Present |
| *C. sakazakii* | 1231 | 4 | Clinical | + | + | R | Present |
| *C. sakazakii* | 1240 | 4 | Clinical | + | + | R | Present |
| *C. sakazakii* | 1241 | 4 | Clinical | + | + | R | N |
| *C. sakazakii* | 4 | 15 CC4 | Clinical | - | + | R | Present |
| *C. sakazakii* | 140 | 40 | Spice | - | + | R | Present |
| *C. sakazakii* | 150 | 16 | Spice | - | + | R | Present |
| *C. sakazakii* | 1586 | 9 | Clinical | + | + | R | N |
| *C. sakazakii* | 1588 | 14 | Clinical | + | + | R | N |
| *C. sakazakii* | 1249 | 31 | Clinical | + | + | R | Present |
| *E.coli* | 1230 | - ve | - | N | N | S | N |
| *Salmonella* | 583 | +ve | - | N | N | R | N |

***** BLAST analysis from PubMLST *Cronobacter* database **N:**Unknown genome **S:** Serum sensitivity **R:** Serum resistance **– :** Absent PCR product **+ :** present PCR product **ST:** Sequence type

**Table 5.3** Correlation of *C. sakazakii* plasminogen activator (*cpa*) gene locus and Serum resistance. In the serum resistance assay the viable counts of cells were obtained at the beginning and after 1, 2, 3 and 4 hours of incubation. All bacterial strains have been assayed in 3 independent assays

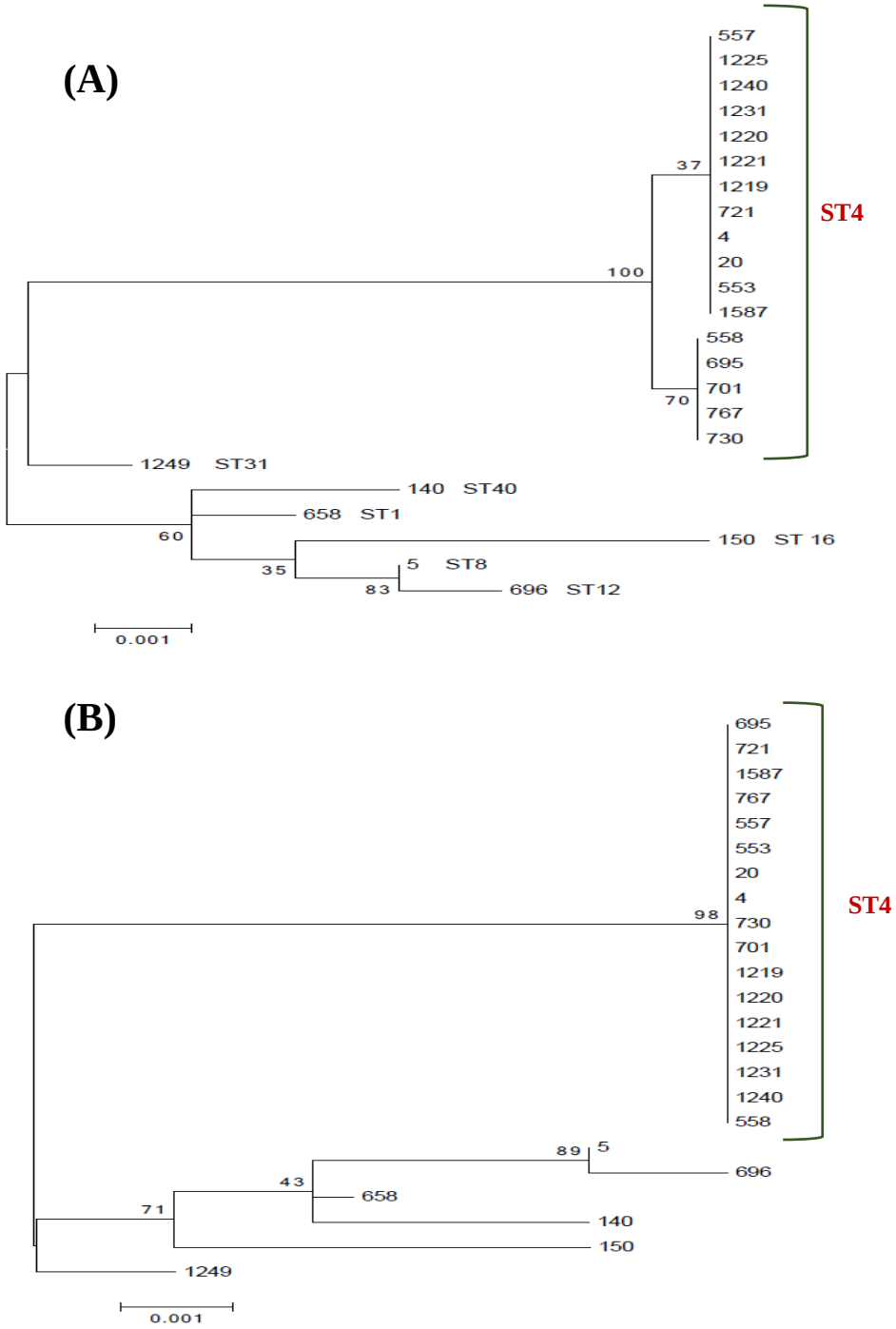


**Figure 5.3** Maximum likehood tree of **(A)** *cpa gene* and **(B)** *Pla* plasminogen activator of *C. sakazakii* clinical strains. The NTU strains IDs are showed at the top of each branches. The tree is drawn to scale using MEGA5, with 1000 bootstap replicates.

### 5.4.3 PLASMID PROFILING

The sequenced strain *C. sakazakii* BAA-894 contains two plasmids; pESA2 (31 kb) and pESA3 (131 kb). Thirty-eight genes were annotated on pESA2 and 127 genes on pESA3 by Kucerova *et al*. (2010). *C. turicensis* z3032 revealed the three plasmids similar to sizes reported by Stephan *et al*. (2011) - pCTU1 (138 kb), pCTU2 (22.5 kb) and pCTU3 (53.8 kb). pESA3 and pCTU1 have been identified several virulence gene clusters encoded on these plasmids *in silico* analysis, such as two iron acquisition system loci (*eitCBAD* and *iucABCD/iutA*), a type six secretion system (T6SS) locus, and a two-partner secretion system (TPS)/filamentous hemagglutinin gene (*fhaB*), and a transporter gene (*fhaC*) and associated putative adhesins (FHA locus). Also, Power *et al*. (2013) published the complete genome of *Cronobacter sakazakii* SP291, with the sequences of three plasmids 118 kb, 52 kb, and 4.4 kb. SP291 have been identified interesting genes associated to the resistance to toxic and antimicrobial and bacterial stress response. Recently Choi *et al*. (2014) identified in *C. sakazakii* ATCC29544 a new plasmid sequenced pCSA2, which encodes *mcp* ( methyl-accepting chemotaxis protein) gene. *Mcp* of *C. sakazakii* ATCC29544 has been reported as essential encoded gene regulated biofilm formation and motility. Furthermore this study demonstrated that the putative *mcp* encoded in pCSA2 was essential for adhesion and invasion.(Choi *et al*. 2014)

In this study, plasmid profiling experiments were carried out on 34 *Cronobacter sakazakii* clinical strains of different sequenced type. It was found that the 34 clinical strains harboured a single or more than two plasmids sized between 138-2.5 kb as summarized in Table 5.3. Because of the large sizes of the *Cronobacter* plasmids, appropriate size DNA ladders could not be used and hence the well-characterized plasmid profiles of the genomes of *C. sakazakii* BAA-894 and *C. turicensis* z3032 were used as reference markers.

Figure 5.4 was showed there is no correlation observed between sequence type and presence or absence of the plasmid. Also, plasmid DNA analysis showed that there was no specific plasmid profiling among clinical strains.

The results showed the presence of the three plasmids or more across *C. sakazakii* strains. A third unstated plasmid similar in size to pCTU3 (53.8 kb) was detected, suggesting the probability that this had not been sequenced with the rest of the genome. The high-size plasmid (molecular weight between 138 and 131 kb) of pCTU1/pESA3 was observed as common in 27/34 of all strains, except *C. sakazakii* 520, 696 (ST12) and strain 6, 1220,1241,4 (ST4).

An intact plasmid corresponding to the size of pCTU3 53.8-52 kb was observed in the strains 658 (ST1), 1219,1220,1221,1223,1240,1241,1225,1585,1587 (ST4) and 1588,150,140 ( non ST4).

An intact plasmid matching to the sizes of pCTU2/pESA2 (22.5- 31 kb) was showed by all the *C. sakazakii* strains except 5 (ST8), 520 (ST12), 553,557, 695,767,6,730 (ST4) and 1586 (non ST4)

A slightly smaller sized plasmid was also detected in the profiles of strain 1, 5 (ST8), 558,721,1221,1222,1223,1231,1242,1225,1587( ST4) and 1586,140 (non ST4).

Both strains 6 (ST4) and 520 (ST12) were plasmid less strains. This most significant observation of missing virulence associated genes is in agreement with results in this chapter (Section 5.4.1). The aim of next chapter was to verify the important of presence of plasmids associated with virulence of *C. sakazakii* by transforming the large plasmid to plasmid-less strains and observe any phenotypic change.

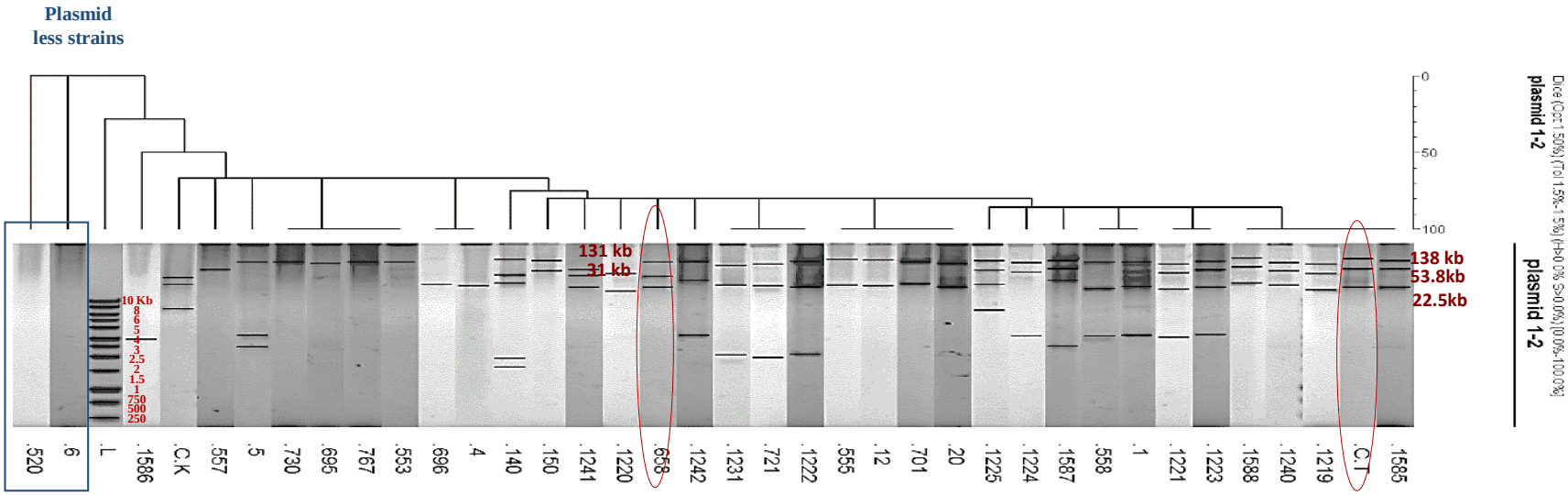


**Figure 5.4** The agarose gel was analysed using BioNumerics software, version 3.5. Dice coefficient, unweight pair group method with arithmetic mean (UPGMA) for cluster analysis of the plasmid profiles of the *Cronobacter* spp. strains sequenced in this study. The plasmid profiles of the strains *C. sakazakii* BAA-894 and *C. turicensis* z3032 (indicated by the red circles) were used as markers, as their sizes had been accurately determined by sequencing studies (Kucerova *et al*. 2010; Stephan *et al*. 2011).

