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Isolation and characterisation of *Enterobacter sakazakii*.

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ABSTRACT

Enterobacter sakazakii is a bacterial contaminant of powdered infant formula milk that has been associated with necrotising enterocolitis, bacteraemia and a rare form of infant meningitis. The presence, persistence and growth of the organism in infant formula milk needs to be better understood to limit the occurrence of infection, and improved isolation methods need to be developed in order for companies to implement appropriate food safety management systems.

A collection of *E. sakazakii* isolates from diverse clinical, food and environmental sources was compiled. Isolates identified biochemically as *E. sakazakii* formed four genomic clusters when housekeeping gene sequences (16S rDNA and *hsp*60 loci) were compared. The reliability of presumptive isolate identification using commercial biochemical galleries was investigated in comparison to identification by 16S sequencing. The Biolog GN2 system appeared to be the most reliable identification gallery.

A novel chromogenic medium, based on the α-glucosidase reaction, was developed to improve the efficiency of *E. sakazakii* isolation methods and is commercially available as Chromogenic *Enterobacter sakazakii* medium, Druggan-Forsythe-Iversen formulation (DFI), CM1055, Oxoid Ltd. The sensitivity and specificity of the DFI medium was compared with other proposed media. Also 486 food samples were tested for the presence of *E. sakazakii.* The organism was isolated from 67 samples using the DFI medium compared with only 19 using the conventional method. A novel enrichment medium was also investigated to improve recovery of *E. sakazakii.*

Preliminary investigation of factors that may be associated with increased risk of acquiring *E. sakazakii* infection from contaminated infant formula indicated that *E. sakazakii* strains are able to survive in a desiccated state for over 6 months. They are also able to form biofilms on infant feeding equipment, can attach and invade human epithelial (CaC0-2) cells *in vitro* and can survive in human serum. Some strains may persist in macrophages, and many produce exopolysaccharide capsules which enhance biofilm formation and may contribute to evasion of host immune defences.

CHAPTER 1 – INTRODUCTION

1.1 PHENOTYPIC AND GENOTYPIC CHARACTERISTICS

Enterobacter sakazakii is a motile, non-spore forming, Gram-negative, oxidase negative, facultative anaerobe. It was formerly known as 'yellow pigmented *Enterobacter cloacae*'. However, in 1976 Steigerwalt *et al.* found the DNA of yellow-pigmented strains were 83-100% related to each other but only 47-54% related to non-pigmented *Enterobacter cloacae* strains. In 1980 *E. sakazakii* was designated as a new species by Farmer, Asbury, Hickman and Brenner in honour of the Japanese bacteriologist Riichi Sakazaki. They reported that DNA-DNA hybridization studies found no clear generic assignment for *E. sakazakii* as it was 53-54% related to *Enterobacter* and *Citrobacter* species. A comparison of the type strains of these two genera showed that *E. sakazakii* was 41% related to *Cit. freundii* and 51% related to *E. cloacae*. Subsequently, since it was also phenotypically closer to *E. cloacae*, Farmer *et al.* (1980) assigned the organism to the *Enterobacter* genus. Additional hybridisation studies by Izard *et al.* (1983) confirmed that *E. sakazakii* strains were highly related (72-97%) and detected no close relationships to other members of the genus *Enterobacter*. It has recently been shown that isolates identified biochemically as *E. sakazakii from* separate genetic groups using 16S rDNA sequence analysis (Iversen *et al.* 2004; Lehner *et al.* 2004). However, it is recognized that 16S rDNA sequences are of limited value for species delineation, and DNA-DNA hybridization, including membrane filter, spectrophotometric and microplate hybridization, is currently the acknowledged golden standard (De Ley *et al.* 1970; Stackebrandt *et al.* 2002; Mehlen *et al.* 2004).

In 1990 Clark *et al.* found that plasmid analysis, ribotyping with *Hind*III, chromosomal restriction endonuclease analysis and multilocus enzyme electrophoresis were equally effective for epidemiological typing. Nazarowec-White and Farber (1999) found RAPD and PGFE to be the most effective typing methods; food and clinical isolates with the same ribotypes had different PFGE profiles with *Xba*1 and *Spe*1 indicating they were different strains; these isolates were also distinguishable by RAPD. In 2001, Clementino *et al.* reported that tDNA-PCR resulted in profiles of *E. sakazakii* that were distinct from *E. cloacae* and *E. aerogenes*. Drudy *et al.* (2006) describe characterisation of *E. sakazakii* isolates from environmental and food sources. Analysis of RAPD banding patterns revealed three major clusters of *E. sakazakii* indicating that RAPD may be useful for direct comparison of *E. sakazakii* isolates enabling traceability through the infant formula food chain.

Farmer *et al.* (1980) found 15 biogroups of *E. sakazakii* based on biochemical profiles with the wild type Biogroup 1 being the most common. Biogroup 1 strains were positive

for the Voges-Proskauer reaction, motility, ornithine decarboxylase, reduction of nitrate, production of gas from glucose, and production of acid from methyl-α-D-glucoside and inositol. They were negative for the Methyl Red test, production of indole, production of acid from dulcitol and utilization of malonate. It was suggested that further investigation may lead to *E. sakazakii* being reorganised into a new genus with Biogroups 1-14 being strains of one species and Biogroup 15 being a separate species (indole, malonate and dulcitol positive, α-methyl glucosidase negative) within the genus. Goullet and Picard (1986) reported that the high degree of enzyme polymorphism in *E. sakazakii* enabled the differentiation of different strains. The organism commonly metabolises dextrin, N-acetyl-D-glucosamine, L-arabinose, D-cellobiose, D-fructose, D-galactose, α-D-glucose, maltose, D-mannitol, D-mannose, D-mellibiose, β-methyl-D-glucoside, D-psicose, D-raffinose, sucrose, D-trehalose, citric acid, D-galactonic acid, lactone, D-gluconic acid, lactic acid, L-asparagine, glycerol, and α-glycerol phosphate. In addition, 75-90% of strains also metabolise m-inositol, α-D-lactose, L-rhamnose, methyl pyruvate, L-alanine, L-aspartic acid, L-serine, inosine, uridine, and thymidine. Only 6% are reported to metabolise sorbitol and 4% D-arabitol. The organism produces D-lactic acid from lactose and is mucate negative.

Muytjens, *et al.* (1984) found α-glucosidase activity in all *E. sakazakii* strains tested (n=129) but not in 97 other *Enterobacter* strains of *E. aerogenes*, *E. cloacae*, and *E. agglomerans*. *E. sakazakii* also uniquely lacked phosphoamidase. Aldova *et al.* (1983) found 97.3% of *E. sakazakii* isolates contained Tween esterase activity, with six strains isolated from dried milks producing Tween 80 esterase after 7 days at 25 and 37°C and suggested this enzyme could be used to distinguish *E. sakazakii* from *E. cloacae*. The organism also produces a delayed extracellular DNase reaction on toluidine blue agar (36°C, 7days). *E. sakazakii* grows on media used to isolate enteric organisms such as MacConkey, eosin methylene blue and deoxycholate agar. It has been reported that some strains of *E. sakazakii,* decolourise acid-base indicators. This may cause problems for biochemical identification tests based on colour reactions to pH changes. On agar plates the organism may form two colony types (glossy and matt) depending upon media and strain. Growth on tryptone soya agar at 25°C usually produces a non-diffusible, yellow pigment although this can be absent, or transient on sub-culturing, for some strains.

E. sakazakii produces a heteropolysaccharide comprising 29-32% glucuronic acid, 23-30% D-glucose, 19-24% D-galactose, 13-22% D-fucose and 0-8% D-mannose (Harris and Oriel, 1989). Optimal production is under high carbohydrate, nitrogen-limited growth conditions (C:N ratio 20:1 with glucose as the carbon source) and an incubation temperature of 27°C (Scheepe-Leberkühne and Wagner, 1986). The unique biophysical properties of the capsule have lead to patents being filed for the exploitation of *E. sakazakii* heteropolysaccharide as a thickening and/or friction

reducing agent to replace xanthan gum (Harris and Oriel, 1989), and as an immunostimulant for treatment of cancer and other diseases associated with immune deficiency (Yang, 2002).

E. sakazakii has been reported to be more sensitive than other *Enterobacter* species to antibiotics, including aminoglycosides, ureidopenicillins, ampicillin and carboxypenicillins (Monroe and Tift, 1979; Farmer *et al.* 1980; Adamson and Rogers, 1981; Jimenez and Gimenez, 1982; Muytjens and van der Ros-van De Repe, 1986; Willis and Robinson, 1988; Hawkins *et al.* 1991; Nazarowec-White and Farber, 1999; Stock and Wiedemann, 2001). Farmer *et al.* (1980) found *E. sakazakii* strains were susceptible to gentamicin, kanamycin, chloramphenicol, and ampicillin; 87% or over were susceptible to nalidixic acid, streptomycin, tetracycline, and carbenicillin; 71 and 67% were susceptible to sulfadiazine and colistin respectively; only 13% were susceptible to cephalothin. All strains were resistant to penicillin; only 1 of over 100 strains tested showed multiple antibiotic resistance. Muytjens and van der Ros-van der Repe (1986) found the MICs for 90% of 195 *E. sakazakii* strains tested against 25 antibiotics were at least twofold lower than those for *E. cloacae*. There was however resistance to cephalothin and sulfamethoxazole. Willis and Robinson (1988) recommended the combined use of ampicillin and gentamicin for the treatment of *E. sakazakii* meningitis, however gentamicin may be unable to achieve adequate concentrations in the cerebral spinal fluid. Nazarowec-White and Farber (1999) found the type strain (ATCC 29544), 5/8 food and 8/9 clinical strains were only resistant to sulphisoxazole and cephalothin. The other clinical strain showed susceptibility to all agents while the other three food isolates were also resistant to chloramphenicol. Two of the chloramphenicol resistant food isolates were also resistant to tetracycline and one was also resistant to ampicillin. Kuzina *et al.* (2001) found *E. sakazakii* isolated from the guts of Mexican fruit flies resistant to ampicillin, cephalothin, erythromycin, novobiocin, and penicillin. Lai (2001) found all *E. sakazakii* isolates were resistant to ampicillin, cefazolin and extended spectrum penicillins, but were susceptible to the aminoglycosides and trimethoprim-sulfamethoxazole, whereas sensitivity to 3rd generation cephalosporins and the quinolones was variable. Subsequently Lai (2001) proposed the use of carbapenems or 3rd generation cephalosporins with an aminoglycoside or trimethoprim with sulfamethoxazole. This treatment regime has improved the outcome of *E. sakazakii* meningitis though the resistance of *Enterobacter* spp. to these antibiotics is increasing (Lai, 2001). Dennison and Morris (2002) have reported an *E. sakazakii* infection that was resistant to multiple antibiotics, including ampicillin, gentamicin and cefotaxamine. In their study of the antibiotic resistance of bacteria isolated from dairy farm soil, Burgos and Varela (2002) found genomic DNA from *E. sakazakii* contained multiple antibiotic resistance (mar) operons.

The temperature growth range of *E. sakazakii* was reported by Farmer *et al.* (1980) for 57 strains. There was no growth at 4°C or 50°C but most strains grew at 47°C. Gavini *et al.* (1983) reported 100% of strains (n=14) grew at 41°C whereas only 86% grew at 44°C. Nazarowec-White and Farber (1997c), using five clinical isolates, five food isolates and the species type strain, found the maximum growth temperature was between 41-45°C (visible growth at 24h) and the minimum growth temperature was 5.5-8°C. Breeuwer *et al.* (2003) reported growth of all strains (n=22) up to 47°C in BHI broth. Iversen *et al.* 2004 found that maximum growth temperatures were dependant on the suspending media. All strains (n=70) grew in tryptone soya broth at 44°C after 24 h but no growth was observed at 47°C, however minimal growth occurred for 34% of strains after 48 h incubation at 47°C.

Decimal reduction times and z-values for the organism in infant formula have been determined by Nazarowec-White and Farber (1997b), Edelson-Mammel and Buchanan (2004) and Iversen *et al.* (2004). While the D values varied, 4.2, 0.51-9.87 and 1.3-1.7 min for D_{58} respectively, z values were similar (5.82, 5.6 and 5.7.5.8°C) and were within the range reported for most non-spore forming bacteria (4-6°C). Breeuwer *et al.* (2003) determined z values of 3.1-3.6 for *E. sakazakii* strains in phosphate buffer indicating a protective effect of infant formula against thermal inactivation. Given the values reported for infant formula, a minimum HTST pasteurisation of 15s at 71.7 °C would result in >11D kill of *E. sakazakii*. Therefore the organism should not survive the pasteurisation process and it is likely contamination occurs as a post-processing event.

Kindle *et al.* (1996) found *E. sakazakii* and *Kleb. pneumoniae* had higher growth rates in reconstituted infant formula than other organisms tested (*Pseudomonas aeruginosa*, *Esch. coli*, *Staphylococcus aureus*, *Mycobacterium terrae* and *Candida albicans*). Nazarowec-White and Farber (1997c) found the generation and lag times for *E. sakazakii* to be similar to those reported for other *Enterobacter* but shorter than those of other organisms found in milk products. The generation time of *E. sakazakii* in infant formula was 40 min at room temperature. Iversen *et al.* (2004) found the optimum growth range for *E. sakazakii* was 37-43°C and the mean doubling time in infant formula was 20 min at 37°C. The doubling time of the strains in infant formula at 6°C and 21°C were 13.7 h and 1.7 h respectively.

Skladal *et al.* (1993) found UHT milk cartons inoculated with 10-15 *E. sakazakii* cells per 500ml and incubated at 30°C supported good bacterial growth with a reduction in the pH from 6.6 to 5.6 in less than 20 h. Concentrations of L-lactate and D-lactate reached 0.40 mM and 10.7 mM respectively. Breeuwer *et al.* (2003) reported a growth rate of 2.2 h^{-1} in BHI broth and growth in BHI containing 1.2 mol l^{-1} sodium chloride (a_w 0.96) at 37°C. The pH growth range was reported to be between pH 4.5 and 10 in BHI at 37°C. Edelson-Mammel and Buchanan (2004) examined resistance to acid in TSB adjusted to pH 3.0 and 3.5 with HCl. Over a 5-h period at 37 °C, 10/12 strains showed

less than a 1 log decline at pH 3.5 while reductions at pH 3.0 were 4.9 to > 6.3 log cfu ml^{-1}.

1.2 CLINICAL SIGNIFICANCE

In general *Enterobacter* organisms are responsible for around 50% of nosocomial infections, mostly in immunocompromised patients (Leclerc *et al.* 2001). *E. cloacae*, along with other *Enterobacter* spp. such as *E. aerogenes*, *E. hormaechei*, *E. gergoviae* and *E. sakazakii* have been associated with infections in neonates (Willis and Robinson, 1988; Nazarowec-White and Farber, 1997a; Weir, 2002). A review of 17 cases of neonatal meningitis revealed that patients with *E. sakazakii* infections suffered worse outcomes than those with meningitis attributed to *E. cloacae* and other Gram-negative bacteria (Willis and Robinson, 1988).

Adult infections due to *E. sakazakii* usually occur in compromised patients with underlying conditions and there are no reports of meningitis. Pribyl *et al.* (1985) described a case where the organism was one of three isolated from a foot ulcer. Other cases have included urosepsis (Jimenez and Gimenez, 1982) and bacteraemia (Hawkins *et al.* 1991). More recently, Lai (2001) reported four adult cases between 1995 and 1996, and Dennison and Morris (2002) reported a multiple antibiotic resistant *E. sakazakii* wound infection. In 2002, Ongradi reported a vaginal infection apparently resulting from swimming in a warm fresh water lake.

Although it is possible for infection to be acquired by previously healthy newborn infants in the home environment (Adamson and Rogers, 1981; Kleiman *et al.* 1981), the majority of reported cases have occurred in Neonatal Intensive Care Units (NICU). Although *Enterobacter* infections in newborns are often acquired from the mother during birth, some *E. sakazakii* infections occur in neonates born by Caesarean section (Urmenyi and Franklin, 1961; Muytjens *et al.* 1983; Muytjens and Kollee, 1990; Bar-Oz *et al.* 2001). Therefore it is thought risk of infection is probably related to ingestion, exacerbated poor hygiene practices and prolonged continuous enteral feeding, and not through vertical transmission from the mother (Muytjens and Kollee, 1990). Prolonged hang-time and repeated use of enteral feed bags have been identified as risk factors (Levy, 1989; Oie and Kamiya, 2001) with the possibility of biofilm formation leading to increased oral dose. *E. sakazakii* has been isolated from a wide range of clinical sources including cerebrospinal fluid, blood, bone marrow, sputum, urine, inflamed appendix, intestinal and respiratory tracts, eye, ear, wounds and faeces. The organism has also been isolated from a doctor's stethoscope (Farmer *et al.* 1980) and from nursery food preparation equipment such as spoons and a blender (Simmons *et al.* 1989; Noriega *et al.* 1990; Bar-Oz *et al.* 2001). Smeets *et al.* (1998) used PFGE to confirm the epidemiological evidence that a contaminated dish brush used for cleaning bottles was the source of three cases in 1981 and was not related to an isolate from a

1979 case. In Iceland three cases were reported linked to milk formula contaminated with *E. sakazakii* (Biering, *et al.* 1989). Two groups (Simmons *et al.* 1989 and Clark *et al.* 1990) reported on four neonates with *E. sakazakii* infections in Tennessee. Three patients had sepsis, and three had bloody diarrhoea. *E. sakazakii* was isolated from faeces from all four patients, a used can of infant formula milk and the blender (which had heavy growth of the organism). In this outbreak identical biotypes, antibiograms and plasmid profiles were obtained for patients and environmental isolates. Although the level of contamination was probably low, there was evidence of prolonged incubation in bottle heaters between 35-37°C before use. Nazarowec-White and Farber (1999a) studying three isolates obtained from one hospital over 11 years showed that they had indistinguishable ribotype patterns indicating persistence in the environment.

The first reported cases of meningitis due to 'yellow-pigmented *E. cloacae*' occurred in England (Urmenyi and Franklin, 1961). No source for the infection was identified although the report does not mention epidemiological investigation of infant formula or feed preparation equipment. Since then cases have been reported worldwide including countries such as Denmark (Jøker *et al.* ,1965), Netherlands (Muytjens *et al.* 1983 Muytjens and Kollee, 1990), Greece (Arseni *et al.* 1987), Iceland (Biering *et al.* 1989), Portugal (Lecour *et al.* 1989), Spain (Reina *et al.* 1989) Iceland (Clark *et al.* 1990), Germany (Ries *et al.* 1994), Israel (Bar-Oz *et al.* 2001, Block *et al.* 2002), Belgium (van Acker *et al.* 2001; IBFAN 2002), New Zealand (www.news-medical.net/?id=9016, 2004), France (Institute de Veille Sanitaire, 2006), Switzerland (Mange *et al.* 2006),Canada and the US (Kleiman *et al.* 1981; Burdette and Santos, 2000; Gallagher and Ball, 1991; Simmons *et al.* 1989; Naqvi *et al.* 1985; Noriega *et al.* 1990; Willis and Robinson, 1988).

Meningitis due to *E. sakazakii* has a high mortality rate 40-80% (Lai, 2001) and most reports are from hospital nurseries and neonatal intensive care units. It is probable that premature infants and those with underlying medical conditions are at highest risk for developing an *E. sakazakii* infection. However, in 1981, Kleiman *et al.* and also Adamson and Rogers reported separate cases of previously healthy 5-week-old infants admitted to hospital with meningitis due to *E. sakazakii*, showing that the infection can also be acquired in the home environment. Muytjens *et al.* (1983) re-evaluated *Enterobacter* strains from blood and CSF and uncovered several cases of meningitis and bacteraemia due to *E. sakazakii* infection suggesting that the organism had been under reported.

1.2.1 Meningitis

Meningitis is an acute inflammation of the meninges surrounding the brain and the spinal chord. Infections can increase inner cranial pressure, requiring aspiration of fluid and drainage of cerebral infarction by insertion of ventriculoperitoneal shunts to prevent

cerebral damage (Muytjens *et al.* 1983). *Esch. coli*, *Enterobacter*, *Citrobacter* and *Listeria* (Kline, 1988a and 1988b; Nazarowec-White and Farber, 1997a) are reported causes of neonatal meningitis however infection of the meninges in children less than 5 years is most frequently caused by *Haemophilus*, *Neisseria meningitidis* and *Streptococcus pneumoniae*. It is probable that, like these three major pathogens, *E. sakazakii* has a developmental dependence on access to the central nervous system. Cases of *E. sakazakii* meningitis have been reported in children between the ages of 3 days to 4 years, with half occurring in the first week after birth and almost three-quarters during the first month (Lai, 2001). A retrospective study of forty-six infants indicated that meningitis is more prevalent in infants of normal gestational age and birth weight with onset of disease usually occurring within the first week following birth. In contrast low birth weight, premature infants were more likely to develop bacteraemia with no progression to CNS disease and the age of onset was usually over one month (Bowen and Braden, 2006). A higher mortality rate and adverse sequelae in survivors were associated with meningitis cases. A head CT scan is recommended early in the management of cases with *E. sakazakii* in the blood or cerebral spinal fluid. Almost all cases have abnormalities due to bacterial invasion and hydrocephalus may later develop in cases with no initial CT abnormalities (Lai, 2001). The bacterium causes cystic changes, abscesses, fluid collection, dilated ventricles and infarctions. *E. sakazakii* meningitis leads to cerebral abscess similar to those due to *Cit. koseri* infections, therefore a similarity in the cascade of pathogenic events induced by the two organisms has been suggested (Willis and Robinson, 1988). Kline *et al.* (1988b) suggest that in both types of meningitis, infection extends directly from the ventricle not from vasculitis with infarction and liquefactive necrosis as previously proposed by Foreman *et al.* (1984). Neonatal meningitis due to *Cit. koseri* occurs sporadically in small outbreaks in nurseries throughout the USA and Europe and is fatal in approximately 34% of cases. *Cit. koseri* is a normal inhabitant of the human intestinal tract and there are no reports that infection has occurred via infant formula, however Muytjens *et al.* (1988) found *Cit. koseri* in two samples of infant formula from Germany and meningitis due to *Cit. freundii* in prepared infant formula has been documented (Thurm and Gericke, 1994; Doran, 1999).

In order to cause meningitis an organism has to colonise mucosal surfaces, translocate into the bloodstream, avoid host defence mechanisms, cross the blood/brain barrier and survive in the cerebral spinal fluid. The route by which most pathogens enter the cerebral spinal fluid to produce meningitis is not yet established, though it is thought the choroid plexus is the most likely entry site and that methods of invasion include paracellular and transcellular mechanisms. Circulating microbial products such as cell wall glycopeptides, endotoxins, proteases, collagenases, and elastases have been shown to induce permeability of the blood/brain barrier. Kline *et al.* (1988b) found 14/17

Cit. koseri strains (82%) from CSF produced a 32-kDa OMP compared to 2/21 (10%) from other body sites. Li *et al.* (1990) found this OMP occurred in a wide variety of genotypically diverse clones with 8/9 isolates from CSF producing the 32-kDa OMP compared to 1/12 other clinical isolates, which suggested it to be a virulence factor in itself not just a marker for a family of clones likely to cause meningitis. Kline *et al.* (1988a) found that a *Cit. koseri* strain possessing the 32-kDa OMP was more likely to cause ventriculitis and brain abscess in infant rats than a strain lacking the OMP, however the OMP-negative strain was more likely to cause bacteraemia, meningitis and death. The persistence of large numbers of bacteria-filled macrophages and free bacteria in the ventricles of the infant rats suggests that resistance to phagocytosis or to intraphagocytic killing may play a role in abscess development. Also, the age of the rats was significant with those at 5 days being unsusceptible to meningitis and those at 2 days prone to the disease suggesting access to the CNS is dependent on the stage of development of the host's blood brain barrier. No correlation has been found between serotype, biotype, plasmid profiles, chromosomal restriction endonuclease digests, presence of pili and strains that cause meningitis (Morris *et al.* 1986; Kline *et al.* 1988b).

1.2.2 Necrotizing enterocolitis (NEC)

Necrotizing enterocolitis is the most common important gastrointestinal illness in the newborn and can be caused by a variety of bacterial pathogens (Beaugerie *et al.* 2003; Yalaz *et al.* 2006, Afroza, 2006). In a study of 125 neonates with NEC, *Enterobacter* spp. were the most common organisms, being isolated from 29% of patients (Chan *et al.* 1994). As with cases of meningitis, necrotizing enterocolitis (NEC) due to *E. sakazakii* has a high mortality rate (10-55%). NEC has an incidence of 2-5% in premature infants and 13% in those weighing less that 1.5kg at birth and is 10 times as common in babies fed formula milk compared with those fed breast milk (Lucas and Cole, 1990). The pathogenesis is associated with neonatal intestinal ischaemia, microbial colonization of the gut and excess protein substrate in the intestinal lumen (the latter being associated with formula feeding). While in breast-fed infants enterobacteria are out numbered by bifidobacteria in a ratio of 1000:1 after 6 days, in formula-fed neonates enterobacteria are the predominant colonisers during the first month (Yoshioka *et al.* 1983; Mackie *et al.* 1999). Preterm neonates especially, often have insufficient lactase and therefore malabsorb lactose allowing it to be fermented by colonic bacteria. *Lactobacillus* spp. ferment lactose primarily to lactic acid, which can be readily absorbed from the intestinal tract, whereas Enterobacteriaceae produce carbon dioxide and hydrogen as well as organic acids from lactose. The hydrogen produced can dissect into the wall of the intestine (pneumatosis intestinalis), enter the blood stream as portal air, or escape through a perforation to become free abdominal gas (Clark *et al.* 1984). Involvement of bacteria in NEC is further implied by the

increased D-lactate found in the urine of NEC patients. Westra-Meijer *et al.* (1983) found the results of three studies showed colonisation with *Klebsiella* increased the risk of NEC. Carbonaro *et al.* (1988) found a *Kleb. pneumoniae* isolate in which β-galactosidase and lactose permease was induced by tryptone, a pancreatic digest of casein. The isolate was found to be pathogenic in the ileal loop rabbit model for NEC whereas a mutant lacking the genetic determinant for tryptone-mediate induction was not. Clark *et al.* (1985) demonstrated mucosal thinning and haemorrhage in rabbit intestinal loop models for NEC after 6 hours, which were necrotic after 18h, and found an increase in disruption of intestinal mucosa when both protein and organic acid were present. They speculated that the decreased pH could increase disassociation of protein bound divalent cations, change the spatial configuration of proteins and trigger release of vasoactive substances altering intestinal microcirculation.

Van Acker *et al.* (2001) described 12 cases of NEC in neonates that occurred in 1998. Eleven strains of *E. sakazakii* were isolated from stomach aspirate, anal swabs and blood samples and 14 strains were isolated from infant milk preparations. Arbitrary primed PCR (AP-PCR) was used to type all the *E. sakazakii* isolates and determine common sources of the outbreak. Three AP-PCR profiles were obtained for patient and milk isolates with the 14 milk isolates matching the profile from three patients. Four years earlier *E. sakazakii* had been isolated from a gastrostomy tube of a neonate fed the same type of milk. This original isolate was subsequently shown to have an AP-PCR profile almost identical to the 14 milk and patient isolates, thus demonstrating a persistent contamination problem.

Pagotto *et al.* (2003) found *E. sakazakii* to be pathogenic for suckling mice inoculated orally and interperitoneally and that *E. sakazakii* appears to produce an enterotoxin-like compound. Duffy *et al.* (1997) found *Enterobacter* spp. to be strongly associated with elevated levels of endotoxin (lipopolysaccharide), an indicator of NEC disease, in stool filtrates from neonates. Adegbola and Old (1983) found 2/4 *E. sakazakii* strains tested produced no haemagglutinins while the other 2 strains produced only mannose-sensitive haemagglutinins and were coated by the type-1 fimbrial antiserum of *E. cloacae* strain 035 but not that of '*Klebsiella aerogenes*' strain 55.

1.3 ENVIRONMENTAL AND FOOD SOURCES

E. sakazakii has been isolated from a diverse range of environments and foods other than infant formula. Environmental sources include soil and rhizosphere (Neelam *et al.* 1987; Emilani *et al.* 2001), crude oil (Assadi and Mathur, 1991), cutting fluids (Suliman *et al.* 1997), hydrothermal springs, sediment, wetlands, water and pipelines (Mosso *et al.* 1994; Espeland and Wetzel, 2001; Bartolucci *et al.* 1995; Al-Hadithi *et al.* 1995; Lee and Kim, 2003). Contaminated foods have been reported, including tofu (No *et al.*

2002), lettuce (Soriano *et al.* 2001), sour tea (Tamura *et al.* 1995), cured meats (Watanabe and Esaki, 1994), cheese, minced beef, sausage meat (Leclercq *et al.* 2002), fufu (pounded cassava with plantain and yam), fish, salad and tomato stew (Mensah *et al.* 2002). *E. sakazakii* has also been found in Khamir bread due to the organism being part of the sorghum seed surface flora (Gassem, 1999) and has also been isolated from rice seeds (Cottyn *et al.* 2001). Robertson *et al.* (2002) isolated *E. sakazakii* from mung bean sprouts in Norway and Cruz *et al.* (2004) isolated 9 strains from 50 samples of alfalfa sprouts in Mexico. The organism has also been isolated from rice starch, rice flour and eggs (Gurtler *et al.* 205).

E. sakazakii can be isolated from processing plant environments and contaminated UHT milk (Skladal *et al.* 1993). DuPont Qualicon describe on their web site the use of the RiboPrinter (Microbial Characterisation System) to trace *E. sakazakii* contamination within three European factories producing infant formula. Khandai *et al.* in 2004 reported isolation of *E. sakazakii* from factory and household dust samples. *E. sakazakii* was isolated from 18/152 (11.8%) and 14/68 (21%) samples from powdered milk factories. Additionally, 2/8 (25%) samples from chocolate, 4/ 9 (44%) from cereal, 4/15 (27%) from potato flour and 6/ 26 (23%) from pasta manufacturing plants were positive for *E. sakazakii*. No positive samples were found in 5 samples from a spice factory. Vacuum cleaner bags in Dutch households yielded 5/16 (31%) positive *E. sakazakii* samples indicating the organism may be ubiquitous in the environment.

The organism is not reported to be part of the normal animal and human gut flora although it can be isolated from faeces (Farmer *et al.* 1980; Zogaj *et al.* 2003) and it is probable that soil, water and vegetables are the principal sources of contaminated food. In coliform positive samples of drinking water in the Netherlands, *E. sakazakii* was one of the most frequent isolates along with *Citrobacter freundii*, *Escherichia coli*, and *E. cloacae* (van der Kooij, 1997). In addition rats and flies may also be carriers (Gakuya *et al.* 2001; Kuzina *et al.* 2001; Hamilton *et al.* 2003).

Despite such widespread occurrence of the organism, Muytjens and Kollee (1990) did not isolate the organism from raw cow's milk, cattle, rodents, grain, bird dung, domestic animals, surface water, soil, mud or rotting wood.