| | | | **Plasmid sizes (kb)** | | | | |
|---|---|---|---|---|---|---|---|
| **Strain No** | **STs** | **Origin** | **(138-131-118)** | **(53.8-52)** | **(31-22.5)** | **(8-4-2.5)** | **Plasmid No** |
| 658*b | ST1 | Non-infant formula | √ | √ | √ | | 3 |
| 555 | ST1 | Clinical | √ | | √ | | 2 |
| 12 | ST1 | Faecal- Clinical | √ | | √ | | 2 |
| 1* | ST8 | Throat – Clinical | √ | | √ | √ | 3 |
| 5* | ST8 | Clinical | √ | | | √ | 2 |
| 520* | ST12 | Clinical | | | | | - |
| 696* | ST12 | Faecal-Clinical (NECII) | | | √ | | 1 |
| 553* | ST4 | Clinical | √ | | | | 1 |
| 557* | ST4 | Clinical | √ | | | | 1 |
| 558* | ST4 | Clinical | √ | | √ | √ | 3 |
| 695* | ST4 | Trachea-Clinical (Fatal NECII) | √ | | | | 1 |
| 701* | ST4 | Peritoneal- Clinical (Fatal NECII) | √ | | √ | | 2 |
| 767* | ST4 | Trachea-Clinical(Fatal meningitis) | √ | | | | 1 |
| 6* | ST4 | Clinical | | | | | - |
| 20* | ST4 | Faecal-Clinical | √ | | √ | | 2 |
| 721* | ST4 | CSF –Clinical | √ | | √ | √ | 3 |
| 1219* | ST4 | CSF-Clinical (Fatal meningitis) | √ | √ | √ | | 3 |
| 1220* | ST4 | CSF –Clinical (Brain abscess) | | √ | √ | | 2 |
| 1221* | ST4 | CSF –Clinical (Meningitis) | √ | √ | √ | √ | 4 |
| 1222 | ST4 | Blood- Clinical | √ | | √ | √ | 3 |
| 1223 | ST4 | Blood- Clinical | √ | √ | √ | √ | 4 |
| 1231* | ST4 | Faecal-Clinical (Meningitis) | √ | | √ | √ | 3 |
| 1240* | ST4 | CSF –Clinical | √ | √ | √ | | 3 |
| 1241 | ST4 | Blood- Clinical | | √ | √ | | 2 |
| 1242 | ST4 | Brain- Clinical | √ | | √ | √ | 3 |
| 1225* | ST4 | Blood -Clinical(Fatal meningitis) | √ | √ | √ | √ | 4 |
| 730* | ST4 | Clinical(NECI) | √ | | | | 1 |
| 1585 | ST4 | Blood –Clinical (Bacteraemia) | √ | √ | √ | | 3 |
| 1587* | ST4 | CSF –Clinical (Sever anatomical damage brain) | √ | √ | √ | √ | 4 |
| 4* | ST15 | Clinical | | | √ | | 1 |
| 1588 | ST14 | Blood- Clinical | √ | √ | √ | | 3 |
| 1586 | ST9 | Blood –Clinical | | | | √ | 1 |
| 150* | ST16 | Spice | √ | √ | | | 2 |
| 140* | ST40 | Spice | √ | √ | √ | √ | 4 |

*Strain have been genome sequenced in the *Cronobacter* PubMLST database **ST:** Sequence type

**Table 5.4** Plasmid profile patterns of *Cronobacter sakazakii* strains isolated from clinical sources.

## 5.4.4 IDENTIFICATION OF *CRONOBACTER* IRON ACQUISITION SYSTEM

Whole genomes have been studied in the last stage of the current thesis in order to advance our understanding of the *Cronobacter* genus (specific *C. sakazakii* in this study) and to verify the important of specific associated virulence trait such as iron acquisition system by using BLAST facility(http://pubmlst.org/perl/bigsdb/bigsdb.pl?db=pubmlst_cronobacter_isolates&page=plugin&name=BLAST).

Bacterial strains used in this study are detailed in Table 2.1 Chapter 2 Section (2.2). The *Cronobacter* and closely related genomes strains species screened for iron acquisition system including plasmids carry several putative virulence genes *eitCBDA* (ABC transporter genes cluster) and *iucABCD/iutA* (aerobactin sidrophore receptor genes). Also non-plasmid iron acquisition genes (ferric dicitrate transport system) targets that have been consisted of *C. sakazakii* (n=70), *C. malonaticus* (n=14), *C. turicensis* (n= 6), *C. dublinesis* (n=8), *C. universalis* (n=1), *C. muytjensii* (n=3) and *C. condimenti* (n=1). In addition, closely related species (n=4) *Franconibacter pulveris* (n=3) *Siccibacter turicensis*, (n=6) and *Franconibacter helveticus* were included for comparative reasons.

The present study was shown in Figure 5.5, that *iucABCD/iutA* is a siderophore present in 97% of *C. sakazakii* (n= 70). The strains missing these genes were plasmid-less strain 6 (ST4) and 520 (ST12) which were confirmed later as a negative for siderophore production in the selected numbers of *C. sakazakii* (n=16 ) as shown using the CASAD assay; Figure 5.7.

Moreover, one of striking features of ferric dicitrate transport system investigation was found especially in small subset of *C. sakazakii* 18 % (n= 70) (plasmid-less strain, non-ST4) and 30 % (n=14) *C. malonaticus*. Most of these were isolated from clinical sources. This system is capable of maintaining bacterial growth in the absence of other iron uptake system. In contrast, this system have been missing in other species; Figure 5.6.

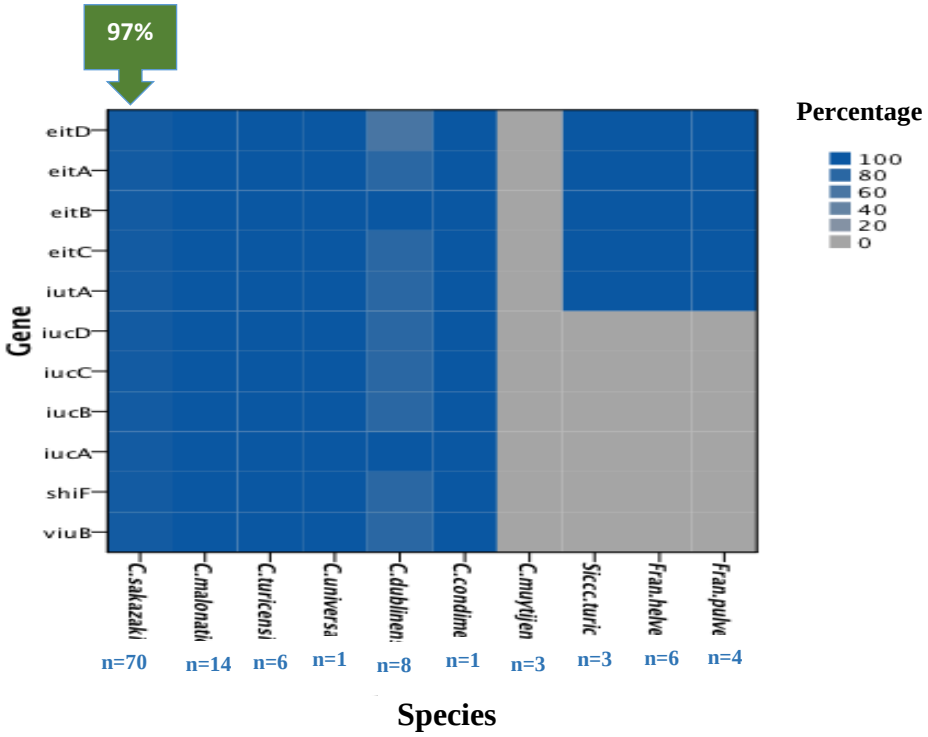


**Figure 5.5** The heat map showing the presence/absence of potentially virulence associated traits for iron acquisition system including plasmids carry several putative virulence genes *eit*CBDA (ABC transporter genes cluster) and *iuc*ABCD/*iut*A (aerobactin sidrophore receptor genes) in the *Cronobacter* and closely related species genomes strains. The difference in colour indicates the presence/absence or percentage gene based on BLAST analysis from PubMLST *Cronobacter* database. The heat map was generated using SPSS (version 21).

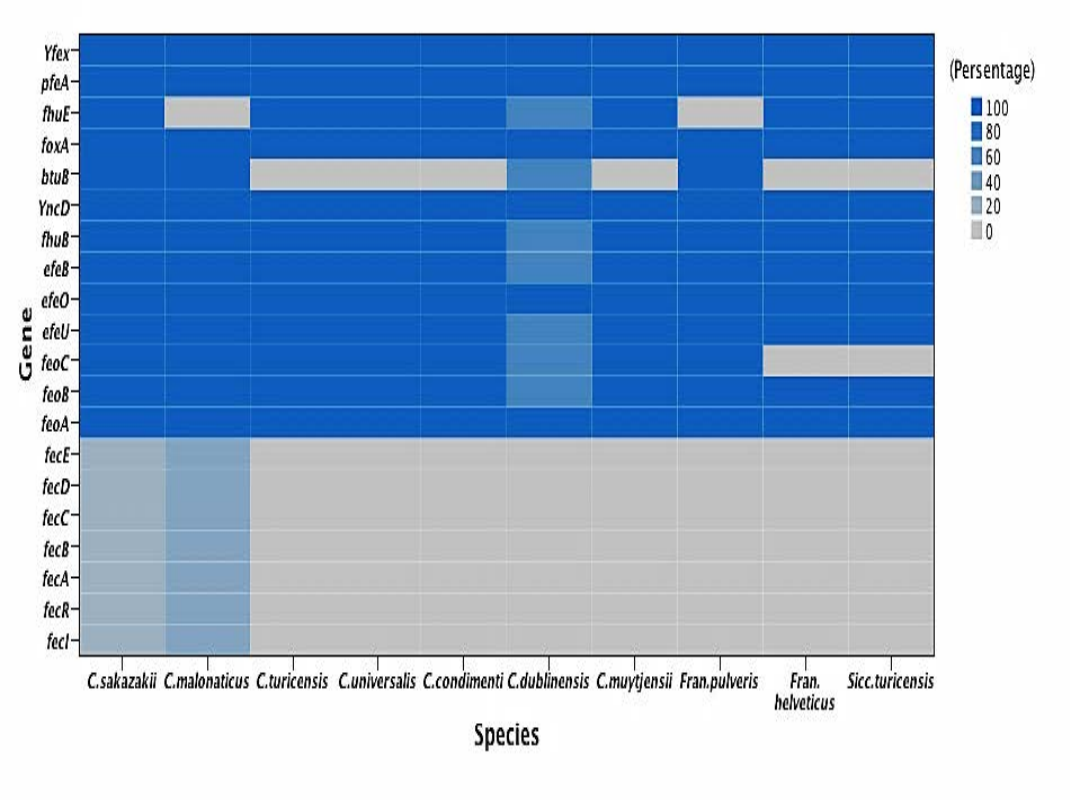


**Figure 5.6** The heat map showing the presence/absence of potentially virulence associated traits for iron acquisition system including non- plasmid iron acquisition genes (ferric dicitrate transport system) in the *Cronobacter* and closely related species genomes strains. The difference in colour indicates the presence/absence or percentage gene based on BLAST analysis from PubMLST *Cronobacter* database. The heat map was generated using SPSS (version 21).