1.3.1 Contamination routes for infant milk formula.

It has been demonstrated that, unlike commercially available liquid feeds, powdered infant formula is not sterile and may contain opportunistic pathogens including *E. sakazakii.* Rehydrated powdered infant formula has been implicated as the source of *E. sakazakii* in several neonatal infections (Biering *et al.* 1989; Simmons *et al.* 1989; Clark *et al.* 1990; Muytjens *et al.* 1983 and 1988; Noriega *et al.* 1990; Smeets *et al.* 1998; van Acker *et al.* 2001; Block *et al.* 2002). The first association of *E. sakazakii* infection with contaminated infant formula powder was by Muytjens *et al.* (1983) in the

Netherlands studying eight retrospective cases of neonatal meningitis and sepsis. *E. sakazakii* was isolated from prepared milk formula, a dish brush and a stirring spoon. However the first outbreak of *E. sakazakii* linked to powdered infant formula from an unopened can was in 2001 (Himelright *et al.* 2002 and Weir, 2002).

To simulate breast milk, cow's milk is modified to reduce protein and mineral content, increase the amount of whey protein, increase the carbohydrate content and increase the Ca/P ratio. The fat is modified and vitamins are added. In the 'dry procedure' for producing infant formula, all ingredients are blended in the dry form. Skimmed milk is pasteurised and then evaporated, reducing the volume of the liquid, before fat, whey, vitamins, emulsifiers, and stabilisers are added and blended. The mixture is pasteurised at 110°C for 60s and then spray dried. This method is more prone to bacterial contamination and the ingredients are harder to mix uniformly and can separate on vibration of the cans. In the 'wet procedure' the liquid skimmed milk and premix of skimmed milk and fat are treated at 80 and 82°C respectively for 20s; the total mixture is heated at 107-110°C for 60s, concentrated using a falling-film evaporator, and heat treated again at 80°C before finally being spray dried. These 'wet' and 'dry' methods can be combined with the more soluble components added to milk before drying.

The risk of contamination is not only related to the manufacturing processes but also to the age and design of individual factories as this influences control of the microbial population in the manufacturing environment. Post-processing contamination in the plant is possible with the drying and filling areas often the principle contamination source for dried products (Caric, 1993). Pathogens can gain access from the environment or from the addition of extra ingredients at the powder stage. Members of the Enterobacteriaceae, such as *Yersinia enterocolotica* and *Salmonella*, have a history of causing foodborne infection associated with spray dried milk (Mettler, 1994). In Jan 1997 Milupa/Nutricia was asked by the UK authorities to withdraw its infant formula *Milumil* from sale following evidence that a number of Milupa-fed infants had been infected with *Salmonella* Anatum, however the source of infection at the factory was not found (IBFAN, 2002). A major outbreak of salmonellosis associated with dried milk powder arose from contamination of infant food with *S.* Ealing, which entered the factory in raw milk, disseminated through the plant environment and entered insulation material via cracks in the dryer wall (Varnam and Sutherland, 1994). In 1985, *Salmonella* infected 4 out of every 267 packets when holes in a spray dryer caused intermittent contamination at a Farley's factory (Rowe *et al.* 1987).

US Centers for Disease Control and Prevention (Himelright *et al.* 2002) reported an investigation into the 2001 Tennessee outbreak of *E. sakazakii* in a neonatal intensive care unit in which 10 cases were identified. The use of infant formula milk was the only factor associating the cases. Following the cultivation of *E. sakazakii* from a batch of

the same product the company voluntarily recalled the batch in March 2002. Further infant formula recalls in the USA due to contamination with *E. sakazakii* occurred in November 2002 (1.5 millions cans) and Jan 2003 (3,030). No outbreaks have been reported with the withdrawn batches. The paper by Farmer *et al.* (1980) defining *E. sakazakii* species includes a strain (NCTC 8155) that was originally isolated from dried milk by Thornley (1960). Therefore *E. sakazakii* has been present in dried milk products for many decades including the period of the first meningitis case in 1958 (Urmenyi and Franklin, 1961).

1.3.2 Microbial criteria

The FAO's code of hygienic practice for foods for infants and children (Codex Alimentarus CAC/RCP 21-1979) requires a minimum of 4/5 samples with <3 coliforms/g and a maximum of 1/5 control samples with >3 but ≤20 coliforms/g. These criteria have not been exceeded by the numbers of *E. sakazakii* present in infant formula including those associated with outbreaks. In 1988, Muytjens *et al.* found 52.2% of 141 powdered infant formula samples from 35 countries were contaminated with Enterobacteriaceae, with 25% containing *E. agglomerans*, 21% containing *E. cloacae* and 14% containing *E. sakazakii*. In total *E. sakazakii* was cultured from unused infant formula products from 13 countries. The level of contamination ranged from 0.36-66.0 cfu/100g. This is similar to the value of 8 cells/100g estimated by Simmons *et al.* (1989) for an open can of powdered milk formula used during the time of an outbreak on an neonatal intensive care unit. Nazarowec-White and Farber (1997b) tested 120 cans of infant formula from five different companies in Canada and found 6.7% contained *E. sakazakii*. The levels of *E. sakazakii* in positive samples were frequently 0.36 cfu/100g, but they did not enumerate Enterobacteriaceae or coliforms. Heuvelink *et al.* (2001), using a present/absence test for 25g quantities, detected *E. sakazakii* in 1 of 40 infant formula powders and 7 out of 170 milk powders. Nestlé upgraded their facilities and applied more stringent release criteria for dietetic specialities of <0.3 coliforms/g, 0 *E. sakazakii* isolates/10g following an outbreak of NEC due to a batch of Alfaré infant formula milk powder which met the FAO standards (van Acker *et al.* 2001).

In March 2002, a fatal case of neonatal meningitis due to *E. sakazakii* was linked to infant formula in Belgium and involved a previously healthy 5-day old boy who became ill on release from hospital. Although the level of contamination (1 sample 20 coliforms/g, 4 samples <1/g) was well below the acceptable international standard it was above Belgium statutory limits (<1 coliform/g in all samples; van Acker *et al.* 2001). An outbreak in 2001 of meningitis in Tennessee (USA) resulted in the recall of a nutritional product for adults, toddlers and infants with rare digestive diseases (Himelright *et al.* 2002). The microbiological quality of each batch was assessed using validated methods, and its product specifications were consistent with those proposed

by the U.S. Food and Drug Administration and other U.S. regulatory organizations. Hence it is probable that infant formula powders associated with the various *E. sakazakii* outbreaks have met the current statutory criteria.

Recently the International Commission for Microbiological Specifications for Foods (ICMSF, 2002) ranked the organism as 'Severe hazard for restricted populations, life threatening or substantial chronic sequelae or long duration'. Subsequently it has the same ranking as more familiar food and waterborne pathogens such as *Listeria monocytogenes*, *Clostridium botulinum* types A and B and *Cryptosporidium parvum*.

Due to epidemiological studies and the fact that powdered infant formulas are not commercially sterile products, the US FDA has proposed interim recommendations on the preparation of powdered infant formula in neonatal intensive care units. These recommendations include the preparation of the feed by trained personnel, immediate refrigeration (temperature not stated), to discard the feed if not used within 24 h, continuous enteral feeding (hang time) should not exceed 4 h and that alternatives to the powdered from should be chosen when possible. However there is clearly a need for a review of the accepted criteria for *E. sakazakii* in infant food products and for a rapid test that is feasible for use in the food manufacturing industry.

1.3.3 Isolation and identification methods

The U.S. Food and Drug Administration, (FDA, 2002) developed a method for the isolation and enumeration of *E. sakazakii* from dehydrated powdered infant formula that takes 6-7 days (URL: vm.cfsan.fda.gov/~comm/mmesakaz.html). This method is similar to that first used by Muytjens *et al.* (1988) and Nazarowec-White and Farber (1997) except the FDA resuspend the infant formula in water rather than buffered peptone water. Also enriched samples (0.1 ml) are streaked on violet red bile glucose (VRBG) agar rather than pour-plating 1.0 ml in VRBG agar. The FDA method is based on the most probable number (MPN) approach, using a total of 333 g of product (3x100g, 3x10g, 3x 1g), and follows the conventional steps of pre-enrichment, enrichment in Enterobacteriaceae Enrichment (EE) broth and isolation using Violet Red Bile Glucose agar (VRBGA). Heuvelink *et al.* (2001) used a presence/absence test instead of the MPN approach by analysing 25g sample size pre-enriched in 225ml BPW, enrichment in EE and isolation on VRBGA. However VRBGA is only selective for Enterobacteriaceae and is not specific for *E. sakazakii*. Therefore five red colonies are picked from VRBGA and subcultured on TSA at 25°C for 48-72h for pigment production, followed by confirmation using the API 20E biochemical identification system (bioMérieux UK Ltd.) and the oxidase test. This approach however suffers from the possibility that other Enterobacteriaceae could outgrow *E. sakazakii* during the pre-enrichment and enrichment stages, leading to relatively few *E. sakazakii* colonies on VRBGA and subsequently a reduced chance of picking the organism onto TSA.

Isolation methods based on raised temperature (~44°C) have been used for 'faecal coliforms' (Alonso *et al.* 1999). This approach would be inappropriate for *E. sakazakii* however since previous workers reported that not all strains grew at this temperature (Gavini *et al.* 1983; Nazarowec-White and Farber, 1997c).

During the past decade there has been a significant increase in the use of chromogenic substrates in isolation media (Manafi, 2000). In particular the indolyl-substrates have been used to differentiate a number of important organisms such as *Esch. coli* from their competitors (van Poucke and Nelis, 2000). Halogen substituted analogs of 3-indolyl-β-D-glycopyranosides are used to monitor the formation or presence of β-glycosidases by release of an aglycone that is converted rapidly to indigo or an indigo analog. A major advantage of these substrates is that strong colours are produced that do not diffuse out from colonies and therefore even small positive colonies are visible in the presence of more numerous competitors. Media for presumptive detection of *E. sakazakii* in infant formula have been developed using the fluorescent substrate 4-methyl-umbelliferyl α-D-glucoside (Leuschner *et al.* 2004; Leuscher and Bew, 2004; Oh and Kang, 2004). However as this substrate readily diffuses across bacterial membranes it is not as specific as the chromogen and diffusion from positive colonies may interfere with the correct assessment of nearby negative colonies.

A method for cationic-magnetic-bead capture of *E. sakazakii* in infant formula has been described which can detect 1 to 5 cfu of *E. sakazakii* in 500g of IMF (Mullane *et al.* 2006). After a short enrichment (6h in BPW) the homogenate is transferred to the Pathatrix (Matrix Microscience Ltd., Newmarket, UK) patented capture system which uses cationic (positively charged) paramagnetic beads to electrostatically attract the negatively charged lipopolysaccharide on the surface of gram-negative bacteria. The beads are then plated directly onto chromogenic agar and presumptive colonies identified.

The confirmation of presumptive positive colonies isolated on growth media is traditionally performed using phenotypic tests. Numerous commercial systems are available for the biochemical characterisation of microorganisms. These usually consist of biochemical test galleries interpreted using databases of negative and positive predictabilities for different species and genera. The Enterotube II as well as the API 20E systems have been used for presumptive-positive confirmations of *E. sakazakii* via biochemical characteristics (Biering *et al.* 1989; Gassem, 1999; Kandhai *et al.* 2004; Monroe and Tift, 1979; Mosso *et al.* 1994; Muytjens *et al.* 1983; Nazarowec-White and Farber, 1999; No *et al.* 2002; Simmons *et al.* 1989; Van Os *et al.* 1996; Willis and Robinson, 1988). Cottyn *et al.* (2001) used fatty acid methylester (FAME) analysis, API 20E strips and Biolog GN2 to confirm *E. sakazakii*. Lee and Kim (2003) identified *E.*

sakazakii using m-Endo agar and whole-cell fatty acid methyl ester profiles generated by the Microbial Identification (MIDI) system in addition to API 20E kits.

Molecular identification methods targeting conserved regions of *E. sakazakii* DNA are being developed and applied to speed up diagnostic procedures. Seo and Brackett (2005) developed a real-time PCR assay for the detection of *E. sakazakii* in infant formula using primers and probes based on the *rps*U and *dna*G genes from the *E. sakazakii* macromolecular synthesis operon. The assay was able to discriminate *E. sakazakii* from all other *Enterobacter* and non-*Enterobacter* strains tested. A detection limit of 100 cfu ml^{-1} was determined in pure culture and reconstituted infant formula in 50 cycles of PCR without enrichment. Liu *et al.* (2006) developed species-specific PCR and oligonucleotide array assays to detect the 16S-23S rDNA internal transcribed spacer (ITS) of *E. sakazakii*. The methods were tested against 88 different bacterial strains with no false negative or false positive reactions. With selective enrichment, sensitivity of detection was 1.3 cfu per 100 g infant formula and the entire procedure could be completed within 48 h. Mohan Nair and Venkitanarayanan (2006) cloned and sequenced the outer membrane protein A gene (*ompA*) from *E. sakazakii* ATCC 51329, along with its flanking sequences. Based on regions of the *ompA* gene unique to *E. sakazakii*, primers were synthesized to develop and optimize an *E. sakazakii*-specific PCR which amplified a 469-bp DNA product from all *E. sakazakii* strains tested but not from other bacteria. Experiments to determine the sensitivity of the PCR indicated that it could detect as few as 10^3 cfu ml^{-1} of *E. sakazakii* bacteria in infant formula directly and 10^{-1} cfu ml^{-1} after an 8-h enrichment step. Lehner *et al.* (2006a) developed an α-glucosidase based PCR system that exclusively targets the gene responsible for the α-glucosidase activity in *E. sakazakii*. Primers generating a product of 1680 bp in size were designed for open reading frames encoding enzymes with the potential to hydrolyze the fluorescent substrate 4-methylumbellyferryl-α-D-glucoside. All *E. sakazakii* target strains were identified with no false positives, including no false positives from organisms that exhibit presumptive positive α-glucosidase activity on chromogenic media.

Further work is needed to clarify the taxonomy of *E. sakazakii* genomic groups. Also accurate and efficient methods need to be developed to protect the health of neonates and to facilitate monitoring of *E. sakazakii* in food products. Additionally research is needed into the mechanisms which lead to *E. sakazakii* pathogenicity.

AIMS

The aims of this project were to investigate the definition and identification of *E. sakazakii,* to improve on existing culture-based isolation methods and to assess the presence of the organism in infant formula and related food products. Also, to investigate persistence and growth of the organism in infant formula powder and to explore potential virulence factors including attachment and invasion, serum survival and persistence within macrophages.

CHAPTER 2 - GENERAL MATERIALS AND METHODS

2.1 MANUFACTURERS, SUPPLIERS AND SOURCES OF STRAINS.

AES	AES Laboratoire, Rue Maryse Bastié, Ker Lann, F-35172 Bruz, France.
ATCC	American Type Culture Collection (ATCC), P.O. Box 1549, Manassas, VA 20108 USA.
BDH	BDH Laboratory Supplies, Poole, UK
Biolog	Biolog, Inc., Hayward, CA, USA
bioMérieux	bioMérieux S.A., Chemin de l'orme, 69280 Marcy l'Etoile, France
CDC	Centre for Disease Control, Atlanta, GA, USA.
CR	Hospital Děské Budéjovice, Czech Republic.
DIFCO	DIFCO Laboratories, Detroit, USA
DW	Don Whitley Scientific Ltd., Shipley, UK.
Fisher	Fisher Scientific, Loughborough, UK
Glycosynth	Glycosynth Ltd., Warrington, UK
HCSC	Food Directorate Microbiology Research, Health Products and Food Branch, Health Canada.
LA	Children's Hospital Los Angeles, CA, USA.
JLU	Institut für Tierärztliche Nahrungsmittelkunde Milchwissenschaften, Justus-Liebig-Universität Gießen, Germany.
NCH	Nottingham City Hospital Trust, Hucknall Road, Nottingham, UK.
NCIMB	National Collection of Industrial and Marine Bacteria (NCIMB) Ltd, AURIS Business Centre, University of Aberdeen Campus, Old Aberdeen, Scotland.
NCTC	National Collection of Type Cultures (NCTC), PHLS Central Public Health Laboratory, London, UK.
NF	Northern Foods, Nottingham, UK.
NRC	Nestlé Research Center, Vers-Chez-Les-Blanc, Lausanne, Switzerland.
NTU	Nottingham Trent University, School of Biomedical and Natural Sciences, Clifton Lane, Clifton, Nottingham, UK.
Oxoid	Oxoid Ltd., Basingstoke, Hampshire, UK.
RAD	Department of Medical Microbiology, Radboud, Nijmegen, Netherlands.
Sigma	Sigma-Aldrich, Poole, Dorset, UK.
VWR	VWR International, Lutterworth, Leicestershire, UK

2.2 ORIGIN OF CULTURES

The origin and identities of the bacterial isolates used in this study are given in Table 2.1. The number after the *E. sakazakii* isolates designates 16S rDNA group – biogroup (where available). Also, PIF = powdered infant formula, [a] 16S rDNA sequence and [b] hsp60 sequence. [c]The source details are listed on the previous page.

Abbreviations for genera;

B.	*Bacillus*	*Lec.*	*Leclercia*
But.	*Buttiauxella*	*Pant.*	*Pantoea*
Cit.	*Citrobacter*	*Prot.*	*Proteus*
Ced.	*Cedacea*	*Prov.*	*Providencia*
Chr.	*Chrysomonas*	*Ps.*	*Pseudomonas*
E.	*Enterobacter*	*Ra.*	*Raoultella*
Esch.	*Escherichia*	*Rh.*	*Rhanella*
Haf.	*Hafnia*	*Ser.*	*Serratia*
Kleb.	*Klebsiella*	*St.*	*Staphylococcus*

Table 2.1 The origin of strains used in this study.

NTU	Strain identity		Source	
1[ab]	NCTC 11467	*E. sakazakii* 1-1	NCTC[c]	clinical
2[ab]	ATCC 12868	*E. sakazakii* 1-1	ATCC	unknown
3[ab]	ATCC 51329	*E. sakazakii* 3-15	ATCC	unknown
4[ab]	HPB 2856	*E. sakazakii* 1-1	HCSC	clinical
5[ab]	HPB 2852	*E. sakazakii* 1-4	HCSC	clinical
6[ab]	HPB 2853	*E. sakazakii* 1-1	HCSC	clinical
7[ab]	FSM 307	*E. sakazakii* 1-2	NRC	environment
8[ab]	CR 4344	*E. sakazakii* 1-9	CR	clinical
9[ab]	BF006BB	*E. sakazakii* 2-16	NTU	baby food
10[a]	DF020BB1	*E. sakazakii* 1-7	NTU	dried food
11[a]	DF026BB	*E. sakazakii* 1-2	NTU	dried food
12[ab]	CR 3753	*E. sakazakii* 1-2	CR	clinical
13[a]	CH15B	*E. sakazakii* 1-2	NTU	herb/spice
14[ab]	FSM 309	*E. sakazakii* 1-1	NRC	environment

NTU	Strain identity		Source	
15 [ab]	CR 2422	*E. sakazakii* 1-8B	CR	clinical
16 [ab]	J11B1	*E. sakazakii* 3-15	NTU	herb/spice
17 [ab]	HT2	*E. amnigenus*	NF	food
18 [ab]	CR 5220	*E. sakazakii* 1-9	CR	clinical
19 [ab]	CR 5243	*E. sakazakii* 1-5	CR	clinical
20 [ab]	CR 5660	*E. sakazakii* 1-7	CR	clinical
21 [ab]	CR 5780	*E. sakazakii* 1-9	CR	clinical
22 [ab]	FSM 321	*E. sakazakii* 1-8A	NRC	environment
23 [ab]	FSM 322	*E. sakazakii* 1-2	NRC	environment
24 [a]	FSM 323	*E. sakazakii* 1-2	NRC	environment
25 [ab]	IFMK5B	*E. sakazakii* 1-2	NTU	PIF
26 [ab]	IFMK8B	*E. sakazakii* 1-2	NTU	PIF
27 [ab]	MP040aB	*E. sakazakii* 1-2	NTU	milk powder
28 [a]	DF014BB	*E. sakazakii* 1-5	NTU	dried food
29 [a]	DF038BB1	*E. sakazakii* 1-2	NTU	dried food
30 [ab]	DF042BB	*E. sakazakii* 1-5A	NTU	dried food
31 [a]	DF052BB1	*E. sakazakii* 1-1	NTU	dried food
32 [a]	BF033BB	*E. sakazakii* 1-1	NTU	baby food
33 [ab]	BF042BB	*E. sakazakii* 1-14A	NTU	baby food
34 [a]	BF043Bw	*Enterobacter* sp.	NTU	baby food
35 [ab]	BF046BB2	*E. sakazakii* 1-8C	NTU	baby food
36 [a]	CH28bB	*Pantoea* sp.	NTU	herb/spice
37 [a]	CH7B1	*Enterobacter* sp.	NTU	herb/spice
38 [a]	J37B	*E. sakazakii* 1-1	NTU	herb/spice
39 [a]	CH22B	Unidentified 6b	NTU	herb/spice
40 [a]	J14B3	Unidentified 6b	NTU	herb/spice
41 [a]	J34B	Unidentified 6a	NTU	herb/spice
42 [a]	J3B	Unidentified 6a	NTU	herb/spice
43 [ab]	MP037B	Unidentified 6b	NTU	milk powder
44 [ab]	IFMK2W	*Pantoea* sp.	NTU	PIF
45 [ab]	ATCC 25408	*Cit. koseri*	ATCC	clinical
46 [a]	CK4277	*Cit. koseri*	LA	clinical
47 [a]	CK4036	*Cit. koseri*	LA	clinical
48 [a]	CKSMT319	*Cit. koseri*	LA	clinical
49 [a]	ATCC 13047	*E. cloacae*	ATCC	clinical

NTU	Strain identity		Source	
50 [ab]	OCC F141	*E. cloacae*	Oxoid	clinical
51 [a]	CH32DB	Unidentified 6b	NTU	herb/spice
52 [a]	MP023W	*Enterobacter* sp.	NTU	milk powder
53 [a]	FF07BB	*But. noakiae*	NTU	food
54 [ab]	CH50B2	Unidentified 5a	NTU	herb/spice
55 [ab]	JOAB1	Unidentified 5a	NTU	herb/spice
56 [a]	CH45DB1	*E. pyrinus*	NTU	herb/spice
57 [ab]	MP039B	*E. sakazakii* 2-16B	NTU	milk powder
58 [ab]	JOCB1	Unidentified 5b	NTU	herb/spice
59 [ab]	JOHB2	Unidentified 6a	NTU	herb/spice
60 [a]	CH49B1	Unidentified 5a	NTU	herb/spice
61 [a]	CH5B	Unidentified 6a	NTU	herb/spice
62 [a]	DF013BB	*E. sakazakii* 1-5	NTU	dried food
63 [a]	CKJB62	*Cit. koseri*	LA	clinical
64 [a]	DF016BB	*E. sakazakii* 1-2	NTU	dried food
65 [a]	DF040BB1	*E. sakazakii* 1-1	NTU	dried food
66 [a]	DF008BB	*Lec. adecarboxylata*	NTU	dried food
67 [a]	DF056BB1	*E. sakazakii* 1-1	NTU	dried food
68 [a]	CH4B2	*E. sakazakii* 1-1	NTU	herb/spice
69 [a]	HPB 2848	*E. sakazakii* 1-8A	HCSC	clinical
70 [ab]	HPB 2873	*E. sakazakii* 1-4	HCSC	clinical
71 [a]	HPB 2874	*E. sakazakii* 1-4	HCSC	clinical
72 [a]	HPB 2882	*E. sakazakii* 1-8C	HCSC	clinical
73 [a]	CH6DB2	*Esch. hermannii*	NTU	herb/spice
74 [a]	CH12B	*E. sakazakii* 1-2	NTU	herb/spice
75 [a]	CH8B	Unidentified 6b	NTU	herb/spice
76 [a]	CH13B	*E. sakazakii* 1-2	NTU	herb/spice
77 [a]	CH14	*E. sakazakii* 1-2	NTU	herb/spice
78 [a]	CH16B	*E. sakazakii* 1-2A	NTU	herb/spice
79 [a]	CH17B	*E. sakazakii* 1-2	NTU	herb/spice
80 [a]	CH18B1	*E. sakazakii* 1-1	NTU	herb/spice
81 [a]	CH19	*E. sakazakii* 1-2	NTU	herb/spice
82 [a]	CH20	*E. sakazakii* 1-2	NTU	herb/spice
83 [a]	CH25DB	*E. sakazakii* 1-2	NTU	herb/spice
84 [ab]	CH31DB	*E. sakazakii* 4-6	NTU	herb/spice

NTU	Strain identity		Source	
85 [a]	CH37B	*E. sakazakii* 1-9	NTU	herb/spice
86 [ab]	CH38B	*E. sakazakii* 1-5A	NTU	herb/spice
87 [a]	CH39B	*E. sakazakii* 1-1	NTU	herb/spice
88 [a]	CH40B1	*E. sakazakii* 1-2	NTU	herb/spice
89 [a]	CH42DB	*E. sakazakii* 1-1	NTU	herb/spice
90 [ab]	CH43B	*E. sakazakii* 1-5A	NTU	herb/spice
91 [a]	CH47B1	*E. sakazakii* 1-1	NTU	herb/spice
92 [ab]	CH48B1	*E. sakazakii* 2-16A	NTU	herb/spice
93 [ab]	J1B	*E. sakazakii* 1-5	NTU	herb/spice
94 [a]	J9B1	*E. sakazakii* 1-4	NTU	herb/spice
95 [a]	J10B	*E. sakazakii* 1-5	NTU	herb/spice
96 [ab]	J46DB	*E. sakazakii* 2-16	NTU	herb/spice
97 [ab]	J14B1	*E. sakazakii* 2-16	NTU	herb/spice
98 [a]	J17B2	Unidentified 6a	NTU	herb/spice
99 [a]	J28B	*E. sakazakii* 1-1	NTU	herb/spice
100 [a]	J33B	*E. sakazakii* 1-1	NTU	herb/spice
101 [a]	J50B	*E. sakazakii* 1-13C	NTU	herb/spice
102 [ab]	J44DB	*E. sakazakii* 1-14	NTU	herb/spice
103 [a]	J51B2	*E. sakazakii* 1-1	NTU	herb/spice
104 [a]	JOAB2	*E. sakazakii* 1-1	NTU	herb/spice
105 [ab]	JOCB2	*E. sakazakii* 2-16	NTU	herb/spice
106 [a]	JOEB2	*E. sakazakii* 1-1	NTU	herb/spice
107 [a]	JOFB1	*E. sakazakii* 1-1	NTU	herb/spice
108 [a]	JOGB1	*E. sakazakii* 1-1	NTU	herb/spice
109 [ab]	JOJB2	*E. sakazakii* 1-5	NTU	herb/spice
110 [a]	JOKB2	*E. sakazakii* 1-2	NTU	herb/spice
111 [a]	TSDB	*E. sakazakii* 2-16	NTU	herb/spice
112 [a]	BF046BB1	*E. sakazakii* 1-9	NTU	baby food
113 [a]	DF020BB2	*Kleb. pneumoniae*	NTU	dried food
114 [a]	OCC 1888	*E. sakazakii* 1-3	Oxoid	clinical
115 [a]	DF052BB2	*E. sakazakii* 1-3	NTU	dried food
116 [a]	BF033VY	*E. sakazakii* 1-1	NTU	baby food
117 [a]	BF042VY	*E. sakazakii* 1-14	NTU	baby food
118 [a]	DF026VY	*E. sakazakii* 1-2	NTU	dried food
119 [a]	BF046VY	*E. sakazakii* 1-9	NTU	baby food

NTU	Strain identity		Source	
120 [a]	V121Y	*E. sakazakii* 1-2	NTU	PIF
121 [a]	V125Y	*E. sakazakii* 1-2	NTU	PIF
122 [a]	J22B2	Unidentified 6b	NTU	herb/spice
123 [a]	J18B2	Unidentified 6b	NTU	herb/spice
124 [a]	J19B2	Unidentified 6b	NTU	herb/spice
125 [a]	J32B	Unidentified 6b	NTU	herb/spice
126 [a]	J25B	Unidentified 6a	NTU	herb/spice
127 [a]	TCFB	*But. noakiae*	NTU	herb/spice
128 [a]	J23B2	Unidentified 6b	NTU	herb/spice
129 [a]	TRDB	Unidentified 5b	NTU	herb/spice
130 [a]	a-LACB	Unidentified 5a	NTU	lactose
131 [a]	CH34B	*E. sakazakii* 1-2	NTU	herb/spice
132 [a]	FF03BK	*Cit. braakii*	NTU	food
133 [a]	J39VY	*E. sakazakii* 1-5A	NTU	herb/spice
134 [a]	CH4B	*E. sakazakii* 1-1	NTU	herb/spice
135 [a]	FF16BK	*Citrobacter* sp.	NTU	food
136 [a]	CH18B2	*E. sakazakii* 1-1	NTU	herb/spice
137 [a]	CH42B	*E. sakazakii* 1-1	NTU	herb/spice
138 [a]	[a]J1SCMB	*E. sakazakii* 1-2	NTU	herb/spice
140 [a]	J11B3	*E. sakazakii* 3-15	NTU	herb/spice
141 [a]	J41B	*E. sakazakii* 1-14	NTU	herb/spice
142 [a]	CH7B2	*E. sakazakii* 1-1	NTU	herb/spice
143 [a]	CH28bDB	*Pantoea* sp.	NTU	herb/spice
144 [a]	JOFB2	*E. sakazakii* 1-1	NTU	herb/spice
145 [a]	JOJVY	*E. sakazakii* 2-16	NTU	herb/spice
146 [a]	JOAVY	*E. sakazakii* 1-1	NTU	herb/spice
147 [a]	JLDB	Unidentified 5a	NTU	herb/spice
148 [a]	JMDB	Unidentified 5a	NTU	herb/spice
149 [a]	JNDB	Unidentified 5b	NTU	herb/spice
150 [a]	JQB	*E. sakazakii* 1-1	NTU	herb/spice
151 [a]	JUB	Unidentified 5a	NTU	herb/spice
152 [a]	CH11B	Unidentified 6a	NTU	herb/spice
153 [a]	IFMK6BK	*Cit. freundii*	NTU	PIF
154 [a]	TRDBK	*Citrobacter* sp.	NTU	herb/spice
155 [a]	CH26B1	Unidentified 6b	NTU	herb/spice