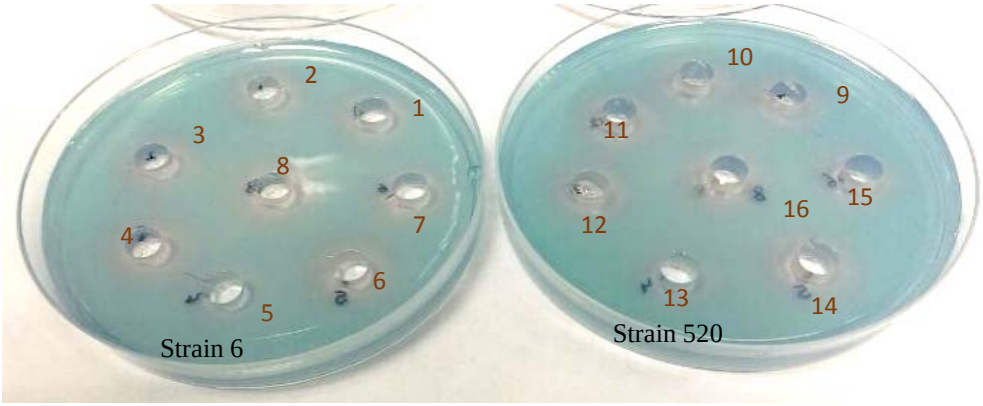


**Figure 5.7** Siderophore activity using CASAD assay, wells were filled with cell free culture supernatant of different clinical strains of *C. sakazakii* (1- 16) shows all of these strains have been able to produce iron sidrophores CAS agar showing orange halo around the site of inoculation, however NTU #6 and 520 strains was negative.

## 5.5 DISCUSSION

Bacterial plasmids are found to be encode a wide-ranging of pathogenic factors which include resistance to antibiotics, toxins, factors causing adherence, and secretion systems (types 3, 4 and 6) (Johnson and Nolan 2009).

The study of the difference in plasmid content extends from our group's earlier CGH studies (Kucerova *et al*. 2010). It was reported that the publicly available *C. sakazakii* BAA-894 plasmid pESA3 (131 kb) or *C. turicensis* z3032 pCTU1 (138 kb) made a conserved backbone, with one copy being present in most of the strains and this was confirmed by both the plasmid profiling as well as by *in silico* analysis. In the earlier laboratory studies of the plasmid regions ( Franco *et al*. 2011), showed the presence of this plasmid in 97% of their 229 *Cronobacter* spp. strains using by PCR. They also recognised these plasmids to belong to RepFIB incompatibility group, characterized by the *repA* gene as an origin of replication. This plasmid backbone is especially essential for this bacterium as they have been considered to be virulence plasmids, with genes encoding for potential virulence traits for example, iron acquisiton systems (*eitCBAD* and *iucABCD/iutA*).

The finding of this research indicate that 34 clinical strains harboured a single or more than two plasmids sized between (138-2.5 kb).The high-size plasmid (molecular weight between 138 and 131 kb) was observed as common in (27/34) of all strains which known to encode an assortment of virulence factors such as iron acquisition and *cpa* plasminogen activator genes. This finding suggested the present of large plasmid may be essential for systemic survival of *C. sakazakii* in a host ( Franco *et al*. 2011).

Also, plasmid DNA analysis showed that there was no specific plasmid profiling among clinical strains. This could be due to a high rate of plasmid transfer or instability amongst strains. Furthermore, it shown there is no correlation observed between sequence type and present or absent the plasmid.

The present study showed also a group of interesting clinical strains ST4 (1223,1221,558,1224 and 1242) and ST8 (1 and 5) which have been isolated from CSF, blood and throat encoding smaller plasmid molecular weight between 6 and 2 bp. This research confirmed the findings of previous studies by Choi *et al*. (2014) that identified a new plasmid sequenced pCSA2 in *C. sakazakii* ATCC29544 (5,103 bp), which encoding *mcp* ( methyl-accepting chemotaxis protein) gene. This putative *mcp* was essential virulence factor regulated genes of adhesion and invasion, biofilm formation and motility. Also in agreement with Power *et al*. (2013) published study of complete genome of *Cronobacter sakazakii* SP291, which contain small plasmid pSP291-3 (4.4 kb). SP291 carries interesting genes associated with the resistance to toxic and antimicrobial and bacterial stress response.

### 5.5.1 *CPA* (PLASMINOGEN ACTIVATOR)

Plasmid pESA3 of *Cronobacter sakazakii* BAA 894 contains a *Cronobacter* plasminogen activator gene (*cpa*), an outer membrane protease reported to provide serum resistance to *C. sakazakii* possibly enhancing its invasion and ability to spread within the host. The *cpa* gene is closely related to plasminogen activators in *Salmonella enterica* and *Yersinia pestis* (Franco *et al.* 2011). This suggests possible horizontal genetic transfer to *C. sakazakii*.

There are protective mechanisms in invasive microorganisms which work against serum-mediated killing. Bacterial structures, comprising of outer membrane proteases and proteins were known to prevent such bactericidal action (Schwizer *et al*. 2013; Rautemaa and Meri 1999; Taylor 1983). Lately, a study by Franco *et al*. (2011b) indicated that one such plasminogen activator is the *Cronobacter* outer membrane protease Cpa. It is quite significant with regard to serum resistance. In this research, a group of *C. sakazakii* strains were tested to determine their capability to resist human serum. Most of the strains were considered to be resistant and able to replicate in serum and they seem to entirely refractory to serum killing. that is similar to *S.* Enteritidis the positive control strains for this experiment. The negative strains included *C. sakazakii* strains 6 (ST4), 680 and 1 (ST8), 520 (ST12) and *E. coli* K12 (negative control), which was sensitive. Resistance of

serum killing is a significant factor that results in survival in the host blood. It could also be involved in development of bacteraemia. Serum bactericidal activity were resisted by all the strains which contain the *cpa* gene, whilst the sensitive strains were 680,1 (ST8),520 (ST12) and 6 (ST4) as they did not have the *cpa* gene (Table 5.3). An interesting point in this study, some of the isolates lacking plasmid pESA3 which encoding *cpa* gene such as strain NTU 520, 696 (ST12) and 1220, 1241, 4 (CC4) showed serum resistance. Therefore *cpa* is not necessarily a plasmid borne trait but could also be inserted in the chromosome. In addition, strains lacking plasmid pESA3 were associated with severe clinical cases; 696 NECII and 1220 with meningitis. Plasmid profiles showed the discrimination by molecular size only, not the gene content of each strains.

There were other genes which could be contributing to serum resistance such as *kpsA* (polysaccharide capsular gene) and *rcsA* (responsible for colonic acid production) (work unpublished). The interest of studies in this field has also increased regarding serum resistance mechanism. But there is not much published data regarding *C. sakazakii*. Schwizer *et al*. (2013) performed a study using transposon knock out mutants, which observed that serum sensitivity decreased due to removal of some structural and regulatory genes of *C. sakazakii* strain ES5. They stated that eradicated expression of the major element of type 1 fimbriae occurred due to the removal of the ybaJ element, which is part of anti-toxin pair YbaJ-Hha. This results in increased survival in the human serum. Nevertheless, this observation does not include the pathogenicity of the bacterium, since type 1 fimbriae is a significant aspect for adhesion. It has also been associated to *E. coli* K1 invasion of human brain micro vascular cells (Adegbola and Old, 1983 and Teng *et al*. 2005).

### 5.5.2 TYPE IV SECRETION SYSTEMS

Secretion systems, commonly known as type VI secretion systems, which have the ability to transport proteins and nucleoprotein complexes are understood to be essential virulence factors as they had been revealed in *C. sakazakii* and *C. turicensis* as a plasmid-borne (pESA2/pCTU2) gene cluster (Franco *et al*. 2011b). On the other hand, quite a few newly identified type VI secretion

systems (T6SSs) were present in the chromosomes of many *Cronobacter* spp. and on pESA3 which is carried by *C. sakazakii*, and include pathogenic *C. sakazakii* ST4 strains (Joseph *et al*. 2012c). It is important to note that the T6SS was also discovered to be essential for *E. coli* K1 invasion of the BBB (Zhou *et al*. 2012).

Type VI secretion system (T6SS) is a recently discovered system which may challenge different bacteria in host-cell invasion, adherence, growth inside macrophages, and survival inside the host. One specific T6SS related gene which was found isolated from the central T6SS gene cluster, *vgrG*, encodes for a lipoprotein (ESA_00292-4). In the *Cronobacter* genomes, six putative T6SS clusters were found (Joseph *et al*. 2012), of which some had been reported before by Kucerova *et al*. (2010, 2011).

### 5.5.3 IRON ACQUISITION SYSTEM

It was shown by Franco *et al*. (2011a) that *Cronobacter* RepFIB plasmids encode two iron acquisition systems – a siderophore-mediated system for iron acquisition (*iucABCD/iutA* operon) and ATP-binding cassette transport- mediated iron uptake and a system of siderophore (*eitCBAD* operon) –which suggested that such plasmids are ordinary essential plasmids and might also play an important role in the systematic survival of *Cronobacter*. To develop an infection after they have penetrated the host cell, iron-acquisition abilities are necessary for many pathogens. Franco *et al*. (2011b) explained that the *iucABCD/iutA* siderophore (cronobactin) is the singular functional siderophore which *Cronobacter* possesses. But Grim *et al*. (2012) showed that cronobactin – a hydroxamate-type, aerobactin-like siderophore –was not the singular iron acquisition system which was possessed by *Cronobacter*. In the present study showed that *iucABCD/iutA* is the active siderophore genes present in 97% of *C. sakazakii*. The strains missing these genes were plasmid-less strain. However, due to the presence of iron utilization genes in all of the *Cronobacter* spp., the details of its function still have not been understood (Joseph *et al*. 2012c).

Grim *et al*. (2013) described analysis which were silico sequence targeted of nine *Cronobacter* genomes and demonstrated that there is sharing of iron acquisition systems between the seven

species in *Cronobacter*. This contain iron acquisition genes ferric and ferrous transporters and haem-iron extractors, along with putative TonB-dependent iron receptors and ferric reductases.

This study also showed, the ferric dicitrate transport system had been revealed majorly in a small subset of *C. sakazakii* and *C. malonaticus* strains, many of which had been isolated from clinical samples, describing that this iron acquisition system contributes to the virulence of *Cronobacter*. Such studies also gave proof of the presence of two other receptors, Fct and FcuA, in *C. dublinensis* and *C. muytjensii*, for the heterologous siderophores formed by plant pathogens, suggesting the advantage of these receptors to *Cronobacter* spp. to fight more successfully for iron in a plant niche. It also supports the hypothesis stating that *Cronobacter* arose from a shared ancestor containing a plant-associated lifestyle before it had been species-level bi-directionally divided.

The analysis of this chapter indicated that the pathogenesis of *Cronobacter sakazakii* by determining the presence of a number of key virulence associated genes which included: two iron acquisition system gene clusters, *Cronobacter* plasminogen activator (*Cpa*), and type IV secretion (T6SS) gene were not unique to specific STs. Moreover, the finding of this research indicate that 28 out of 34 clinical strains harboured the large plasmid which is known to encode an assortment of virulence factors. As well as, it shown there is no correlation observed between sequence type and present or absent the plasmid.

Hence, from this point onward, it was decided to investigate whether the presence of plasmids is associated with virulence of *C. sakazakii* by transforming the plasmid pESA3 into a plasmid-less strain and to observe any changes in the phenotypic and virulence associated behaviour. The analysis is presented in Chapter 6.

In summary;

- Number of key traits such as type six secretion systems, iron acquisition systems and serum resistance were found to be scattered across the clinical strains, with varying levels of diversity. Some of the virulence traits were also identified to be plasmid-borne.

- The genome did not reveal any unique virulence traits exclusive to ST4, hinting at the

possibility of gene expression playing a greater role in the virulence phenotype of the organism, rather than just the presence or absence of the virulence associated genes.

# CHAPTER 6

# TRANSFER OF THE VIRULENCE ASSOCIATED PLASMID pESA3 INTO THE PLASMID LESS *C. SAKAZAKII* ISOLATE AND ITS CHARACTERIZATION.

## 6.1 INTRODUCTION

### 6.1.1 VIRULENCE STUDIES

Most plasmids have selectable phenotypic traits, one of the most frequently targeted being resistance to antibiotics. Plasmid genomes contain clusters of genes termed antibiotic cassettes, which play a very essential role in the protection mechanism of the bacterial cell against antibiotic activity. It has been reported that the vast majority of plasmids carry important virulence correlated genes related to adhesion factors, secretion systems, siderophores, serum resistance, and toxin encoding genes. In addition there can be genes linked to metabolic functions. Through the evolutionary process, as a consequence of positive selection, plasmids could at times lose these accessory regions by conjugative transfer to the host chromosome (Frost *et al*. 2005; Johnson and Nolan 2009; Rankin *et al*. 2011).