NTU	Strain identity		Source	
156 [a]	CH41B2	*E. sakazakii* 1-5	NTU	herb/spice
157 [a]	CH34DB	Unidentified 6b	NTU	herb/spice
158 [a]	CH41B1	Unidentified 6b	NTU	herb/spice
159 [a]	DF038BB3	Unidentified 5a	NTU	dried food
160 [a]	DF039BB	Unidentified 6b	NTU	dried food
161 [a]	J29B	*E. pyrinus*	NTU	herb/spice
162 [a]	DF049BB	Unidentified 5a	NTU	dried food
163 [a]	CH47VY	Unidentified 5b	NTU	herb/spice
164 [a]	CH47DB	Unidentified 5b	NTU	herb/spice
165 [a]	CH28aDB	Unidentified 6b	NTU	herb/spice
166	OCC F191	*Prot. vulgaris*	Oxoid	clinical
167	OCC 195	*Prot. vulgaris*	Oxoid	unknown
168	NCTC 4636	*Prot. vulgaris*	NCTC	unknown
169	OCC 112	*Prot. vulgaris*	Oxoid	unknown
170	OCC 217	*Ser. marcescens*	Oxoid	unknown
171	TTFBK	*Salmonella* Arizonae	NTU	herb/spice
172	T33BK	*Prov. stuartii*	NTU	herb/spice
173	FF04BB2	*Prot. vulgaris*	NTU	food
174	T32W	*Prov. rettgeri*	NTU	herb/spice
175	FF02BW	*Prot. vulgaris*	NTU	food
176	DF025BW	*Esch. hermannii*	NTU	dried food
177	JQW	*E. cloacae*	NTU	herb/spice
178	OCC 219	*Ser. marcescens*	Oxoid	unknown
179	OCC 253	*Ser. marcescens*	Oxoid	unknown
180	J6W	*Pantoea* sp.	NTU	herb/spice
181 [ab]	J9W	*Cit. sedlakii*	NTU	herb/spice
182	J10W	*Kleb. pneumoniae*	NTU	herb/spice
183	J14W	*E. amnigenus*	NTU	herb/spice
184	J18W1	*Kleb. oxytoca*	NTU	herb/spice
185	J23W	*E. cloacae*	NTU	herb/spice
186	J29W3	*Kleb. ozaenae*	NTU	herb/spice
187	J29W1	*E. cloacae*	NTU	herb/spice
188	J53W2	*Ser. ficaria*	NTU	herb/spice
189 [ab]	J32W1	*Cit. sedlakii*	NTU	herb/spice
190	J32W2	*Pantoea* sp.	NTU	herb/spice

NTU	Strain identity		Source	
191	J32XSCW	*Ser. ficaria*	NTU	herb/spice
192	J36W	*E. cloacae*	NTU	herb/spice
193	J43W	*E. cloacae*	NTU	herb/spice
194	J44W2	*Pantoea* sp.	NTU	herb/spice
195	J22W	*E. cloacae*	NTU	herb/spice
196	J50W	*E. cloacae*	NTU	herb/spice
197	J51W	*Kleb. ornitholytica*	NTU	herb/spice
198	J52W	*Pantoea* sp.	NTU	herb/spice
199	J53W1	*E. cloacae*	NTU	herb/spice
201	CH11W	*E. cloacae*	NTU	herb/spice
202	CH15W	*E. aerogenes*	NTU	herb/spice
203	CH19W	*Lec. adecarboxylata*	NTU	herb/spice
204	CH20W	*Lec. adecarboxylata*	NTU	herb/spice
205	CH23W	*E. cloacae*	NTU	herb/spice
206	CH25W1	*E. aerogenes*	NTU	herb/spice
207	CH33W2	*Kleb. ozaenae*	NTU	herb/spice
208	CH39W	*E. cloacae*	NTU	herb/spice
209	CH40W	*E. cloacae*	NTU	herb/spice
210	CH41W	*E. cloacae*	NTU	herb/spice
211	CH43W1	*Lec. adecarboxylata*	NTU	herb/spice
212	CH44W1	*E. cloacae*	NTU	herb/spice
213	CH45W1	*Kleb. pneumoniae*	NTU	herb/spice
214	CH46W	*E. cloacae*	NTU	herb/spice
215	CH50W1	*Esch. coli*	NTU	herb/spice
216	OCC 118	*E. cloacae*	Oxoid	unknown
217	OCC 641	*E. cloacae*	Oxoid	unknown
218	ATCC 23355	*E. cloacae*	ATCC	unknown
219	NCTC 10006	*E. aerogenes*	NCTC	clinical
220	NCIMB 702071	*Pant. agglomerans*	NCIMB	food
221	NCIMB 702072	*Pant. agglomerans*	NCIMB	milk
222	NCIMB 702073	*Pant. agglomerans*	NCIMB	milk
223	NCIMB 10341	*Kleb. pneumoniae*	NCIMB	milk
224 [a]	ATCC 27026	*Cit. koseri*	ATCC	clinical
225 [a]	ATCC 25409	*Cit. koseri*	ATCC	clinical
226 [a]	ATCC 25410	*Cit. koseri*	ATCC	clinical

NTU	Strain identity		Source	
227	FF05BW	*Esch. coli*	NTU	food
228	FF06BW	*Esch. vulneris*	NTU	food
229	FF12BW	*Pantoea* sp.	NTU	food
230	FF13BW	*Kleb. oxytoca*	NTU	food
231	FF13BK	*Cit. braakii*	NTU	food
232	FF14BW	*Kleb. pneumoniae*	NTU	food
233	FF17BW	*Kleb. pneumoniae*	NTU	food
234	FF18BW	*Kleb. pneumoniae*	NTU	food
235	FF19BW	*Kleb. oxytoca*	NTU	food
236	T29W	*Haf. alvei*	NTU	herb/spice
237	T37W2	*Pantoea* sp.	NTU	herb/spice
238	T37W1	*Ps. aeruginosa*	NTU	herb/spice
239	T38W2	*Cit. freundii*	NTU	herb/spice
240	TRMW	*Haf. alvei*	NTU	herb/spice
241	FF01BW	*E. amnigenus*	NTU	food
242	FF01BB2	*Esch. coli*	NTU	food
243	FF23BW	*Kleb. pneumoniae*	NTU	food
244	IFM044W	*Chr. luteola*	NTU	PIF
246	DF055VY	*Pantoea* sp.	NTU	dried food
247	DF056BW	*Burkholderia cepacea*	NTU	dried food
248	DF059VY	*Pantoea* sp.	NTU	dried food
249	BF048VY	*Pantoea* sp.	NTU	baby food
250	V110Y	*Ser. ficaria*	NTU	PIF
251	IFMSA1	*Rh. aquatilis*	NTU	PIF
252	V140Y	*Kleb. ozaenae*	NTU	PIF
253	IFMK3W	*Kleb. ozaenae*	NTU	PIF
254	IFMK6W	*E. cloacae*	NTU	PIF
255	V137Y	*Pantoea* sp.	NTU	PIF
256	IFMK7W	*Pantoea* sp.	NTU	PIF
257	V139Y	*Pantoea* sp.	NTU	PIF
258	MPK12W	*Chr. luteola*	NTU	milk powder
259	MPK15W	*E. cloacae*	NTU	milk powder
260	JOAW2	*E. cloacae*	NTU	herb/spice
261	JOAW1	*Chr. luteola*	NTU	herb/spice
262	JOCW2	*E. cloacae*	NTU	herb/spice

NTU	Strain identity		Source	
263	JOCW1	*E. cloacae*	NTU	herb/spice
264	JOEW2	*E. cloacae*	NTU	herb/spice
265	JOFW2	*Pantoea* sp.	NTU	herb/spice
266	JOGW2	*Ced. davisae*	NTU	herb/spice
267	JOGW1	*Ser. ficaria*	NTU	herb/spice
268	JOHW2	*Esch. vulneris*	NTU	herb/spice
269	JOIW2	*E. cloacae*	NTU	herb/spice
270	JOJW2	*Esch. vulneris*	NTU	herb/spice
271	JOKW2	*Esch. coli*	NTU	herb/spice
272	JLW	*E. cloacae*	NTU	herb/spice
273	JPW1	*Kleb. pneumoniae*	NTU	herb/spice
275	JNW1	*Esch. coli*	NTU	herb/spice
276	JNDW	*Rh. aquatilis*	NTU	herb/spice
277	JOW	*E. cloacae*	NTU	herb/spice
278	JPDW(JPW3)	*Kleb. pneumoniae*	NTU	herb/spice
279	JPW2	*Kleb. pneumoniae*	NTU	herb/spice
280	JRW	*Kleb. pneumoniae*	NTU	herb/spice
281	JSW	*Kleb. pneumoniae*	NTU	herb/spice
282	JTW	*Kleb. pneumoniae*	NTU	herb/spice
283	JUW	*Kleb. pneumoniae*	NTU	herb/spice
284	JWW	*E. cloacae*	NTU	herb/spice
285	DF061W	*E. aerogenes*	NTU	dried food
288	BF028VY	*Pantoea* sp.	NTU	baby food
289	BF028BW	*Esch. vulneris*	NTU	baby food
290	BF043VY	*Pantoea* sp.	NTU	baby food
291	BF045BW	*Esch. vulneris*	NTU	baby food
292	BF045VY	*Pantoea* sp.	NTU	baby food
293	DF026VW	*Ra. terrigena*	NTU	dried food
294	DF035VY	*Esch. hermannii*	NTU	dried food
295	DF035BW1	*Pantoea* sp.	NTU	dried food
296	DF039BW1	*E. cloacae*	NTU	dried food
297	DF039BW2	*Kleb. pneumoniae*	NTU	dried food
298	DF040BW	*E. cloacae*	NTU	dried food
300	MP003W	*Pantoea* sp.	NTU	milk powder
301	V162Y	*Pantoea* sp.	NTU	PIF

NTU	Strain identity		Source	
302	MP005aW	*E. cloacae*	NTU	milk powder
303	MP006aW	*Lec. adecarboxylata*	NTU	milk powder
304	V99Y	*Lec. adecarboxylata*	NTU	PIF
305	MP011W	*Pantoea* sp.	NTU	milk powder
306	V98Y	*Pantoea* sp.	NTU	PIF
307	MP013W	*E. cloacae*	NTU	milk powder
309	MP017W	*Pantoea* sp.	NTU	milk powder
311	V86Y	*Esch. hermannii*	NTU	PIF
312	MP019W	*Esch. hermannii*	NTU	milk powder
314	V161Y	*Esch. vulneris*	NTU	PIF
315	MP025aW	*E. cloacae*	NTU	milk powder
316	MP027aW	*E. cloacae*	NTU	milk powder
317	MP028aW	*E. amnigenus*	NTU	milk powder
318	MP029W	*Ra. terrigena*	NTU	milk powder
320	MP033aW2	*E. cloacae*	NTU	milk powder
321	MP037W	*E. cloacae*	NTU	milk powder
322	MP038W	*Chr. luteola*	NTU	milk powder
324	MP040aW	*E. cloacae*	NTU	milk powder
325	MP041W	*E. cloacae*	NTU	milk powder
326	MP005bW	*E. cloacae*	NTU	milk powder
327	MP006bW	*Kleb. ozaenae*	NTU	milk powder
328	MP014bW	*E. cloacae*	NTU	milk powder
330	MP028bW	*E. cloacae*	NTU	milk powder
331	MP033bW	*Ra. terrigena*	NTU	milk powder
332	MP039bW	*Kleb. pneumoniae*	NTU	milk powder
335	X2SDW8-3	*Esch. vulneris*	NTU	PIF
336	X3SDW1	*Rh. aquatilis*	NTU	PIF
337	JMDW	*Pantoea* sp.	NTU	herb/spice
338	DF050VY	*Ced. lapagei*	NTU	dried food
339	DF054VY	*Chr. luteola*	NTU	dried food
340 [a]	FF08BK	*Citrobacter* sp.	NTU	food
341	CH28BK	*Cit. youngae*	NTU	herb/spice
342	CH27VY	*Esch. vulneris*	NTU	herb/spice
343	JVW	*E. cloacae*	NTU	herb/spice
344	DF058VY	*Pantoea* sp.	NTU	dried food

NTU	Strain identity		Source	
345	JNDY	*Pantoea* sp.	NTU	herb/spice
346	DF057VY	*Esch. hermannii*	NTU	dried food
348	BF049VY	*E. cloacae*	NTU	baby food
349	DF051VY	*Pantoea* sp.	NTU	dried food
350	CH24W	*E. cloacae*	NTU	herb/spice
351	MP039W	*E. cloacae*	NTU	milk powder
352	HT1	*E. cloacae*	NF	food
353	HT3	*E. cloacae*	NF	food
354	T28E	*Prot. vulgaris*	NTU	herb/spice
355	FF04BB1	*Prot. vulgaris*	NTU	food
356	HT5	*E. sakazakii* 1-1	NF	food
357	HT7	*E. sakazakii* 1-1	NF	food
358	NCTC 3046	*Salmonella* Enteritidis	NCTC	unknown
359	NTU 305	*Prov. rettgeri*	NTU	unknown
360	V27Y	*Esch. hermannii*	NTU	PIF
361	NCTC 235	*Morganella morganii*	NCTC	unknown
362	HT4	*E. sakazakii* 1-1	NF	food
363	HT6	*E. sakazakii* 1-1	NF	food
364	FF10BW	*Ser. odorifera*	NTU	food
365	FF09VY	*E. amnigenus*	NTU	food
366	DF038BW	*Esch. vulneris*	NTU	dried food
367 [a]	HT10	*E. sakazakii* 1-1	NF	food
368	T32E	*Prot. vulgaris*	NTU	herb/spice
369	V21Y	*Esch. hermannii*	NTU	PIF
370 [a]	JOHVY	*Enterobacter* sp.	NTU	herb/spice
371	DF048VY	*Ser. ficaria*	NTU	dried food
372	NCTC 10396	*Edwardsiella tarda*	NCTC	unknown
373	EC0157	*Esch. coli*	NTU	unknown
374	HT8	*E. sakazakii* 1-1	NF	food
375	HT9	*E. sakazakii* 1-1	NF	food
376 [a]	NCIMB 5920	*E. sakazakii* 1-5A	NCIMB	unknown
377 [a]	NCIMB 8272	*E. sakazakii* 1-1	NCIMB	milk powder
378 [ab]	ATCC 51329	*E. sakazakii* 3-15	ATCC	unknown
379 [a]	ATCC 41365	*Kleb. oxytoca*	ATCC	clinical
380 [a]	FSM 392	*E. sakazakii* 1-1	NRC	environment

NTU	Strain identity		Source	
381	IFMPD2DB	*E. sakazakii* 1-1	NTU	PIF
382	IFMPD3DB	*E. sakazakii* 1-1	NTU	PIF
383	IFMPD4DB	*E. sakazakii* 1-1	NTU	PIF
384	IFMPD2DW	*Pantoea* sp.	NTU	PIF
385	Q04BW	*Esch. coli*	NTU	cheese
386	Q05BW	*E. cloacae*	NTU	cheese
387	Q06BW	*E. cloacae*	NTU	cheese
388	Q07BW	*H. alvei*	NTU	cheese
389	Q09BW	*Haf. alvei*	NTU	cheese
390	Q10BW	*Haf. alvei*	NTU	cheese
391	Q11BW	*Esch. coli*	NTU	cheese
392 [a]	Q12BE	Unidentified 6a	NTU	cheese
393	Q13BW	*Haf. alvei*	NTU	cheese
394	Q14BW	*Cit. youngae*	NTU	cheese
395	Q17BE	*Prot. vulgaris*	NTU	cheese
396	Q20BW	*Ser. liquefaciens*	NTU	cheese
398	Q21BW	*Ra. terrigena*	NTU	cheese
399	Q24BW	*Esch. coli*	NTU	cheese
400	Q25BW	*Kleb. oxytoca*	NTU	cheese
401	Q26BW	*Ser. marcescens*	NTU	cheese
402	Q27BW	*E. cloacae*	NTU	cheese
403	Q28BE	*Prot. vulgaris*	NTU	cheese
404	Q29BW	*Kleb. pneumoniae*	NTU	cheese
405	Q30BW	*Esch. coli*	NTU	cheese
406	Q31BW	*Ser. liquefaciens*	NTU	cheese
407	Q32BW1	*Ra. terrigena*	NTU	cheese
408	Q32BW2	*Ser. liquefaciens*	NTU	cheese
409	Q34BW	*Ser. liquefaciens*	NTU	cheese
410	Q35BW	*Pantoea* sp.	NTU	cheese
411	Q37BW	*Kleb. oxytoca*	NTU	cheese
412	Q39BB	*E. aerogenes*	NTU	cheese
413	Q40BW	*E. cloacae*	NTU	cheese
414	Q41BW	*Prot. mirabilis*	NTU	cheese
416	Q47BW	*Haf. alvei*	NTU	cheese
417	Q48BW	*Ser. marcescens*	NTU	cheese

NTU	Strain identity		Source	
419	Q61BW	*Esch. coli*	NTU	cheese
420 [a]	Q40BB	*E. sakazakii* 1-1	NTU	cheese
422 [a]	Q39DB	*E. sakazakii* 1-1	NTU	cheese
424 [a]	IFMPD2BB	*E. sakazakii* 1-1	NTU	PIF
425 [a]	IFMPD3BB	*E. sakazakii* 1-1	NTU	PIF
426 [a]	IFMPD4BB	*E. sakazakii* 1-1	NTU	PIF
450 [a]	SH1	*E. hormaechei*	NCH	clinical
451 [a]	SH2	*E. pyrinus*	NCH	clinical
456 [a]	SH7	*Cit. koseri*	NCH	clinical
457 [a]	SH8	*E. aerogenes*	NCH	clinical
467 [a]	LM110	*E. sakazakii* 1-1	NTU	PIF
468 [a]	LM325	*E. sakazakii* 1-1	NTU	PIF
469 [a]	LM589	*E. sakazakii* 1-1	NTU	PIF
470 [ab]	ATCC 29004	*E. sakazakii* 1-3	ATCC	unknown
471 [ab]	IFMK33BGY	*E. sakazakii* 1-1	NTU	PIF
472 [ab]	IFMK36X	*E. sakazakii* 1-5A	NTU	PIF
474	IFMK33X	*E. cloacae*	NTU	PIF
476	IFMK33BGP	*E. cloacae*	NTU	PIF
478	IFMK21DFI	*Esch. vulneris*	NTU	PIF
479	IFMK21JKCII	*Ps. fluorescens*	NTU	PIF
480	IFMK21JKCI	*Kleb. pneumoniae*	NTU	PIF
481	IFMK23J	*Pantoea* sp.	NTU	PIF
482	IFMK23DFI	*Ced. lapagei*	NTU	PIF
483	IFMK24J	*Chr. luteola*	NTU	PIF
484	IFMK26JB	*Rh. aquatilis*	NTU	PIF
485	IFMK26JP	*Ced. lapagei*	NTU	PIF
486	IFMK26JPU	*Pantoea* sp.	NTU	PIF
487	IFMK26JDPU	*Pantoea* sp.	NTU	PIF
488	IFMK30JK	*E. cloacae*	NTU	PIF
490	IFMK35J	*Esch. vulneris*	NTU	PIF
491	IFMK37BGA	*E. cloacae*	NTU	PIF
492	IFMK38J	*E. cloacae*	NTU	PIF
493	IFMK39jK	*Ced. davisae*	NTU	PIF
494	IFMK40DFI	*E. cloacae*	NTU	PIF
495 [a]	SH18	*E. cloacae*	NCH	clinical

NTU	Strain identity		Source	
499 [a]	SH22	*Citrobacter* sp.	NCH	clinical
504 [a]	HMA05	*Esch. hermannii*	RAD	clinical
505 [a]	HMA39	*E. hormaechei*	RAD	clinical
506 [a]	HMA42	*E. sakazakii* 1	RAD	clinical
507 [a]	HMA57	*E. sakazakii* 1-13B	RAD	clinical
509 [a]	HMA65	*E. sakazakii* 1	RAD	clinical
510 [a]	HMA68	*E. sakazakii* 1-9	RAD	clinical
511 [a]	HMA71	*E. sakazakii* 1-13A	RAD	clinical
512 [a]	HMA72	*E. sakazakii* 1-2A	RAD	clinical
513 [a]	HMA74	*E. sakazakii* 1-4A	RAD	clinical
514 [a]	HMA75	*E. sakazakii* 1-9	RAD	clinical
515 [a]	HMA76	*E. sakazakii* 1-4A	RAD	clinical
516 [a]	HMA77	*Esch. hermannii*	RAD	clinical
517 [a]	HMA78	*Esch. hermannii*	RAD	clinical
518 [a]	HMA79	*E. sakazakii* 1-3	RAD	clinical
520 [a]	HMA82	*E. sakazakii* 1-3	RAD	clinical
521 [a]	HMA83	*E. sakazakii* 1-9A	RAD	clinical
522 [a]	HMA84	*E. sakazakii* 1-9	RAD	clinical
523 [a]	HMA85	*E. sakazakii* 1-9	RAD	clinical
524 [a]	HMA86	*E. sakazakii* 1-9	RAD	clinical
525 [a]	HMA88	*E. sakazakii* 1	RAD	clinical
526 [a]	HMA89	*E. sakazakii* 1-13A	RAD	clinical
527 [a]	HMMP52	*E. sakazakii* 1-8B	RAD	PIF
528 [a]	HMMP58	*E. sakazakii* 1-1	RAD	PIF
529 [a]	HMMP05	*E. sakazakii* 1-1	RAD	PIF
530 [a]	HMMP13	*E. sakazakii* 3-15	RAD	PIF
531 [a]	HMMP15	*E. sakazakii* 1-8A	RAD	PIF
532 [a]	HMMP61	*E. sakazakii* 1-1	RAD	PIF
533 [a]	HMMP08	*E. sakazakii* 1-5	RAD	PIF
534 [a]	HMMP43	*E. hormaechei*	RAD	PIF
535 [a]	HMMP51	*E. sakazakii* 1-13A	RAD	PIF
536 [a]	HMMP35	*E. sakazakii* 1-1	RAD	PIF
537 [a]	HMMP34	*E. sakazakii* 1-2	RAD	PIF
538 [a]	HMMP26	*E. sakazakii* 1-13	RAD	PIF
539 [a]	HMMP20	*E. sakazakii* 1-2	RAD	PIF

NTU	Strain identity		Source	
540 [a]	HMMP59	*E. sakazakii* 1	RAD	PIF
541 [a]	HMMP67	*E. sakazakii* 1-2	RAD	PIF
542 [a]	HMMP68	*E. sakazakii* 1-2	RAD	PIF
543 [a]	HMMP69	*E. sakazakii* 1	RAD	PIF
544 [a]	HMMP70	*E. sakazakii* 1	RAD	PIF
545 [a]	HMMP71	*E. sakazakii* 1-2	RAD	PIF
546 [a]	HMMP07	*E. sakazakii* 1	RAD	PIF
547 [a]	HMMP39	*E. sakazakii* 1-1	RAD	PIF
548 [a]	HMMP47	*E. sakazakii* 1-1	RAD	PIF
549 [a]	HMMP49	*E. sakazakii* 1	RAD	PIF
550 [a]	HMN01	*Enterobacter* sp.	RAD	clinical
551 [a]	HMN06	*E. sakazakii* 1-1	RAD	clinical
552 [a]	HMN09	*E. sakazakii* 1-1	RAD	clinical
553 [a]	HMN13	*E. sakazakii* 1-1	RAD	clinical
554 [a]	HMN14	*E. sakazakii* 1	RAD	clinical
555 [a]	HMN15	*E. sakazakii* 1-2	RAD	clinical
556 [a]	HMN16	*E. sakazakii* 1	RAD	clinical
557 [a]	HMN20	*E. sakazakii* 1-1	RAD	clinical
558 [a]	HMN21A	*E. sakazakii* 1-1	RAD	clinical
558 [a]	HMN21B	*E. sakazakii* 1-1	RAD	clinical
559 [a]	HMN31	*E. sakazakii* 1-1	RAD	clinical
560 [a]	HMN52	*E. sakazakii* 1	RAD	clinical
561 [a]	HMN61	*E. sakazakii* 1-2	RAD	clinical
562 [a]	HMN71	*E. sakazakii* 1	RAD	clinical
563 [a]	CDC 0743-75	*E. sakazakii* 4-12	CDC	clinical
564 [a]	CDC 5960-70	*E. sakazakii* 4-6	CDC	clinical
565 [a]	CDC 1895-73	*E. sakazakii* 1-14	CDC	clinical
566 [a]	CDC 3523-75	*E. sakazakii* 3-15	CDC	clinical
567 [a]	CDC 9363-75	*E. sakazakii* 1-13	CDC	clinical
570	NCTC 6571	*St. aureus*	NCTC	unknown
571	NCTC 4163	*St. aureus*	NCTC	unknown
572	NCTC 9945	*B. cereus*	NCTC	unknown
573	NCTC 8035	*B. cereus*	NCTC	unknown
574	Lbp	*Lactobacillus plantarum*	NTU	unknown
575	Lcl	*Lactococcus lactis*	NTU	unknown

NTU	Strain identity		Source	
576	Psf	*Ps. fluorescens*	NTU	unknown
577	ATCC 15442	*Ps. aeruginosa*	ATCC	unknown
578	NCTC 10927	*Enterococcus faecalis*	NCTC	unknown
579 [a]	AES1	*Ser. liquefaciens*	NTU	environment
580 [a]	NCTC 9238	*E. sakazakii* 1-7	NCTC	clinical
581 [a]	NCTC 9529	*E. sakazakii* 2-16C	NCTC	environment
582 [a]	NCTC 9844	*E. sakazakii* 4-10	NCTC	unknown
583 [a]	NCTC 9846	*E. sakazakii* 4-2	NCTC	unknown
584 [a]	IND6a	*E. sakazakii* 1-1	JLU	PIF
585 [a]	IND7a	*E. sakazakii* 1-2	JLU	PIF
586 [a]	IND10a	*E. sakazakii* 1-2	JLU	PIF
587 [a]	INDG1a	*E. sakazakii* 1-1	JLU	PIF
588 [a]	IND35a	*E. sakazakii* 1-1	JLU	PIF
589 [a]	IND37k	*E. sakazakii* 1-2	JLU	PIF
590 [a]	IND39a	*E. sakazakii* 1-1	JLU	PIF
591 [a]	IND50n	*E. sakazakii* 1-4	JLU	PIF
592 [a]	IND64i	*E. sakazakii* 1-2A	JLU	PIF
593 [a]	IND65d	*E. sakazakii* 1-1	JLU	PIF
594 [a]	IND24b	*E. sakazakii* 1-1	JLU	PIF
595 [a]	IND36o	*E. sakazakii* 1-5	JLU	PIF
596 [a]	INDG1b	*E. sakazakii* 1-2	JLU	PIF
597 [a]	TGC8	*E. cloacae*	NTU	herb/spice
598 [a]	CK11	*Cit. koseri*	NTU	PIF
605 [a]	SH33	*Esch. coli*	NCH	clinical
610 [a]	MPN7AB	Unidentified 6b	NTU	milk powder
611 [a]	SMT115	*E. pyrinus*	LA	clinical
612 [a]	SMT116	*E. hormaechei*	LA	clinical
613 [a]	SMT117	*E. hormaechei*	LA	clinical
614 [a]	SMT118	*E. hormaechei*	LA	clinical
615 [a]	SMT119	*E. hormaechei*	LA	clinical
616 [a]	SMT120	*E. hormaechei*	LA	clinical
617 [a]	SMT121	*E. hormaechei*	LA	clinical
656 [a]	ATCC BAA893-1	*E. sakazakii* 1-2	ATCC	unknown
657 [a]	ATCC BAA893-2	*E. sakazakii* 1-2A	ATCC	unknown
658 [a]	ATCC BAA894	*E. sakazakii* 1-2	ATCC	unknown

NTU	Strain identity		Source	
659 [a]	ATCC BAA895	*Cit. koseri*	ATCC	unknown
680 [a]	CDC 996-77	*E. sakazakii* 1-3	CDC	clinical
681 [a]	CDC 1058-77	*E. sakazakii* 1-9	CDC	clinical
682 [a]	CDC 1059-77	*E. sakazakii* 1-4A	CDC	clinical
683 [a]	CDC 407-77	*E. sakazakii* 1-4	CDC	clinical
684 [a]	CDC 3128-77	*E. sakazakii* 1-11	CDC	clinical
685 [a]	CDC 1716-77	*E. sakazakii* 1-5	CDC	clinical
686 [a]	CDC 9369-75	*E. sakazakii* 1-7	CDC	clinical
	OCC 9945	*B. cereus*	Oxoid	
	OCC 115	*B. subtilis*	Oxoid	
	OCC 369	*E. faecalis*	Oxoid	
	OCC 534	*Lactobacillus fermentatum*	Oxoid	
	OCC 319	*Lactobacillus arabinosis*	Oxoid	
	OCC 229	*Lactobacillus helveticus*	Oxoid	
	OCC 535	*Lactobacillus casei*	Oxoid	
	OCC 192	*St. aureus*	Oxoid	

2.3 CHEMICALS AND MICROBIOLOGICAL MEDIA

The chemicals and microbiological media used in these studies are given in Table 2.2, along with abbreviation where appropriate. For dehydrated media, enzymes and prepared solutions the product code is given. For chemical compounds the Chemical Abstract Service Registry Number (CAS) is given as a unique global identifier.