The *in silico* analysis of *C. sakazakii* BAA-894 (pESA3) and *C. turciensis* z3032 (pCTU1) was performed by Franco *et al*. (2010). The analysis led to the identification of various virulence gene clusters encoded on these plasmids pESA3 (131 kb, 56% GC), encoding for 127 genes. In contrast, pCTU1 (138 kb, 56% GC), encoding for 136 genes respectively. Amongst them were two iron acquisition system loci (*eitCBAD* and *iucABCD*/*iutA*), a type six secretion system (T6SS) locus, a transporter gene (*fhaC*), a two-partner secretion system (TPS)/filamentous hemagglutinin gene (*fhaB*) and associated putative adhesins (FHA locus). The occurrence of these homologous plasmids among the species groups as well as the distributions of the virulence gene clusters was ascertained through laboratory screening of large collections of *Cronobacter* spp.

The outer membrane protease (*cpa*) was encoded by the pESA3, which has significant identify to the pla sub family of omptins. This protease was observed to increase spread and invasion in the host and ability to resist serum by activating the plasminogen, plasmin inhibitor α 2-AP and cleaving complement (Franco *et al*. 2011).

The fact that *Cronobacter* can attach to human intestinal Caco2 cells and survive in macrophages was reported by Townsend *et al*. (2007a). The ability of the *Cronobacter* to invade the CSF circulation was also proved by these researchers. Their research further claimed that a massive influx of inflammatory cells was caused by the organisms into the ventricles and meninges, thus resulting in the breakdown of adhesion junctions and consequently accessing the brain parenchyma. Also the ability of the *Cronobacter* to invade the capillary endothelial cells was reported. They were also found to resist for 96 hours in the macrophages. There were variations in the ability of the strains to cross the blood brain barrier and cause CNS infections.

Seven *Cronobacter* strains linked to the largest reported NICU outbreak (Caubilla *et al.* 2007) was studied by Townsend *et al.* (2008a) have been caused the most fatalities. As compared to the *E coli* K12 and *Salmonella*, all strains were seen to attach and invade the intestinal cell line Caco2 and survival on macrophage for up to 96h. Recently, Eshwar *et al* (2015) studying the possible influence of macrophage infectivity potentiator (Mip) such as (*fkpA*) gene in the intracellular survival of *Cronobacter* spp in human macrophages as a virulence factor. Also the finding showed a variation in *Cronobacter* spp within macrophage survival and replication as a result to the differences in amino acid of *fkpA* protein (Eshwar *et al*.2015)

The genetic basis of *Cronobacter* virulence needs further study, to date the virulence studies have focused upon the effects of the organism on tissue culture cell lines or *in vivo* models. The role of the outer membrane protein, OmpA, was emphasized in various studies through the invasion of intestinal epithelial and brain microthelial cells (Singamsetty *et al*. 2008; Mittal *et al*. 2009b; Wang & Kim 2002; Kim *et al*. 2010). As compared to wild type strains, *Cronobacter* strains deficient in the *OmpA* gene showed lower invasion of HBMEC. According to the studies, microfilaments were needed in the invasion of intestinal epithelial cells by the bacterium, while microtubules are needed in the invasion of HBMEC.

## 6.1.2 TARGETED GENE DISRUPTION USING λ -RED TECHNIQUE

Various methods can be used to create gene disruptions through targeted gene deletions. A common example is the transformation of linear or plasmid DNA carrying an antibiotic resistance marker flanked by regions of homology to the target gene into bacterial cells. A recombination event between the homologous regions is created. The newly developed antibiotic resistance and genetic disruptions are ascertained through the screening of the cells while identifying the accurate phenotype by PCR and sequencing of the target region.

This strategy has been explored through a wide range of experimental methods. At first strains deficient in the RecBCD nuclease system were only included in the first protocols that used linear DNA for gene replacement (Jasin and Schimmel 1984; Winans *et al*. 1985), while facing the chances of degradation of the linear double stranded phage DNA in the other case (Murphy. 1998).

The combination of the plasmid with the bacterial chromosome and subsequent resolution of the integrated complex is utilized in other methods. These methods have led to the regeneration of the wild type locus or any other alternative. The protocols were limited by the restriction of only using plasmids that do not replicate under the conditions applied for the selection of the required mutants. This meant that temperature sensitive plasmids or phagemid-based vectors had to be used. The cell division resulted in the disappearance of the plasmids carrying the marker. The low frequency of the resolution of cointegrate was also a drawback. In addition, the unintentional gene replacements created by the replacement can also create problems.

Murphy (1998) developed the first gene replacement protocols that were based on bacteriophage λ recombination genes known as"Red" or "λ-Red". The activity of λ bacteriophage genes *exo, bet* and *gam* were used by the λ-Red system. Single stranded DNA overhangs are created by the (5′-3′) exonuclease Exo that binds to the dsDNA in the presence of the host RecA protein. The recombinations between the overhangs and homologous regions of the chromosomes are induced

by the BET protein. The hostRecBCDexonuclease is obstructed by the Gam, which is responsible for the digestion of the phage DNA.

A simple and effective method was developed by Datsenko and Wanner (2000) to improve the replacement technigue of λ-Red gene which have been widely used for generating the chromosomal genes in *Escherichia coli* and other bacteria. The homology is provided in this method in desired chromosomal sequence have been replaced by ~26 nt homology of PCR product at each end to target sequence flanking the gene to be replaced. Gene replacement occurs via λ-Red recombination that have been mediated by pKD46. pKD46 has a sensitive temperature replicon that could eliminate by growth at 37°C.

### 6.1.3 STUDIES IN THE *CRONOBACTER*

Regarding to *Cronobacter* researches, it has been reported by Kim *et al*. (2010) as the first time using lambda red recombination method as described previously by Datsenko and Wanner (2000). This study generated deletion mutants of *C. sakazakii* ATCC29544 for outer membrane protein A (OmpA) and (OmpX). This study showed the presence of OmpX played roles in the invasion of the host cells and translocate into liver and spleen of rats (Kim *et al.* 2010). Another study was undertaken in 2013 showed a deletion mutants of *C. sakazakii* ATCC29544 by modified λ-red recombination method of thermotolerance island (Datsenko and Wanner 2000). This thermotolerance island could promote the survival in production facilities (Orieskova *et al*. 2013).

Recently, Kim *et al.* (2015) generated mutants using lambda red recombination (Datsenko and Wanner 2000) to demonstrate the role of hfq in pathogenesis of *C. sakazakii* ATCC29544. The mutant have been generated indicating defect in invasion and survival within host cells (Kim *et al*. 2015)

In the earlier study by Franco and his colleagues (2011), showed the ability of *Cpa* in the wild type strain of BAA-894 to serum resistance than the construction of Cpa isogenic mutant strain. This mutant have been generated by detected the *Cpa* gene and flanking region in pESA3. The result

indicated that *Cpa* isogenic mutant strain reduced serum resistance in comparison to *C. sakazakii* BAA-894 parent strain. Also the observation made show the over express of Cpa protein caused inactivation of α 2-AP and activation of plasminogen (Franco *et al*. 2011 a)

Another research in 2011 by the same author have determined if pESA3 encoding the functional active siderophore system by curing the pESA3 from *C. sakazakii* BAA-894. This curing method have been done by labelling with ampicillin resistance gene to disrupt specific target gene. The finding was the wild type strain (harbouring plasmid pESA3) possessed siderophore activity as a unique plasmid associated. (Franco *et al*. 2011b).

### 6.1.3 AIMS OF THE CHAPTER

Developing a tool in order to show inserted of plasmid well characterized pESA3 into plasmid less strain (NTU 6) and observing any changes in its phenotypic and virulence associated behaviour including serum resistance, siderophore and tissue culture. Due to the variation within *Cronobacter sakazakii* strains during this research based on plasmid profile and virulence gene contents such as iron acquisition system loci (*eitCBAD* and *iucABCD*/*iutA*), outer membrane protease (*cpa*) and type six secretion system (T6SS). Moreover, it is known in our group at NTU (work unpublished) some of *Cronobacter* spp. resistance to serum which have not encoding *Cpa*.

## 6.2 METHODS AND MATERIALS

The key methods, culture media and culturing condition for this section were described previously in Chapter 2 Materials and Methods section.

### 6.2.1 BACTERIAL STRAINS AND PLASMIDS:

| Strains, plasmid and primer | Features | Source |
|---|---|---|
| *E.coli* K12 (NTU 1230) | HB101 | NTU collection |
| *Salmonella enterica* serovar Enteritidis (NTU 358) | NCTC3046 | NTU collection |
| *Citrobacter koseri* (NTU 48) | SMT319 CSF, meningitis case, USA | NTU collection |
| *C. sakazakii* ATCC BAA-894 (pESA3) (NTU 658) | The first sequenced strain of C. sakazakii -sequence type 1 from formula, 2001,USA | Townsend et al 2007-Kucerova et al 2010 |
| *C. sakazakii* (NTU 6) | Plasmid- less strain, negative siderophore production, non-invasive | NTU collection |
| NTU 6 (pESA3K) | Strain 6 after insertion of a kanamycin resistance cassette. | This study |
| *Y. enterocolitica* 8081 | BT1B O: 8. Sequenced strain of human origin from septicaemic patient | Portnoy *et al*. (1981) |
| *C. sakazakii* (NTU 520) | Plasmid- less strain, negative siderophore production | NTU collection |
| **Primers** | | |
| KanpESA3_F | CGAGCGGCGGCGTCATTAAACCGTTATTAGT GTGTAGGCTTGGACTGCTTC | This study |
| KanpESA3_R | CGCGTGATGTTATATTGCGCCAGCGCCGATCA TATGAATATATCCTCCTTAG | |
| Kan_F | TGTGTAGGCTGGACTGCTTC | Datsenko & Wanner, 2000 |
| Kan_R | CATATGAATATCCTCCTTAG | |
| FusA_F | GAAACCGTATGGCGTCAG | http://pubbmlst.org/ *cronobacter*. |
| FusA_R | AGAACCGAAGTGCAGACG | |
| **Plasmids** | | |
| pAJD434 | 6566 bp Trimethoprim TmR plasmid | Alan Mcnally,UK |
| pKD4 | 3267 bp Kanamycin KR plasmid | Datsenko & Wanner, 2000 |
| pESA3 | The larger plasmid of *C. sakazaki*i ATCC BAA-894 131 KB, 56 % GC-encoding 127 genes | Kucerova et al 2010 |
| pESA3K | pESA3 after insertion of a kanamycin resistance cassette | This study |

**Table 6.1** List of strains used in this study

## 6.2.2 PCR VERIFICATION

To verify the insertion sites in the identified plasmid, specific primers were designed to amplify the insertion site by PCR. Both forward and reverse primers were designed.

**KanpESA3_F**

CGAGCGGCGGCGTCATTAAACCGTTATTAGTGTGTAGGCTGGACTGCTTC

**KanpESA3_R**

CGCGTGATGTTATATTGCGCCAGCGCCGATCATATGAATATCCTCCTTAG

PCR products were generated by using several pairs of 50nt containing a good mixture of A , C , T and G that includes 30nt homology extensions of 658 plasmid pESA3 and 20nt priming sequence for PKD4 as template. PCR products were purified and the correct structure of the amplicons was observed using gel electrophoresis.

## 6.2.3 GENERATION OF LINEAR DNA PCR FRAGMENT

Oligonucleotide primers were either designed manually. All primers were synthesised by eurofins mwg /operon. Primers used in this study are shown in Table 1.6. Each PCR reaction was carry out in a final volume of 50 µl.