Table 2.2 Sources of chemicals and media used in this study

Chemical / media	Abbreviation	CAS / product number	Source
4α-Phorbol 12-myristate 13-acetate	PMA	CAS 63597-44-4	Sigma
5-bromo-4-chloro-3-indolyl -α-D-glucopyranoside	X-α-Glc	70051	Glycosynth
Ammonium iron(III) citrate	$C_6H_{11}FeNO_7$	CAS 1185-57-5	BDH
Ammonium sulphate	$(NH_4)_2SO_4$	A4915	Sigma
Bacteriological agar		LP0011	Oxoid
Bovine serum albumin	BSA	A0281	Sigma
Brain Heart Infusion broth	BHI	CM0225	Oxoid
Brilliant Green agar	BGA	CM0329	Oxoid
Brilliant Green Bile (2%) broth	BGBB	CM0031	Oxoid
Buffered Peptone Water	BPW	CM0509	Oxoid
Calcium chloride	$CaCl_2{:}2H_2O$	CAS 10043-52-4	Sigma
Casein hydrolysate		LP0041	Oxoid
Chromogenic *Enterobacter sakazakii* agar (DFI formulation)	DFI	CM1055	Oxoid
Citric acid		CAS 77-92-9	Sigma
Dimethyl sulfoxide	DMSO	CAS 67-68-5	Sigma
Di-sodium hydrogen phosphate	Na_2HPO_4	CAS 10028-24-7	BDH
Dulbecco's Modified Eagle's Medium	DMEM	D5796	Sigma
Dulcitol		CAS 608-66-2	Sigma
Enterobacter sakazakii isolation agar	ESIA	AEB520010	AES
Enterobacter sakazakii selective broth	ESSB	AEB611448	AES
Enterobacteriaceae Enrichment broth	EE	CM0317	Oxoid
Foetal Bovine Serum	FBS	F2442	Sigma
Giemsa stain solution		GS500	Sigma
Glucose	Glc	CAS 50-99-7	Sigma
HEPES solution		H0887	Sigma
Human Serum, fresh-frozen		H4522	Sigma
Inositol		CAS 87-89-8	Sigma
Iron(II) sulphate	$FeSO_4$	CAS 7782-63-0	Sigma
Lauryl Sulphate broth	LST / LSB	CM0451	Oxoid

Chemical / media	Abbreviation	CAS / product number	Source
L-Glutamine solution		G7513	Sigma
Magnesium sulphate	$MgSO_4:7H_2O$	CAS 7487-88-9	Sigma
Malonate broth		M8802	Sigma
Maltose		CAS 6363-53-7	Fisher
Methyl red		CAS 493-52-7	Sigma
MRS (de Man, Rogosa, Sharpe) agar	MRS agar	CM0361	Oxoid
MRS (de Man, Rogosa, Sharpe) broth	MRS broth	CM0359	Oxoid
MR-VP broth		1.05712.0500	VWR
Methylthiazolyldiphenyl-tetrazolium bromide	MTT	CAS 298-93-1	Sigma
Non-essential Amino Acid Solution		M7145	Sigma
Peptone		LP0085	Oxoid
Phenol red		CAS 143-74-8	Sigma
Phosphate buffered saline	PBS	P4417	Sigma
Plate Count agar	PCA	CM0325	Oxoid
Potassium di-hydrogen phosphate	KH_2PO_4	CAS 16788-57-1	Fisher
Potassium sulphate	K_2SO_4	CAS 7778-80-5	BDH
RPMI-1640 Medium	RPMI	R8758	Sigma
Sodium bicarbonate solution		S8761	Sigma
Sodium chloride	NaCl	CAS 7647-14-5	BDH
Sodium deoxycholate	$C_{24}H_{39}NaO_4$	CAS 302-95-4	Sigma
Sodium pyruvate solution		S8636	Sigma
Sodium thiosulphate	$Na_2S_2O_3$	CAS 7772-98-7	BDH
Trypsin:EDTA solution (10x)		T4174	Sigma
Tryptone Soya agar	TSA	CM0131	Oxoid
Tryptone Soya broth	TSB	CM0129	Oxoid
Tryptose		LP0047	Oxoid
Vancomycin hydrochloride		V2002	Sigma
Violet Red Bile Glucose agar	VRBGA	CM0485	Oxoid
Violet Red Bile Lactose agar	VRBL	CM 0107	Oxoid
Whitley Impedance Broth	WIB	G50001	DW
Water, ≤1.0 EU/mL Endotoxin	H_2O	W3500	Sigma
Xylose-Lysine-Desoxycholate agar	XLD	CM0469	Oxoid
Yeast extract		LP0021	Oxoid

All dehydrated media was prepared according to manufacturers' instructions. ESIA and ESSB were purchased as ready prepared media.

2.3.1 mLST

Modified Lauryl Sulphate broth (mLST) comprised LST with added 29 g l^{-1} NaCl (final concentration 0.5M) and 10 mg l^{-1} vancomycin hydrochloride.

2.3.2 IFM

Cow & Gate Premium 1 (200ml cartons) of sterile ready-to-use infant formula milk were obtained from local commercial retailers within Nottingham City, UK.

2.3.3 IFM agar

IFM agar was prepared as follows: 3.0 g bacteriological agar and 0.4 g ammonium sulphate were dissolved in 40 ml distilled water. After autoclaving, 200 ml of warm (55°C) sterile IFM was added prior to dispensing into Petri dishes.

2.3.4 SCH broth

The high C:N ratio broth (SCH) was based on the method described by Scheepe-Leberkühne and Wagner (1986) for optimisation of capsule formation in *E. sakazakii.*

Base

The base medium comprised casein hydrolysate (2 g l^{-1}), yeast extract (2 g l^{-1}), di-sodium hydrogen phosphate (15 g l^{-1}), potassium di-hydrogen phosphate (4.5 g l^{-1}), di-potassium sulphate (1 g l^{-1}), sodium chloride (1 g l^{-1}). The base medium was autoclaved at 121°C for 15 min after which 100ml 20% glucose solution and 10 ml trace salt solution were added per litre.

Glucose solution

Glucose (20% w/v) was dissolved in distilled water and autoclaved at 121°C for 15 minutes.

Trace salt solution

The trace salt solution comprised citric acid (1 g l^{-1}), $MgSO_4{:}7H_2O$ (20 g l^{-1}), $CaCl_2{:}2H_2O$ (2 g l^{-1}), $FeSO_4$ (0.1 g l^{-1}) in distilled water and autoclaved at 121°C for 15 minutes.

2.4 CELL CULTURE MAINTENANCE AND ENUMERATION

2.4.1 Bacterial cells

2.4.1.1 Storage

Isolates were stored at room temperature in the dark in sealed ½ strength TSA stabs (15-20 ml of 20 g l^{-1} TSA in universal bottles). Isolates were also stored frozen at -20°C on cryobeads (Microbank™, Pro-Lab Diagnostics, Neston, South Wirral, Cheshire, UK).

2.4.1.2 Enumeration

Miles and Misra

Samples were diluted to 10^{-8} and 20 µl spots placed on TSA. After incubation the number of colonies per dilution were averaged, multiplied by 50 and multiplied by the dilution factor to give an estimate of the cfu ml^{-1} in the original sample.

Spread plates

Aliquots of 100 µl of appropriate dilutions were spread on TSA and incubated at 37°C for 18-24 h. The number of colonies was multiplied by 10 and by the dilution factor to give an estimate of the cfu ml^{-1} in the original sample.

MPN

Most Probable Number estimates were calculated using the tables and spreadsheet available from the U.S. Food & Drug Administration Center for Food Safety & Applied Nutrition Bacteriological Analytical Manual *Online* (http://www.cfsan.fda.gov/~ebam/bam-a2.html; Blodgett, 2000).

2.4.1.3 Impedance microbiology

Impedance is the resistance of flow of an alternating current as it passes through a conducting material and when two metal electrodes are immersed in conductive medium the system acts as a resistor and capacitor in series. Application of an alternating sinusoidal potential produces a resultant current which is dependant on the impedance of the system which is a function of the resistance, capacitance and applied frequency. Microbial metabolism causes changes in the bulk electrolyte as weakly charged substrates in growth media are converted to highly charged end products, therefore decreasing impedance by increasing both conductance and capacitance (Bolton, 1991). The composition of the growth media must support the organism under investigation and also allow a significant electrical response. Temperature control is critical to ensure a stable base line. The Rapid Automated Bacterial Impedance Technique (RABIT; Don Whitley Scientific Ltd., UK) comprises a solid block heating system, into which electrode assembly incubation tubes are inserted, and a computer control system. The detection time is the time at which three successive increases or decreases in conductivity occur ≥ a predetermined value. For a given protocol, the

Time to Detection (TTD) is a function of the initial size of the microbial population, the growth kinetics of the organism and the properties of the test medium. The doubling time of an organism can be measured by inoculating tubes with serial dilutions and determining the regression equation from a plot of the TTD against $\log_{10}$ cfu. Alternatively, as log 2 = 0.301 a constant of 0.15 can be substituted to calculate generation time as 0.15xTTD when a 1/100 dilution is employed. Enumeration of a microbial culture is achieved by comparing the TTD of an inoculum to an established calibration curve under the same test conditions.

Direct Impedance

In direct impedance the electrodes are in contact with the microbial culture. The growth media (2 ml) was placed into the electrode assembly incubation tube, inoculated and incubated at various temperatures for 24 h with a detection criteria of +5 µS change monitored at 6 min intervals (Silley and Forsythe, 1996).

Indirect Impedance

Where the composition of a microbial medium has inherent high conductance, such as high salt concentrations, microbial metabolism can be monitored via the production of CO_2. An agar bridge comprising 0.2 ml KOH agar was added to the electrode assembly tube, an inner tube containing 2 ml inoculated media was inserted and the electrode assembly tube tightly plugged. The CO_2 released during metabolism was absorbed by the KOH agar causing a decrease in conductivity. The tubes were incubated at appropriate temperatures for 24 h with a detection criterion of -11 µS change monitored at 6min intervals (Hilton *et al.* 2001).

2.4.1.4 Desiccation of Enterobacteriaceae.

Bacterial strains were grown 48 h on IFM agar (2.3.3) or on TSA at 37°C. The cells were harvested, weighed and resuspended in sterile IFM to give cell densities *ca.* 10^{11} cfu ml^{-1} considering 1 g wet cell mass equivalent to approximately 10^{12} bacterial cells. A 100 µl aliquot of the cell suspension was decimally diluted in BPW to 10^{-10} and the viable count estimated on TSA. The remaining cell suspension was frozen at -80°C for 24 h, immersed in liquid nitrogen for 10 min and freeze dried (Modulyo, Edwards, Crawley, UK). The viable bacterial count in the desiccated samples was estimated using a Most Probable Number (MPN) technique (n=8) in BPW. A 100 mg aliquot of the desiccated sample was decimally diluted to 10^{-11} in BPW and 100 µl aliquots of each dilution dispensed into the wells in individual columns of 96 well microtitre plates. The plates were incubated at 37°C for 48 h and the wells scored for growth either by optical density or by spotting the wells onto DFI agar (the highest concentrations contained milk powder residue and therefore it was not possible to use optical density measurements for these wells). The BAM online MPN tables and spreadsheet (http://www.cfsan.fda.gov/~ebam/bam-a2.html) were used to interpret the number of

positive wells in terms of MPN g^{-1} powder. For spiking samples, the freeze-dried cells were stored for a minimum of four weeks prior to use (to allow the bacterial concentration to stabilize (5.2)) and the MPN technique used to determine the viable bacterial count.

2.4.2 Mammalian cells

2.4.2.1 Caco2

Caco2 cells are human epithelial (colorectal) cells used for bacterial adherence and invasion assays. Caco2 are adherent with a doubling time of approximately 20 h.

Growth medium

DMEM with 2 mM L-glutamine, 1.5 g l^{-1} sodium bicarbonate, 1.0 mM sodium pyruvate. 0.1 mM non-essential amino acids and 20% FBS. For maintenance, cell density is maintained at 50-75% and the medium replaced twice per week.

Subculturing

To harvest cells the medium was removed and the cells washed with 1 ml trypsin:EDTA (0.25%:0.03%). Fresh trypsin:EDTA was added at 1 ml per 25 cm^2 and the flask returned to 37°C until the cells detached (2-10 minutes). Complete culture medium was add to inactivate the trypsin and the cells were harvested and centrifuged at 1200 rpm for 5 min. The cell pellet was resuspend 1 ml culture medium and the cell density counted using trypan blue exclusion (2.4.2.5). Flasks were seeded at $2x10^5$ cells per 25 cm^2 flask; $7x10^5$ cells per 75 cm^3 flask. Plates were seed at $2x10^5$ cells per well of a 24 well plate in 1 ml media and were cultivated for 6-7 days, replenishing media every 48 h, to allow differentiation of a confluent monlayer prior to attachment and invasion experiments.

2.4.2.2 N2a

N2a cells are mouse neuronal and amoeboid stem cells (neuroblasts) used for cytotoxicity assays.

Growth medium

DMEM with 2 mM L-glutamine, 1.5 g l^{-1} sodium bicarbonate, 1.0 mM sodium pyruvate, 0.1 mM non-essential amino acids and 10% FBS. For maintenance, cell density is maintained at 50-75% and the medium replaced twice per week.

Subculturing

N2a are moderately adherent and cells were detached by pipetting culture medium over the base of the flask. The medium containing suspended cells was harvested and centrifuged at 1200 rpm for 5 min. The cell pellet was resuspended in complete medium and the cell density counted using trypan blue exclusion (2.4.2.5). Flasks were

seeded at $2x10^5$ cells per 25 cm^2 flask; $7x10^5$ cells per 75 cm^3 flask. Plates were seed at $2x10^4$ cells per well of a 96 well plate in 100 µl of media.

2.4.2.3 U-937

U-937 cells are human monocyte cells used for macrophage survival assays. They can be induced to terminal monocyte differentiation by supernatants from human mixed lymphocyte cultures, phorbol esters (e.g. PMA), vitamin D3, gamma interferon, TNF and retinoic acid.

Growth medium

RPMI 1640 with 2 mM L-glutamine, 1.5 g l^{-1} sodium bicarbonate, 1.0 mM sodium pyruvate, 10 mM HEPES, 4.5 g l^{-1} glucose and 10% FBS. U-937 grow as a suspension.

Subculturing

The cell density was counted using trypan blue exclusion (2.4.2.5) and the cell concentration maintained at approximately 10^5-10^6 cells ml^{-1}. Medium was replaced twice per week, with up to half the cell suspension being removed and replaced with an equal volume of fresh complete medium. To seed plates, the cell suspension was harvested and centrifuged at 1200 rpm for 5 min. The cell pellet was resuspended in culture medium containing 0.1 µg ml^{-1} PMA and the cell density counted using trypan blue exclusion (2.4.2.5). Plates were seeded at 10^4 cells per well of a 96 well plate in 100 µl of media and 10^5 cells per well of a 24 well plate in 1 ml of medium.

2.4.2.4 Storage

Freezing

The contents of pre-confluent 75 cm^3 flasks fed 24 h previously were suspend (using trypsin:EDTA if necessary). The cell viability was determined and only cell suspensions with viability >90% were frozen in order to achieve good recovery. The cell suspensions were centrifuged at 1200 rpm for 5 min, the supernatant removed and the pellets resuspended at 2-$4x10^6$ cells ml^{-1} in freeze mix (FBS:DMSO, 95:5 v/v). The cells were placed in cryotubes (labelled with cell line, cell concentration, initials and date) and frozen at -80°C for at least 24 h. The contents of one vial were thawed and cultivated to verify the viability and the remaining vials of viable preparations placed in a liquid nitrogen store.

Thawing

Vials were thawed quickly in water equilibrated to 37°C until only a small amount of ice remained in the vial as at temperatures above 4°C the DMSO in the freezing medium is toxic to cells. The vial was wiped with 70% ethanol and opened in a hood over tissue moistened with 70% ethanol. The contents of one vial were add dropwise to 5 ml pre-warmed complete medium in a 25 cm^3 flask. Approximately 50-75% of the media was

replaced every 48 h until the cells were 50% confluent. Cultivation was expanded by resuspending the cells and transferring 5 ml to 75 cm^3 flasks to which a further 20 ml complete medium was added. The cells were maintained in exponential growth by replacing media and removing excess cells as required.

2.4.2.5 Enumeration

Trypan blue exclusion

Homogenous suspension of cells were prepare, using trypsin:EDTA and homogenising clumps by gentle pipetting if necessary. An aliquot of 100-200 µl cell suspension was removed and an equal volume of trypan blue 0.4% solution added, immediately prior to enumerations as trypan blue is toxic to cells on prolonged exposure. Dead cells appear blue as the stain diffuses into the cell, while living cells appear transparent as the stain is pumped out of the cell as it diffuses in. The haemocytometer was prepared and enough squares counted to include >100 viable cells; the number of dead cells taking up the stain was also counted. The number of each type of cell and the number of squares counted were noted and the following calculations used for the concentration of viable and non-viable cells and the percentage viability;

A = mean number of viable cells (total viable cells/no. of squares)

B = mean number of dead cells (total dead cells/no. of squares)

C = the dilution factor (1/2 in trypan blue)

D = correction factor for haemocytometer (Neubauer counting chamber)

square volume = 0.00025 mm^3

multiply by 4000 for 1 mm^3

multiply by 1000 for 1 cm^3

therefore D = 4000000 ($4x10^6$)

Concentration of viable cells = A x C x D

Concentration of dead cells = B x C x D

Total number of viable cells = concentration of viable cells x volume of suspension

Total number of cells = A + B

Percentage viability = A/(A + B) x 100

2.5 SAFETY CONSIDERATIONS

The Health and Safety Code of Practice for Microbiology containment level 2 was complied with. All materials and procedures were assessed with preparation of appropriate departmental COSHH and Risk analysis forms, and all waste was disposed of according to recommended procedures. Human blood products were treated as potential biological hazards and the NTU guidelines on handling and disposal adhered to. Respiratory protection was worn while handling protein, microbiological media and sodium deoxycholate in powdered form.