PCR amplification were carried out as follows:

| | |
|---|---|
| *Tag* DNA polymerase | 0.3 µl |
| 10% buffer | 5 µl |
| d $H_2O$ | 33.7µl |
| Forward primer ( 10 pmol/ µl) | 1 µl |
| Reverse primer ( 10 pmol/ µl) | 1 µl |
| dNTP | 2 µl |
| $MgCl_2$ | 5 µl |
| pKD4 | 2 µl |

**PCR CONDITION:**

| | | |
|---|---|---|
| 1 cycle of: | | |
| Denature | 94°C | 5 minutes |
| 30 cycles of: | | |
| Denature | 94°C | 30 seconds |
| Anneal | 70°C | 30 seconds |
| Elongation | 72°C | 10 minutes |

5 µl was checked on an agarose gel before the remaining PCR reaction was cleaned up with Qiagen AIA quick PCR purification kit. The DNA eluted using 50 µl elution buffer.

### 6.2.4 MINI PREP PLASMID PURIFICATION OF pKD4

Bacteria were grown overnight in 5 ml TSB with appropriate antibiotic at 37°C at 200 rpm. For medium to high copy numbers of plasmids, 1.5 ml of culture was used to purify the plasmid with QIAGEN plasmid miniprep kit (Qiagen, UK) according to the manufacturers instructions. All plasmid prep strains were then reconstituted in a final volume of 50 µl sterile d $H_2O$

### 6.2.5 ETHANOL PRECIPITATION OF DNA

The DNA samples were precipitated by the addition of 0.1 × volume of either 5 M NaCl – 3 M Na acetate and 2.5 × volume of 100% ethanol. The mixture was incubated at -20°C for 60 minutes, before centrifugation at 13,000 rpm for 10 minutes. The pellet was washed with 500 µl ice-cooled 70 % ethanol, followed by centrifugation at 13.000 for 5 minutes. The DNA sample was then re-suspended into 40 µl d $H_2O$ and stored at -20°C until required.

### 6.2.6 ELECTROPORATION OF *C.SAKAZAKII* 658 WITH pAJD434

The pAJD434 plasmid was electroporated into the desired *C. sakazakii* strain and trimethoprim (35 µg/ ml) was used to maintain the plasmid. Five millimetre TSB was inoculated with one colony of *C. sakazakii* harbouring pAJD434 and grown with trimethoprim (35 µg/ ml) overnight at 30C°, 200 rpm. The following day the culture was diluted 1/100 into 50 ml TSB with (35 µg/ ml) and 0.1% L-arbinose and grown at 30C°, 200 rpm until an OD600 of ≈ 0.6 was reached. This culture was used to prepare of electrocompetent cells. Electroporation was done by using a Cell-porator™ (BIO-RAD Gene Pulser X cell) with a voltage booster and 0.2 cm chambers according to manufacture instruction by using 50 µl of cell and 25µl of PCR product. Shocked cells were added to 1 ml SOC, incubated 1h at 37°C and then 100 µl of the electrooperation sample was spread onto TSB agar

plates supplemented with 125 µg/ ml kanamycin for selection of mutants. pAJD434 plasmid is thermosensitive and will not replicate at 37°C.

## 6.2.7 INSERTION OF THE KANAMYCIN RESISTANT CASSETTE INTO THE PLASMID PESA3.

This procedure is based on the λ-Red system which enables us to make insertions in to the *Cronobacter* plasmid without inactivating any gene. The basic strategy is to label a plasmid of *C. sakazakii* (plasmid pESA3) with a selective antibiotic resistance gene from pKD4 using the lambda Red helper plasmid pAJD434, which can then be amplified by PCR (Figure 6.1).

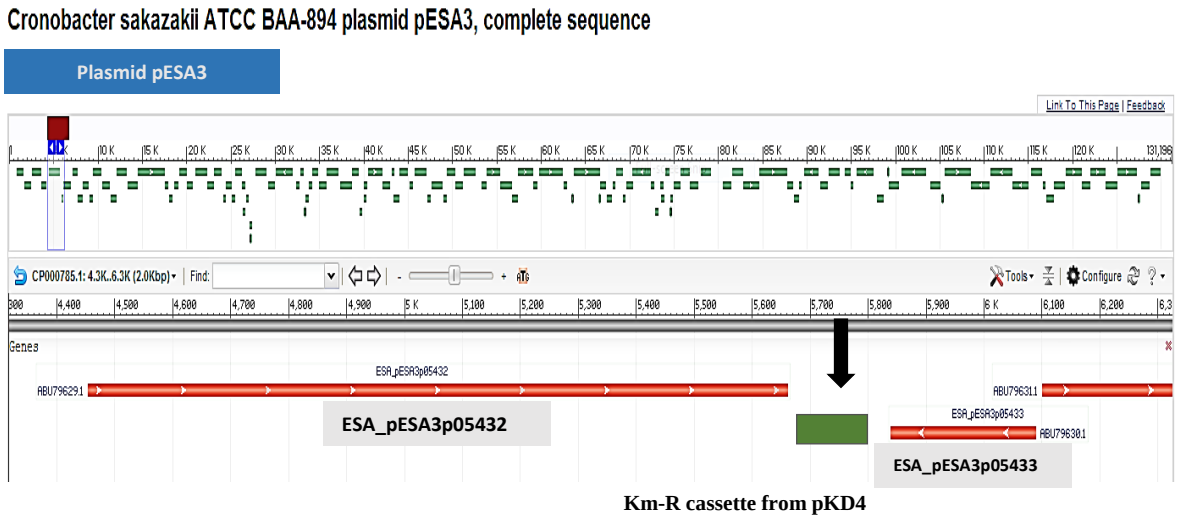


**Figure 6.1** Insertion site of the Km-R cassette from pKD4 into pESA3. Black arrows show the site located midway between 2 convergent genes, pESA3p05432 and pESAp05433. The green box shows location of Km-R cassette into pESA3.

## 6.2.8 TISSUE CULTURE INVESTIGATIONS

### 6.2.8.1 INVASION ASSAYS

Invasion assays were completed to examine the capability of selected bacterial strains to invade mammalian cells Caco 2 (Human colonic carcinoma epithelial cells, ECACC# 86010202),Human brain microvascular endothelial cells (HBMEC) and rat brain capillary endothelial cell line (rBCEC4) as previously described by Townsend *et al*. (2007). Attachment and invasion assays were performed at the same time using the same cell line passage with same inoculum of mammalian cell line and bacterial suspension. *Salmonella enterica* serovar Enteritidis strain number NCTC 3046 (358 NTU) was used as positive control for the Caco-2 cell line. While *Citrobacter koseri* strain number SMT319 (NTU 48) was used as positive control for rBCEC and HBMEC. Both were used in this investigated as standard enteric pathogens capable of attachment and invasion. *E. coli* K12 (NTU 1230) was used as a negative control for all cell lines used in this study, which is non-pathogenic and incapable of invading the Caco-2 cell line (Townsend *et al*. 2008).

### 6.2.8.2 MACROPHAGE ASSAY

As given by Townsend and colleagues (2007), Section 2.9.1 described in detail the growth media for macrophages and then were treated with 0.1µg/ml of the PMA (phorbol 12-myristate 13-acetate) (Sigma Aldrich, UK; P8139) at least 24h prior to infection, and they were placed at 37°C into 75cm$^2$ tissue culture flasks under 5% $CO_2$ for stimulation. For cell adhesion the wells were then filled with the suspension concentration of 4x10$^6$ cfu/well at 37 °C for 72 hours in the presence of 5% $CO_2$.

The concentration of overnight bacterial cell used to infect the macrophages was 4x10$^6$ cfu/ml (MOI 10). This was incubated at 37 °C for 1 hour in 5% $CO_2$. Then the media was replaced by infection media containing 125 µg/ml of gentamicin and incubated at 37°C in the presence of 5% $CO_2$. The plates were washed by PBS and supplied with infection media contain 50 µg/ml of gentamicin. This was then incubated, the plates were completed the process of lysing using 1 % (v:v) Triton X-100 (Fisher Scientific, UK) The final result was diluted and then placed on TSA to

obtain the intracellular bacteria at different points (uptake, 6 h, 24h, 48h, and 72h). Percentage of uptake and persistence was used for data presentation.

### 6.2.9 SERUM RESISTANCE

Bacterial cultures were grown overnight at 37°C in TSB broth and then centrifuged (200 rpm). The pellet was diluted to $10^6$ cfu/ml in 5ml of PBS. Suspended cells 0.5ml were added to 1.5 ml of undiluted human serum, the equal volume was 2.0 ml. The suspended bacterial cells and human serum were mixed and incubated at 37°C (200 rpm) (Hughes *et al*. 1982). The viable counts of cells were obtained at the beginning and after 1, 2, 3 and 4 hours of incubation. Miles and Misra technique were used on TSA plates at 37°C for 18 hours. All strains were analysed in triplicate. All bacterial strains have been assayed in 3 independent assays.

### 6.2.10 IRON SIDEROPHORE DETECTION

For siderophore production, the method of Schwyn and Neilands (1987) was used with slight modification. Chrome azurolsulphate (CAS) agar was prepared by using two solutions. First solution (dark blue liquid) was prepared by using 50 ml of CAS solution (see section 2.4.11), 10ml of iron III solution; section 2.4.10 and 40 ml of HDTMA before autoclaving at 121 °C for 15 minutes (100ml in total of dark solution). The second solution was prepared by mixing 900ml of distilled water, 15g agar, 30.24g PIPES and 12g NaOH and then autoclaving at 121°C for 15 minutes. After autoclaving, the first solution was mixed with the second solution and then the media was poured into the plates. Immediately before use, 5mm diameter holes were punched into the agar. To prepare the bacterial suspension, five colonies were taken from TSA and inoculated into LB broth containing 200µM of 2, 2´-dipyridyl (31.236mg in 1L of LB broth) and incubated with shaking (170 rpm) at 37°C overnight. After incubation the sample was centrifuged at 5000 rpm for 10 minutes and 70 µl of the supernatant was added into the holes. The agar was incubated at 37°C for 4-8 hours. The observance of an orange zone around the hole indicated that the strain is

positive for siderophore production. *Yersinia enterocolitica* strain 8081 was used as a positive control.

## 6.3 RESULTS

### 6.3.1 CONSTRUCTION OF *C.SAKAZAKII* 658 USING PAJD434

This method is based upon the plasmid pAJD434 and the system described by Datsenko and Wanner (2000) however**,** this method involves designing two primers that amplify a kanamycin resistance cassette. Linear fragments were generated directly in a single step by PCR amplification of a kanamycin cassette with the plasmid pKD4 as the source of the template using primer pairs KanpESA3_F and KanpESA3_R. The priming sites used on pKD4 incorporates 50 bp of DNA flanking the deletion site on the chromosome; Figures 6.2- 6.3. PCRs were carried out as detailed in 6.2.2. The transfare procedure is detailed in 6.2.6 and 6.2.7.

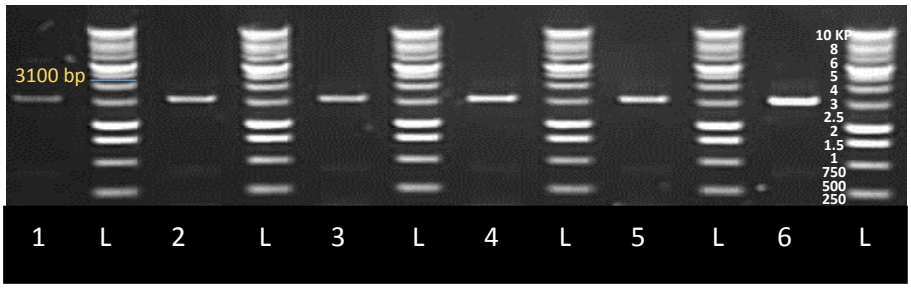


**Figure 6.2** PCRs were used to show that all PCR products have the correct structure of primer designing

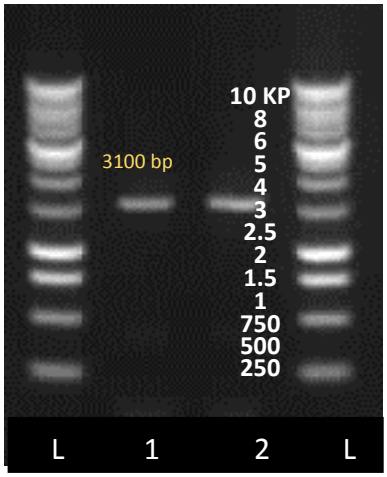


**Figure 6.3** PCR cleaning up of pKD4 with $Kan^R$ cassette, transformed into bacteria carrying Red helper plasmid

## 6.3.2 CONFIRMATION OF KANAMYCIN CASSETTE INSERTION BY PCR

As further confirmation that the correct chromosomal region had been disrupted by the insertion of kanamycin cassette (3267 bp). Specific PCR probes were carried out in this study. Sequence specific probes were generated for detection of the kanamycin cassette using primers kan_F - Kan_R and pKD4 as the template. For confirmation that the kanamycin cassette had been inserted into the correct CDS, specific probes were generated which amplified the kanamycin cassette using pESA3_F and pESA3_R primers within *C. sakazakii* 658 (Figure 6.4).

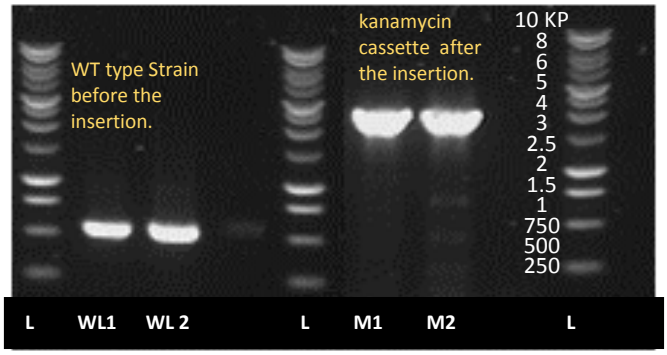


**Figure 6.4** Confirmation of insertion of kanamycin cassette- Ladder: 1 kb - WT1-2: Wild Type *C. sakazakii* (NTU 6) - M 1-2: NTU 6 (pESA3K).

## 6.3.3 TISSUE CULTURE INVESTIGATIONS

### 6.3.3.1 CACO-2 INVASION

The gentamicin protection assays were performed to investigate the ability of the virulence plasmid-containing *C. sakazakii* strain 658 (ATCC BAA-894), NTU 6 (lacking pESA3) and NTU 6 (pESA3K) to invade the Caco-2 cell line. *S.* Enteritidis (NTU 358) and *E. coli* K12 were used as positive and negative controls, respectively for comparative data. Figure 6.5 shows both strains ATCC BAA-894 (658) and NTU 6 (pESA3K) have a significantly greater ability to invade Caco-2 cells than NTU 6 ($P < 0.003$).

The number of recovered cells of 658 and strain 6 with plasmid were in the range between 5.8 $\log_{10}$ to 6 $\log_{10}$ CFU/ml. However the original plasmid-less strain NTU 6 displayed low invasive rates of about 4.3 $\log_{10}$ CFU/ml.

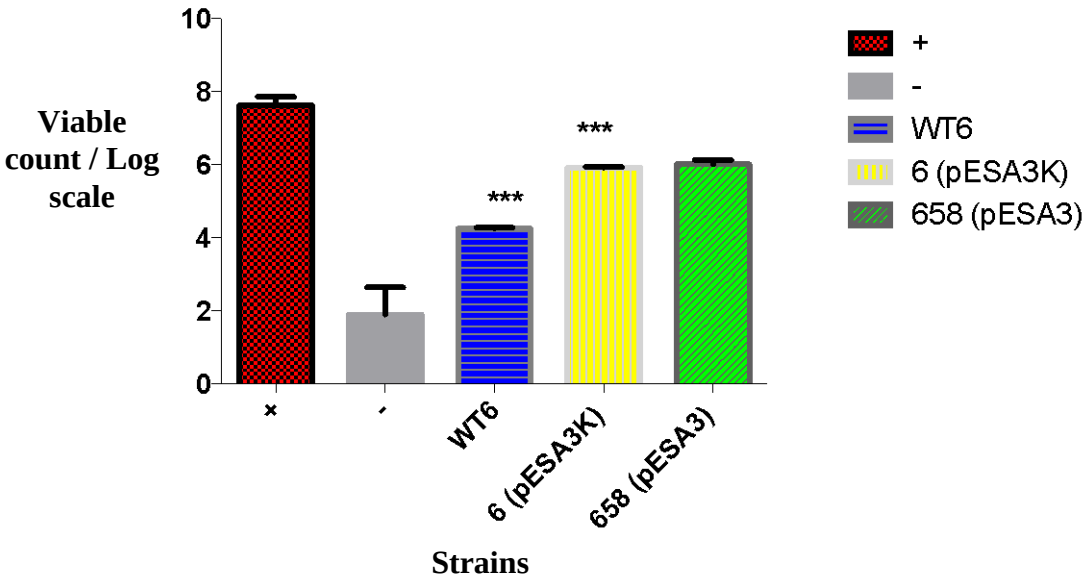


**Figure 6.5** Invasion of Caco-2 cells by wild Type *C. sakazakii* NTU 6, NTU 6 (pESA3K) and 658 (pESA3). *S.* Enteritidis 358 and *E. coli* K12 were used as positive and negative controls respectively.

### 6.3.3.2 HBMCE INVASION

Figure 6.6 displays the ability of NTU 658, NTU 6 and NTU 6 (pESA3K) to invade the HBMEC. The gentamicin protection assay used *C. koseri* (NTU 48) as an invasive positive control for this cell line and *E. coli* K12 as a negative control and the number of recovered cells was approximately 2 $\log_{10}$ CFU/ml. Both strains 658 and NTU 6 (pESA3K) were significantly higher invaders compared with NTU 6 (P = 0.0294), which were recovered in the range 5 $\log_{10}$ to 6 $\log_{10}$ CFU/ml. However the original plasmid-less strain NTU 6 displayed low invasive rates of about 4 $\log_{10}$ CFU/ml.

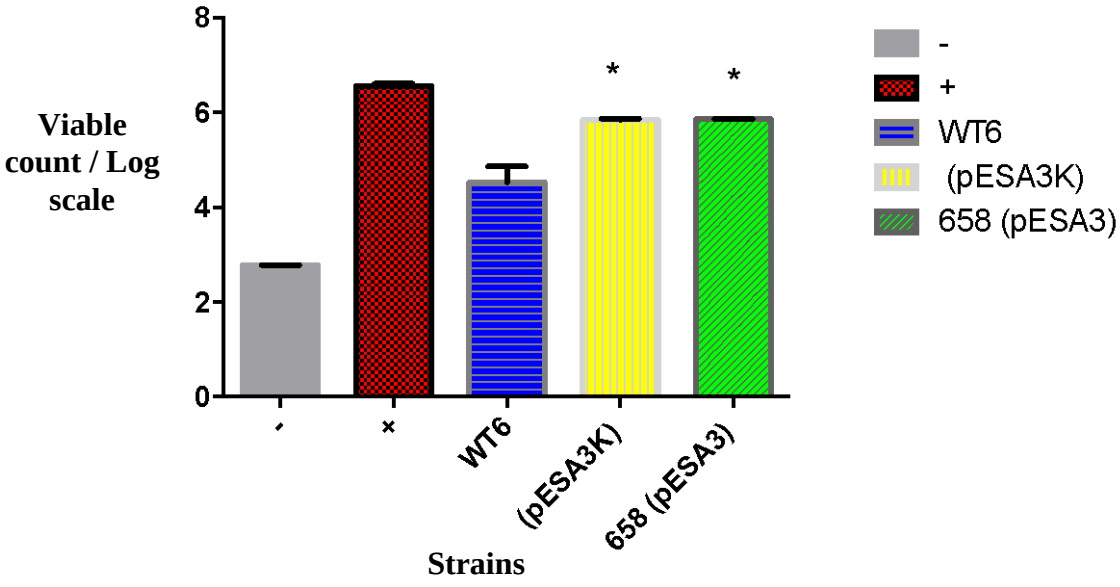


**Figure 6.6** Invasion to HBMCE cells by wild Type *C. sakazakii* NTU 6, NTU 6 (pESA3K) and 658 (pESA3). *S.* Enteritidis 358 and *E. coli* K12 were used as positive and negative controls respectively

### 6.3.3.3 rBCEC INVASION

Figure 6.7 displays the abilities of NTU 658, NTU 6 and NTU 6 (pESA3K) to invade the rat brain capillary endothelial cell line (rBCEC4). The gentamicin protection assay used *C. koseri* NTU 48 as an invasive positive control for this cell line and *E. coli* K12 as a negative control and the number of recovered cells was approximately 3 $\log_{10}$ CFU/ml.

Both strains 658 and NTU 6 (pESA3K) were significantly higher invaders compared with the original plasmid-less strain NTU 6 (P = 0.0009), which were recovered in the range 5 $\log_{10}$ to 6 $\log_{10}$ CFU/ml. However the original plasmid-less strain NTU 6 displayed low invasive rates of about 4 $\log_{10}$ CFU/ml.

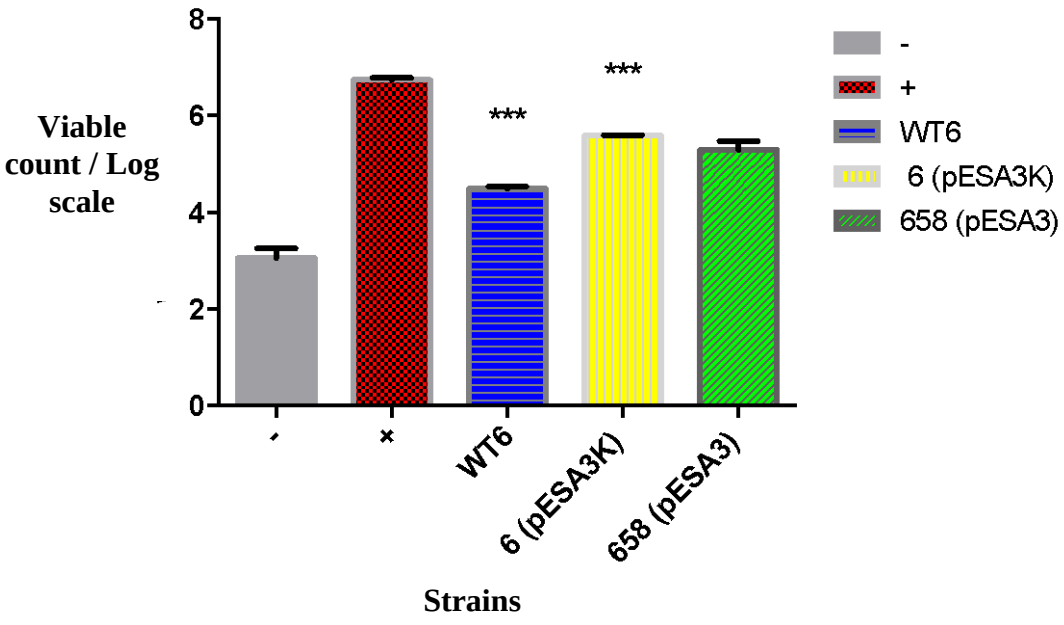


**Figure 6.7** Invasion to rBCEC cells by wild type *C. sakazakii* NTU 6, NTU 6 (pESA3K) and 658 (pESA3). *S.* Enteritidis 358 and *E. coli* K12 were used as positive and negative controls respectively

### 6.3.3.4 UPTAKE AND PERSISTENCE INTO MACROPHAGE CELL LINE (U937)

Figure 6.8 shows the results of the same strains bacteria used with previous cell lines which were investigated for their ability to persist in human macrophages after phagocytosis. This was performed using the U937 macrophage cell line. Strains of *C. koseri* SMT319 (NTU 48) and *E. coli* K12 (NTU 1230) were used as positive and negative controls respectively (Townsend *et al.* 2008).

Both bacterial strains NTU 658 and NTU 6 (pESA3K) were shown to persist and replicate inside macrophages following the initial 45 min incubation, the macrophages internalised about 10 % of the inoculated cells of these strains which duplicated and increased to 23 % within 24 h, the number of survival cells has been reduced to 3 % from the initial inoculum of 48 hours which ultimately reduced to 0.1 % after 72 h.

In contrast, the wild type strain of *C. sakazakii* NTU 6 were higher only in their persistent within the macrophage which able to uptake 16 % from the initial inoculum and decreased to 10 % within 24h, the number of survival cells has been reduced to 3% within 48h. While *Cit. koseri* strain SMT319 (NTU 48) continued to replicate until the end of the observation time of 27 hour, however *E.coli* k12 (NTU 1230) was killed by human macrophage. Furthermore, it is interesting to note that the persistence of *C. sakazakii* strains within macrophages is likely to be higher than positive control of *Cit. koseri* as showed in figure (6.8) ANOVA one way was used to obtain the consistence of the independent experiments, the significance was set at $p < 0.0001$.

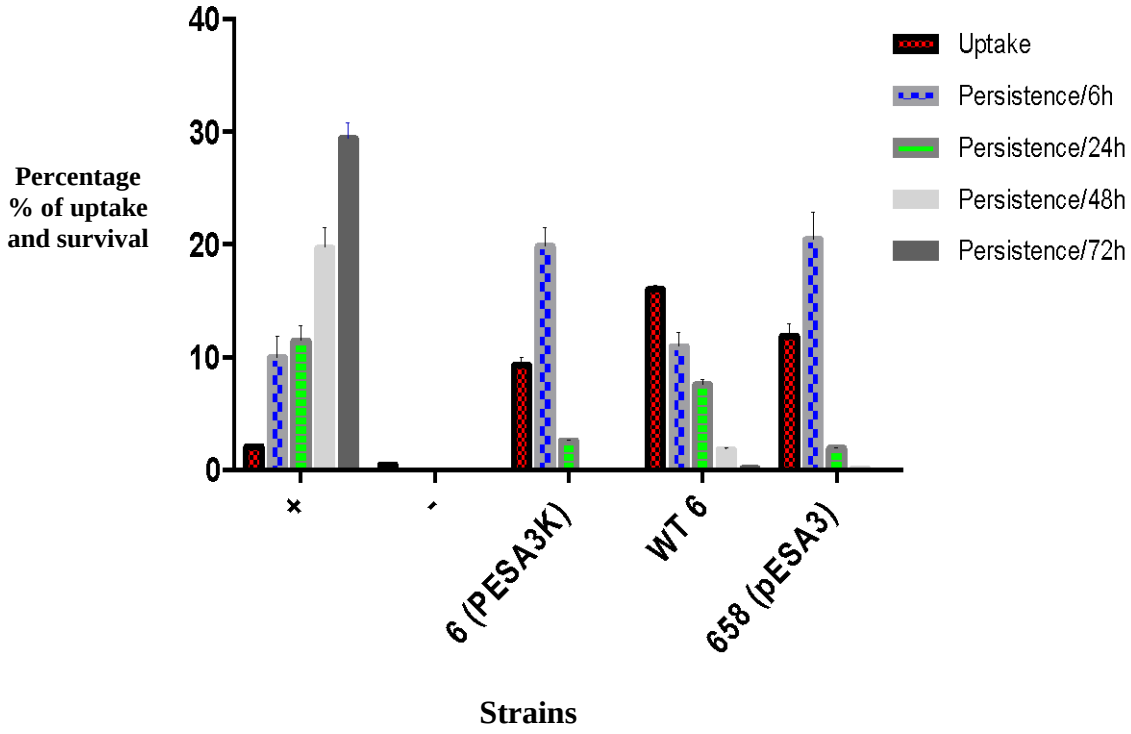


**Figure 6.9** Level of uptake and survival of *C. sakazakii* strains by U937 macrophage cells were calculated and determined after 45, 24, 48 min and 72h incubation. Three of *C. sakazakii* strains uptake in higher count than positive control. Strain plasmid less strain 6 reduced gardually after the uptake.*cit. koseri* 48 *and E. coli* K12 were used as positive and negative controls. Error Bar represent ANOVA of three independent experment.

### 6.3.4 SERUM RESISTANCE

The survival of *C. sakazakii* strains after incubation for 4h in undiluted human serum was determined for all the same strains used in the previous tissue culture experiment (Figure 6.9). Variable count over an extended period of time of the strains, 658 and 6 (pESA3K) were characterized as serum resistant showing survival rate of about 8 $\log_{10}$ CFU/ml or higher, however NTU 6 was sensitive to the killing action of undiluted human serum with low survival range of 4 $\log_{10}$ CFU/ml comparing to 658 and 6 (pESA3K).

The positive control of *S.* Enteritidis showed increased growth rates overtime representing their tolerance to human serum. However, *E. coli* K12 the negative control strain revealed a declined growth levels and that is a sign for serum sensitivity.

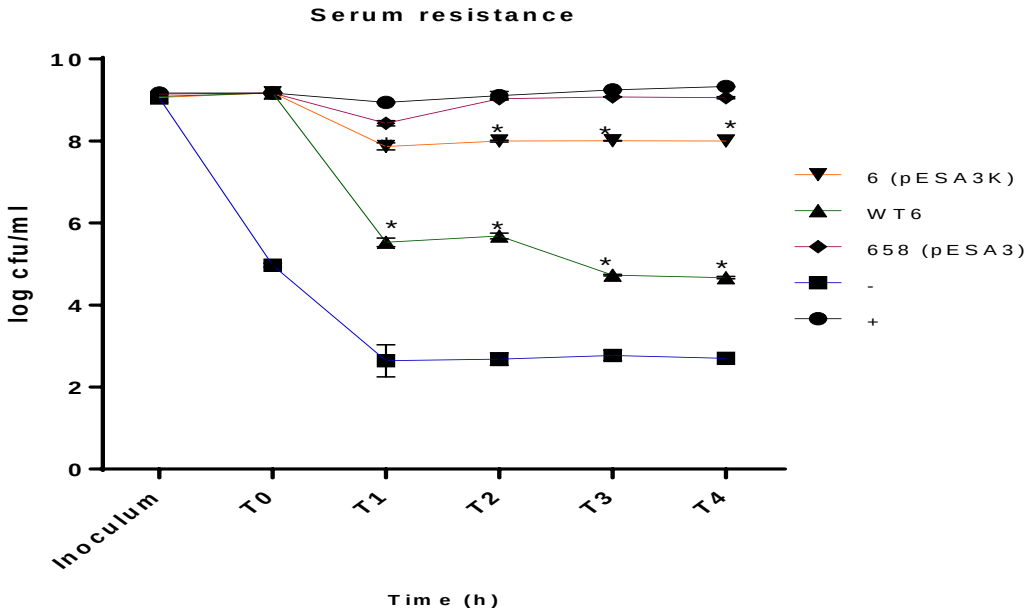


**Figure 6.9** Grades of response to undiluted human serum of *C. sakazakii* strains over 4 hours of incubation. The strains 658, 6 (pESA3K) and showed increase in their viable counts, however strain 6 and *E.coli* showed significantly values declined. *S.* Enteritidis 358 and *E. coli* K12 were used as positive and negative controls respectively

### 6.3.4 IRON SIDEROPHORE PRODUCTION BY *C.SAKAZAKII* STRAINS USING CASAD ASSAY

Figure 6.10 shows both strains 658 and NTU 6 (pESA3K) have been able to produce iron siderophores. CAS agar showed orange halo around the site of inoculation. However NTU #6, the plasmid less strain was negative for siderophore production. Strains of *Y. enterocolitica* (8081) and *C.sakazakii* NTU 520 were used as positive and negative controls respectively. Strains 6 (ST4) and 520 (ST12) were not able to produce iron siderophores. This is in agreement with the results that have obtained previously in chapter 5 (Section 5.4.4) in the absence of the iron acquisition genes in their genomes using *Cronobacter* BLAST research facility at http://pubmlst.org/cronobacter/.

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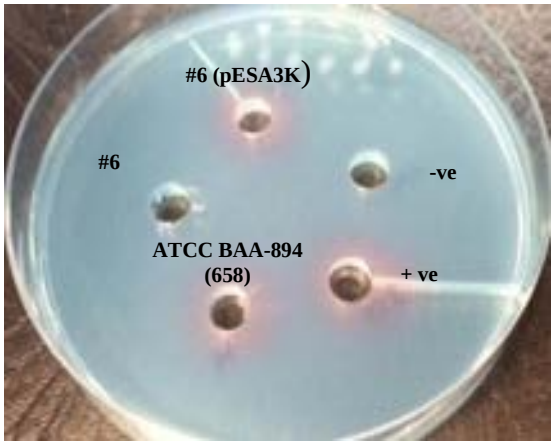


**Figure 6.10** Iron siderophore activity using CASAD assay. Wells were filled with bacterial suspension, contain five colonies and inoculated into LB broth containing 200µM of 2, 2´-dipyridyl. 70 µl of the supernatant was added into the holes. The agar was incubated at $37^0$C for 4-8 hours. The observance of an orange zone around the hole indicated that the strain is positive for siderophore production. *Yersinia enterocolitica* strain 1880 was used as a positive control.

## 6.4 DISCUSSION

The present study clarifies the transfer of plasmid well characterized pESA3 into plasmid less strain (NTU 6) without inactivating any gene. The basic strategy is to label a plasmid of *C. sakazakii* (plasmid pESA3) with a selective antibiotic resistance gene from pKD4 using the lambda Red helper plasmid pAJD434, which can then be amplified by PCR. This technique have been used for many years in *E.coli* (Datsenko & Wanner. 2000), yeast (Baudin *et al*. 1993) and *Cronobacter* spp. (Kim *et al*. 2015; Orieskova *et al*. 2013; Kim *et al*. 2010). The plasmid PAJD343 express the red system (*exo*, *bet* and *gam*) under control of a well regulated promotor to avoid unwanted recombinational events under noninducing condition. When making gene disruption using the templates and priming sites elimination of the antibiotic resistance gene leaves behind an 50 nt scar in a place of the disruption gene in strain 658. pKD4 (plasmid encoding Kanamycin cassatte) are identical except for the region, an identical scar have been created for the limitation homology using the Red system and new PCR fragment. The transfer of plasmid well characterized pESA3 into plasmid less strain (NTU 6) has been verified by PCR strategy which indicted for the presence of new locus fragments of predicted size by direct transform carring a red helper plasmid with PCR product having short homology extension for the targeted locus. This method has been widely useful based on previous study Datsenko & Wanner (2000) and Baudin *et al.* (1993).

### 6.4.1 TISSUE CULTURE

The tissue culture study of viable account showed the ability of virulence plasmid *Cronobacter sakazakii* pESA3 and NTU 6 (pESA3K) to significantly invade the mammalian cell lines Caco-2 (Human colonic carcinoma epithelial cells), human brain microvascular endothelial cells (HBMEC) and rat brain capillary endothelial cell line (rBCEC4). Nevertheless, the plasmid less-strain NTU 6 was low invade in these cell lines. The detection of viable count of bacterial cells, after exposure to gentamicin could be due to clumping or biofilm formation (Schwarz *et al* 2004). Attachment to host cells might be crucial to cause infection; one attached to the host surfaces then

certain biochemical developments may result in the establishment of infection within the host after invasion. (Wilson *et al*.2002).

Invasion of intestinal epithelial cells is a key characteristic of many microorganisms. It permits them to reach a safe environment where they could consequently proliferate and spread from cell to cell and be less subject to the host immune defenses or therapeutic mediations (Pereira *et al*. 2008).

Macrophage studies were conducted to investigate the ability of the test strains to survive and multiply within this type of immune cell. Both bacterial strains (NTU 658) and NTU 6 (pESA3K) were shown to grow to 23 % within 24 h. In contrast, the wild type strain were decreased to 10 % within 24h. Thus, the plasmid encoded virulence associated traits such as plasminogen activator cpa and iron acquisition genes could be essential for the virulence of *C. sakazakii* to enter and persist within macrophages. The plasmid less strain NTU 6 was unable to survive the intracellular environment.

There is some similarity between the current study and the study by Townsend *et al.* (2008) which has shown that *C. sakazakii* strains which cause severe infections in neonates such as fatal meningitis and necrotizing enterocolitis have the ability to invade intestinal Caco2 cells and survive within macrophage.

Several previous researchers have shown that there has been widely divergent views on the genetic base of *Cronobacter* virulence need and their effects of the organisms on the tissue culture cell (Mittal *et al.* 2009, Wang and Kim 2002 and Kim *et al*.2010). It is predictable that *Cronobacter* possesses various virulence factors which could help in the invasion of a number of human mucosal cell lines as the initial step in the systemic spread of the bacterium. Similarly, such pathogens have developed mechanisms to escape the immune system of defence, for example surviving inside macrophages, and having resistance to serum. *Cronobacter* seems to avoid the host's immune system by adapting to a lifestyle of replication in the intracellular environment limited within macrophages.

Macrophages provide one of the essential defence of innate immune system in microbial uptake to exhibit antibacterial strategy for example oxidative stress, acidic compartmentalization and nutrient deprivation (Townsend *et al.* 2007). The microorganism which evade phagocytic killing have the

ability to persist and replicate within macrophages which is crucial to develop severe diseases such as meningitis and sepsis (Eshwar *et al.* 2015).

### 6.4.2 SERUM RESISTANCE

Bacteria that cause invasive infections such as bacteraemia and sepsis have developed strategies to defend themselves against serum mediated component. Bacterial structures containing cell surface, for example outer-membrane proteins, capsules and LPS have been recognised as being responsible of bacteria for the complement resistance Schwizer *et al*. (2013).

In the earlier study by Joseph *et al*. (2011), the plasmid pESA3 in ATCC BAA-894 (658) encoded plasminogen activator gene *cpa* as a virulence factor providing serum resistance. Moreover, it has previously been showed that plasmid borne genes associated with antibiotic resistance which could increase the degree of serum activity in *E.coli* (Tylor and Hughes 1978, Binns *at al*.1979).

The observation made in this research show that presence of pESA3 plasmid associated virulence could play an important role in pathogenies of *C. sakazakii,* also has been pointed out the ability of strain NTU 6 (pESA3K) after transform the plasmid to resist human serum compared to the wild type plasmid less strain NTU 6. However, as mentioned in the previous chapter (Chapter 5) there were two clinical strain 696 (ST12) and strain 4 (ST15) which do not encode large plasmid (only 31 kb) were serum resistant. Also it is known in our group at NTU (work unpublished) some of *Cronobacter* spp. resistance to serum, which have not encoding *cpa* gene. This finding leads us to believe that there could be additional factors producing serum resistance such as capsules and LPS ,or *cpa* might be located on the chromosome.

### 6.4.3 IRON SIDEREPHORE

Iron is an essential factor for bacterial pathogenesis and it is involved for many process including electron transfer, superoxide metabolism and cellular respiration. It has been reported in 2011 by Franco *et al* that the bacteria under iron limiting condition, can produce siderophore which is a high affinity iron binding molecules to scavenge iron from their environmental. Moreover, it has been

found from Barron (1995) work that siderophore can help the bacteria to scavenge iron from human brain cell.

This research indicates that strain NTU 6 (pESA3K) after transformation with the plasmid, were able to produce iron siderophore. However, NTU 6 the plasmid-less strain was negative. This is evidence in the present study that produce siderophore contributes the benefit to the *Cronobacter* to survive in the human body and support pathogenesis. This results were compatible with those reported in 2011 by Franco and his colleague which have been determined if pESA3 encoding the functional active siderophore system by curing the pESA3 from *C. sakazakii* BAA-894 (Franco *et al*. 2011 b).

In summary;

- The study of this chapter indicates that pESA3 plasmid is positively correlated with invasion or virulence of *C. sakazakii*. However, it must be remembered that some of the isolates lacking plasmid pESA3 such as strain (NTU 520, 696 and 4) showed serum resistance and were obtained from clinical sources.
- Plasmid pESA3 is likely to be crucial for the virulence of *C. sakazakii*; it may not be solely responsible for its virulence.

# CHAPTER 7

# CONCLUSIONS AND FUTURE DIRECTIONS

# 7.1 CONCLUSIONS AND FUTURE DIRECTIONS

*Cronobacter* spp. (previously known as *Enterobacter sakazakii*) is opportunistic pathogen that can cause serious infection in premature neonates and all age groups such as meningitis, bacteraemia and NEC. As a recently recognised bacterial pathogen of considerable importance, regulatory control and appropriate identification methods are required following the association of *Cronobacter* spp. to numerous publicized deadly outbreaks in neonatal intensive care units of bacteraemia, necrotizing enterocolitis and meningitis.

- The first part of this project was in response to FAO-WHO (2004) request to develop a reliable detection and molecular typing methods to facilitate the control of this bacterium. An multilocus sequence typing scheme (MLST) has been established (Baldwin *et al*.2009) and is available as an open access internationally based database (http://www.pubMLST.org/cronobacter/). This is supported by international contributors and currently includes the profiles of >1000 strains and >100 whole genomes. The MLST scheme has advanced our understanding of the *Cronobacter* genus, revealing predominant and stable clones associated with neonatal meningitis.

  The clinical strains have been studied were from USA, Israel and the Czech Republic in which eleven sequence types (STs) were identified spanning the genus of *Cronobacter.* This analysis has contributed to a key aspect of the genus by identifing a clonal lineage for a majority of the neonatal meningitis, NEC and bacteraemia cases- the *C. sakazakii* ST4 clonal complex. This identification of ST4 clonal complex as the lineage responsible for the majority of fatal neonatal infection caused in *Cronobacte*r spp. has reported a unique molecular signature caused by microorganism. In addition this research reported the first meningitis case by *Cronobacter malonaticus* (CC112) which was from an infant (age <1 month) with severe brain damage which led to their death.

- In the second part of this project, the functionality of sialic acid trait in *C. sakazakii* as well as the potential mechanisms of the pathogenicity in the neonatal brain were investigated. Laboratory studies have been published investigating the variation in growth in sialic acid of *Cronobacter* spp. of 19 *Cronobacter* strains were selected which represented the seven recognized species, and included those from reported clinical cases and species type strains. Initial results confirmed *C. sakazakii* is the only species able to utilize the sialic acid. During the late steps of the current project, 107 *Cronobacter* genomes were analysed to expand our research using *Cronobacter* PubMLST (http://www.pubMLST.org/cronobacter). As a result, some of *Cronobacter turicensis* strains were found to also encode the *nanAKT* cluster. For this reason the next step was to examine the growth of all the *Cronobacter turicensis* strains in our database. Ten out of 24 (42%) have been growing in all the M9 supplemented with sialic acid substrates. Also the variation in the gene contents of *Cronobacter* associated with sialic acid utilization have been reported. As a result, the functionality of this trait in *C. sakazakii* and some strains of *C. turiensis* revealed the possible mechanisms of the pathogenicity in the neonatal brain and mucin. The high degree of colinearity of the alignment between different genomes of *Enterobacterice* have been shown. Also the whole cluster is located in a certain location flanked by some conserved housekeeping trait (*gltB*) and starvation gene (*sspA*) suggesting loss from other *Cronobacter* spp. instead of two separate acquisition events.

  Moreover, because of close adaptation of intracellular microorganism to the physiologically stable environments of their host cells, a reductive genome evolution happened that led to the loss of some genes not crucial for life within the host which is called evolution by reduction to assume loss from other *Cronobacter* spp. instead of separate acquisition events.

- Study of clinical strains of *C. sakazakii* have been used in order to gain a better understanding of bacterium pathogenicity. For this purpose in the beginning of my PhD study, 36 representative clinical strains, 23 were ST4 and 13 strains were non-ST4 strains

of which three strains were ST1, three strains were ST8 and two strains were ST12. A number of key traits such as plasmid profiling, PCR probes for virulence genes including Type six secretion system, iron acquisition system and *Cronobacter* plasminogen activator *Cpa* were found to be scattered across the *C. sakazakii* genus. Some of the virulence traits varied in their level of the diversity, and were also identified to be plasmid borne. This observation making the plasmids one of the obvious targets for future studies especially the strains lacking the large plasmid and encoding most of virulence genes such as strain 696 (ST12) isolated from a NECII case. Diversity of the physiological traits and *in vitro* tissue culture assay were being individually characterized by laboratory studies as part of PhD projects in our group, in order to gain a better understanding of the pathogenicity of the organism. *C. sakazakii* is a newly emerging pathogen, and understanding the mechanism of serum resistance could assist in clarifying the difference in resistance between the strains. In addition, this could also increase the information about this organism's pathogenicity and present other potential methods of virulence. The chances of surviving and multiplying in human blood is present in the strains which have resistance against serum bactericidal activity and many virulence genes like the iron acquisition genes. This can result in increased pressure and organs could have collateral damage, particularly to the CNS since the bacterium has been known since long to have caused meninges and brain damage cases.

Some factors of *C. sakazakii* virulence which increases the infection and the pathogenic process have been reviewed in this study. To result in a successful infection, it is important for *C. sakazakii* to have invasion potential. Different iron acquisition systems were encoded by 97% *C. sakazakii* to attain iron from the host. They were also able to survive and spread in the host under a little supply of iron which is associated with a rise in virulence (Raymond *et al*.2003). Majority of *C. sakazakii* were serum resistance (32/36), and thus, they had the ability to survive in blood by preventing serum-mediated killing. Also, the organism can survive and even multiply inside macrophages. This will enable the development of bacteraemia through dissemination in the entire body and through

intracellular reproduction. Correlation between T6SS and virulence function in some of *C. sakazakii* has been detected in the most of clinical cases such as NECII and severe meningitis strains 696 (ST12), 5 (ST8), 553, 721, 1219, 1220, 1221, 1225, 1231, 1240, 1251 (ST4). These results are being analysed by other member of our group includes host cell adhesion and invasion, cytotoxicity, and macrophage survival study.

- It is believed *C. sakazakii* strains vary in plasmid profile and gene contents. Thus, some of the virulence traits need more investigation. the plasmid pESA3 as the largest plasmid which contains several potential virulence-associated traits have been chosen as well characterized plamid in this study. It was of interest to insert plasmid pESA3 as well characterized plasmid into plasmid less strain. Stains encoding large plasmid were able to invade human intestinal epithelial cells Caco-2, brain endothelial cells HBMEC and rBCEC4. Also have been reported significant observation in siderophore production and serum survival values whereas plasmid less strain and not shown less significant associated virulence.

In summary, the impact of this research work to the existing knowledge of *Cronobacter sakazakii* are represented by the following:

I. First to study the whole genus population to better understand how this microorganism could cause irreparable destruction to neonate brain.

II. Determined the ability of this organism could help us to answer the destructive pathogenic mechanism of *Cronobacter* spp., that could afterward assistance to put better control procedures in place.

III. Proved the capacity of some *Cronobacter sakazakii* strains to survive inside the brain, intestinal cell and blood as a result of the invasion process.

## 7.2 FUTURE DIRECTIONS

Consequently, this is not the end of this research, now the *Cronobacter* MLST database contains records of 1007 isolates indicating the source, geographic and temporal diversity of the genus. Now genomes are available for further analysis in the PubMLST database. Different strains of *Cronobacter* have been isolated from 36 different states and from various sources. The huge study by Forsythe *et al*. (2014) was the end of the beginning to better understand this microorganism which can effect severe damage to a newborn baby's brain. This research has also opened up many new ways into the investigation of this opportunistic food-borne bacterium, that could thereafter help to put improved control measures in place.

I. The effect of the presence of a large plasmid of most significant STs (ST4) associated with virulence of *C. sakazakii* by transform the large plasmid into another sequence type strain such as ST12 and ST8 to determine of unique phenotypic characterization.

II. To mutate (knock out) and functionally characterise selected virulence genes from those identified using bioinformatics analysis which could be involved in bacterial pathogenesis for example, genes responsible for siderophore production and *nanAKT* gene cluster of sialic acid utilization. These can be used to find mechanisms of invasion and spreading in the infected host and observe significant changes in the phenotypic traits including tissue culture of different cell lines and virulence associated behaviour.

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