2.6 MATHEMATICAL ANALYSIS

2.6.1 Sensitivity, specificity and predictive values

Sensitivity was calculated as the number of true positives divided by the sum of true positives plus false negatives, expressed as a percentage, and indicates the occurrence of false negatives with 100% meaning no false negatives occurred. Specificity was calculated as the number of true negatives divided by the sum of false positives plus true negatives, expressed as a percentage, and indicates the occurrence of false positives with 100% meaning no false positives occurred (Greenhalgh, 1997).

The positive predictive value (PPV) is the proportion of tests with positive results that are correct and is calculated as the number of true positives divided by the sum of true positives plus false positives, expressed as a percentage. Likewise, the negative predictive value (NPV) is the proportion of tests with negative results that are correct and is calculated as the number of true negatives divided by the sum of true negatives plus false negatives, expressed as a percentage (Altman and Bland, 1994).

Table 2.3 Calculation of sensitivity, specificity, PPV and NPV.

		Gold Standard		
		Positive	Negative	
Test Outcome	Positive	True Positive (TP)	False Positive (FP)	PPV = (TP/(TP+FP))x100
	Negative	False Negative (FN)	True Negative (TN)	NPV= (TN/(TN+FN))x100
		Sensitivity = (TP/(TP+FN))x100	Specificity = (TN/(TN+FP))x100	

2.6.2 McNemar test

The McNemar test is a non-parametric method used on nominal data in 2 x 2 contingency tables of matched pairs of tests to determine whether the row and column marginal frequencies are equal and therefore whether there is any difference in the results obtained with the two tests (McNemar, 1947). Marginal homogeneity occurs when the row totals are equal to the column totals (Table 2.4) and indicates there is no difference obtained with the two tests.

Table 2.4 McNemar test for marginal homogeneity.

		Test 1 +	Test 1 −	Row totals
Test 2	+	a	b	a + b
	−	c	d	c + d
Column totals		a+c	b+d	n

The McNemar test calculates a Probability value (*P*) using only the number of discordant pairs, i.e. the number of pairs for which Test 1 was positive but Test 2 was negative and vice versa. A chi-squared statistic with 1 degree of freedom is then calculated using the following equation:

$$X^2 = (b - c)^2/(b + c)$$

A correction for discontinuity results in the following equation:

$$X^2 = (|b - c| - 1)^2/(b + c)$$

Statistical significance is determined by evaluating the probability of X^2 using a table of cumulative probabilities of the chi-squared distribution. The test is inherently two-tailed and a significant result implies that marginal frequencies are not homogeneous and therefore a difference exists between the two tests.

2.6.3 Student's t-Test

The TTEST function in Microsoft™ Excel was used to estimate whether significant differences existed between datasets. The syntax TTEST(1stt dataset,2nd dataset,tails,type) was used where tails = 1 or 2 indicates a one-tailed or two-tailed distribution respectively, and type = 1, 2 or 3 indicates Paired, Two-sample equal variance (homoscedastic), or Two-sample unequal variance (heteroscedastic) tests respectively. Unless otherwise indicated a Probability <0.05 was considered significant.

CHAPTER 3 - IDENTIFICATION OF *ENTEROBACTER SAKAZAKII*

Enterobacter sakazakii was originally considered a yellow-pigmented variant of *E. cloacae*. In 1976 Steigerwalt *et al.* suggested that these strains should be designated as a new species based on DNA–DNA hybridization which showed yellow-pigmented strains shared less than 50% similarity with non-pigmented strains. *E. sakazakii* was defined as a new species by Farmer *et al.* in 1980 based on differences in DNA relatedness, pigment production, biotyping, and antibiotic susceptibility patterns. Using DNA-DNA hybridization *E. sakazakii* was shown to be 41-54% related to *Enterobacter* and *Citrobacter* species. In 1983, Izard *et al.* found *E. sakazakii* strains (n=13) formed a tight DNA-hybridization group (72-97% relative binding ratio at 59.5°C – RBR) in contrast to other *Enterobacter* strains (n=38) which were below 45% RBR to the labelled *E. sakazakii* strain. Sequencing of housekeeping genes, in particular the 16S rDNA locus, has become an important tool in the investigation of species relationships and for species descriptions (Stackebrandt *et al.* 2002). There is no consensus on the degree of difference that defines a species or genus and these values may vary for different groups of organisms. Previously, values of ≥99% similarity have often been used to describe species, and ≥97% similarity has been used to delineate genera (Clarridge, 2004; Stackebrandt and Goebel, 1994; Drancourt *et al.* 2000). Increasingly other housekeeping genes are being used to determine taxonomic differences. Harada and Ishikawa (1997) used DNA sequence analysis of the *gro*EL operon to determine the phylogenetic relationship among *Enterobacter*, *Pantoea*, *Klebsiella*, *Serratia* and *Erwinia* species. Also Hoffmann and Roggenkamp, 2003, used *hsp*60 (*gro*EL homologue) DNA sequence variation to investigate *E. cloacae* polyphyletic groups.

Phenotypic characterisation and differentiation based on biochemical traits is often the first approach to distinguish characteristics of isolates. In the original study fifteen biogroups of *E. sakazakii* were described based on eleven discriminating tests, motility, Voges-Proskauer, Methyl Red, indole, ornithine decarboxylase, reduction of nitrate, production of gas from glucose, malonate utilization and production of acid from methyl-α-D-glucoside, inositol and dulcitol. The wild type, Biogroup 1, was the most common.

Numerous commercial systems are available for the biochemical characterisation of microorganisms. These usually consist of biochemical test galleries interpreted using databases of negative and positive predictabilities for different species and genera. The API20E gallery from bioMérieux is popularly used throughout European laboratories for identification of Gram-negative, oxidase negative rods. This kit consists of a strip of twenty test plus an additional requirement to test for oxidase. The ID32E gallery is also from bioMérieux and comprises thirty-two miniature biochemical tests thus theoretically

providing more accuracy through the increased number of variables under consideration. In both these methods dehydrated media is rehydrated using a pure culture suspended in physiological saline. The growth of the microorganism either creates a change in pH/composition of the media that is detected by incorporated indicators, or produces end metabolites that are detected by the addition of reagents at the end of the 24h incubation period. The Biolog GN2 system (Biolog Inc., Hayward, CA.) consists of 96 well plates containing ninety-five different carbon sources and a negative control. The production of NADH by respiring cells in wells where the nutrient can be metabolised reduces a tetrazolium dye and a characteristic pattern of positive wells creates a metabolic "fingerprint".

In order to develop accurate isolation and identification methods for the detection, monitoring and epidemiology of emerging pathogens it is necessary to clearly define the target species. It is also necessary to have accurate, accessible methods for confirmation of presumptive isolates. In this study a collection of 191 presumptive *E. sakazakii* strains were analysed using partial 16S rDNA sequencing, hsp60 sequencing and phenotypic analysis to determine their relatedness to the *E. sakazakii* type strain ATCC 29544 (NCTC 11467; NTU 1). The genotypes and phenotypes of *E. sakazakii* isolates were compared to the biogroups originally determined by Farmer *et al.* 1980. The results obtained with commercial phenotypic identification kits were compared to the 16S rDNA sequence analysis to determine the sensitivity and specificity of the kits. Additionally, the positive and negative predictive values (PPV and NPV) of key phenotypic characteristics were calculated, which could improve the identification of *E. sakazakii* in respect to non-target Enterobacteriaceae.

3.1 MATERIALS AND METHODS

The strains analysed in this study were from the culture collection at Nottingham Trent University, Nottingham, UK (Table 2.1).

3.1.1 Genotypic identification.

3.1.1.1 Partial16S rDNA gene sequencing

Partial16S rDNA gene sequencing (528 bp) was performed by Accugenix (Newark, DE, USA). Single colonies from pure cultures were harvested into ampoules containing 100 µl PrepMan™ Ultra DNA preparation reagent (Applied Biosystems, CA), vortexed for 15 seconds, heated at 95-100°C for 10 min in a boiling water bath, cooled and shipped in appropriate packaging to Accugenix. The isolates were analysed using the MicroSeq™ 500 16S rDNA Bacterial Sequencing Kit (Applied Biosystems). The kit was composed of PCR and cycle sequencing modules, analysis software, and a 500 bp 16S rDNA library of bacterial nucleic acid sequences. DNA was prepared for PCR by quick-heat lysis by removing one colony into a tube of PrepMan Ultra™ (Applied Biosystems) and placed at 99°C for 10 min. Two microlitres of genomic DNA was amplified in 50 µl of a master mixture consisting of 0.4 µM TGGAGAGTTTGATCCTGGCTCAG and TACCGCGGCTGCTGGCAC primers, 200 mM deoxynucleoside triphosphates, PCR buffer, 0.3 U of AmpliTaq DNA polymerase, and 10% glycerol. PCR conditions were 95°C for 10 min; 30 cycles each of 95°C for 30 s, 60°C for 30 s, and 72°C for 45 s; and a final step at 72°C for 10 min. Purification of the PCR product to remove excess primers and nucleotides was performed using Montage SEQ_{96} filter plates (Millipore). Cycle sequencing was performed with the sequencing module, and after removal of excess dyes using Montage SEQ_{96} filter plates (Millipore), the labelled extension products were separated on an ABI 3100 16 capillary genetic analyzer (Applied Biosystems). All sequence files for the samples were assembled, edited, and compared to those in a modified version of the MicroSeq 500 bacterial database, which was updated with current taxonomic changes. The sequences were also compared to a supplemental database compiled by Accugenix. The collective database contained 1602 entries. Sequences were aligned and genetic distances calculated using the MicroSeq® Microbial Analysis Software (Applied Biosystems, Foster City, CA). Neighbor-Joining trees were also generated using the MicroSeq® Microbial Analysis Software to illustrate the ten closest matching database entries and identification was provided for each isolate based on proximity to species type strains. Where no close species match could be obtained the isolate remained unidentified.

3.1.1.2 *hsp*60 gene sequencing

*hsp*60 gene sequencing was performed by AGOWA (Berlin, Germany) using the same primers as previously reported (Hoffman and Roggenkamp, 2003). The forward and

reverse primers were: GGT AGA AGA AGG CGT GGT TGC and ATG CAT TCG GTG GTG ATC ATC AG. These generated a 341 nucleotide PCR product.

3.1.1.3 Phylogenetic trees

Neighbor-Joining (Figures 3.1 and 3.2) and Maximum parsimony (Figures 3.3) phylogenetic trees were generated using Bionumerics (Applied Maths, Belgium). Gaps were not considered an extra state. The Jukes-Cantor correction was used to compensate for divergence being a logarithmic function of time due to the increased probability of a second substitution at a nucleotide site slowing the increase in the count of differences as divergence time increases (Kimura and Ohta, 1972). Felsenstein bootstraps (1000 simulations) were applied to assess the level of confidence for each clade of the observed trees based on the proportion of bootstrap trees showing the same clade (Felsenstein 1992). The topology of the maximum parsimony tree was optimized using simulated annealing. This is a heuristic that occasionally accepts a worse tree during the course of the search allowing it to escape local optima (Barjer, 2004). This method is more economical than the more usual heuristic searches (stepwise addition and hill-climbing), which can require many random re-starts, especially with large data matrices.

3.1.2 Phenotypic identifications.

3.1.2.1 API20E and ID32E

API20E and ID32E (bioMérieux UK Ltd.) standardized biochemical test strips were inoculated following the manufacturer's instructions. A single colony from a non-selective agar plate was suspended in sterile physiological saline and the suspension used to inoculate/rehydrate microtubules containing dehydrated media. The test strips were incubated at 37°C for 24 h during which time metabolism of the organisms produced colour changes in the media either spontaneously or revealed by addition of reagents. The colour reactions were interpreted according to the Reading Tables provided with the test kits, and identification for the test organism obtained by reference to the Analytical Profile Index and APILAB identification software (version 3.3.3 bioMérieux SA, France). Tests were performed on at least two separate occasions.

3.1.2.2 Biolog GN2 MicroPlates

Biolog GN2 MicroPlates were inoculated (150 µl per well) using pure cultures grown on TSA 24 h at 37°C suspended in Gram negative/Gram positive inoculating fluid (GN/GP-IF plus thioglycolate) at a density of 63% ±3% as determined by comparison to appropriate Biolog turbidity standards (Biolog, CA). After incubation of the GN2 Microplates at 37°C for 24 h the reduction of tetrazolium redox dye due to microbial respiration in the presence of the 95 different carbon sources was assessed

spectrophotometrically and the metabolic 'fingerprint' compared to the MicroLog database for identification.

3.1.2.3 Additional tests

Additional tests were performed using standard microbiological methods. Motility was determined at 37°C after 24 h and 48 h using motility medium (tryptose 10 g l^{-1}, NaCl 5 g l^{-1}, agar 5 g l^{-1}, pH 7.2 ±0.2). Acid production from carbohydrates was tested in phenol red broth base (10 g l^{-1} peptone, 1 g l^{-1} yeast extract, 5 g l^{-1} NaCl, 0.018 g l^{-1} phenol red) with addition of filter-sterilized carbohydrate solution (final concentration 0.5%). Gas production was determined by collection in Durham tubes. Malonate utilization was determined using sodium malonate broth (sodium malonate 3 g l^{-1}, ammonium sulphate 2 g l^{-1}, bromothymol blue 0.025 g l^{-1}, NaCl 2 g l^{-1}, yeast extract 1 g l^{-1}, dipotassium hydrogen phosphate 0.6 g l^{-1}, potassium dihydrogen phosphate 0.4 g l^{-1}). The Methyl Red test was performed by addition of indicator (0.1 g methyl red per 300 ml 95% ethanol) to cultures grown for 48 h in 4 ml MR-VP broth (VWR, 1.05712.0500). The Voges-Proskauer test was performed by addition of 40% potassium hydroxide in water and 5% 1-naphthol in ethanol to cultures grown for 24 h in MR-VP broth. Indole production was measured by addition of Kovacs reagent (5 g p-dimethyl-amino-benzaldehyde, 25 ml HCl, 75 ml pentanol-1-ol) or James Reagent (70542 BioMérieux) to cultures grown for 24 h in Peptone Water (CM0009 Oxoid). Nitrate reduction was measured by addition of 1% sulphanilamide in 1M HCl and 0.02% N-1 naphthylene diamine HCl in water to glucose test broths. Zinc dust was added to negative tubes to confirm the presence of unreduced nitrate. Constitutive metabolism of 5-bromo-4-chloro-3-indolyl-α,D-glucopyranoside (X-α-D-glucoside, Glycosynth Ltd., Warrington, UK) was determined by formation of blue-green colonies on TSA containing 0.1 g l^{-1} of this substrate and also on commercial media (Chromogenic Enterobacter sakazakii medium, DFI formulation, CM1055, Oxoid Ltd.; and ESIA, AES Laboratoire, France).

3.1.3 Sensitivity and specificity of identification methods

The partial 16S rDNA sequence identification was used as the standard to determine true positive identification as *E. sakazakii* to which the commercial biochemical test kit identifications were compared to determine sensitivity and specificity of the kits (Table 2.3). The results obtained with the different biochemical galleries were also compared using the McNemar test (Table 2.4) to further determine differences in performance. The negative and positive predictabilities of different individual biochemical tests were estimated to determine appropriate tests for the differentiation of *E. sakazakii.*

3.2 RESULTS

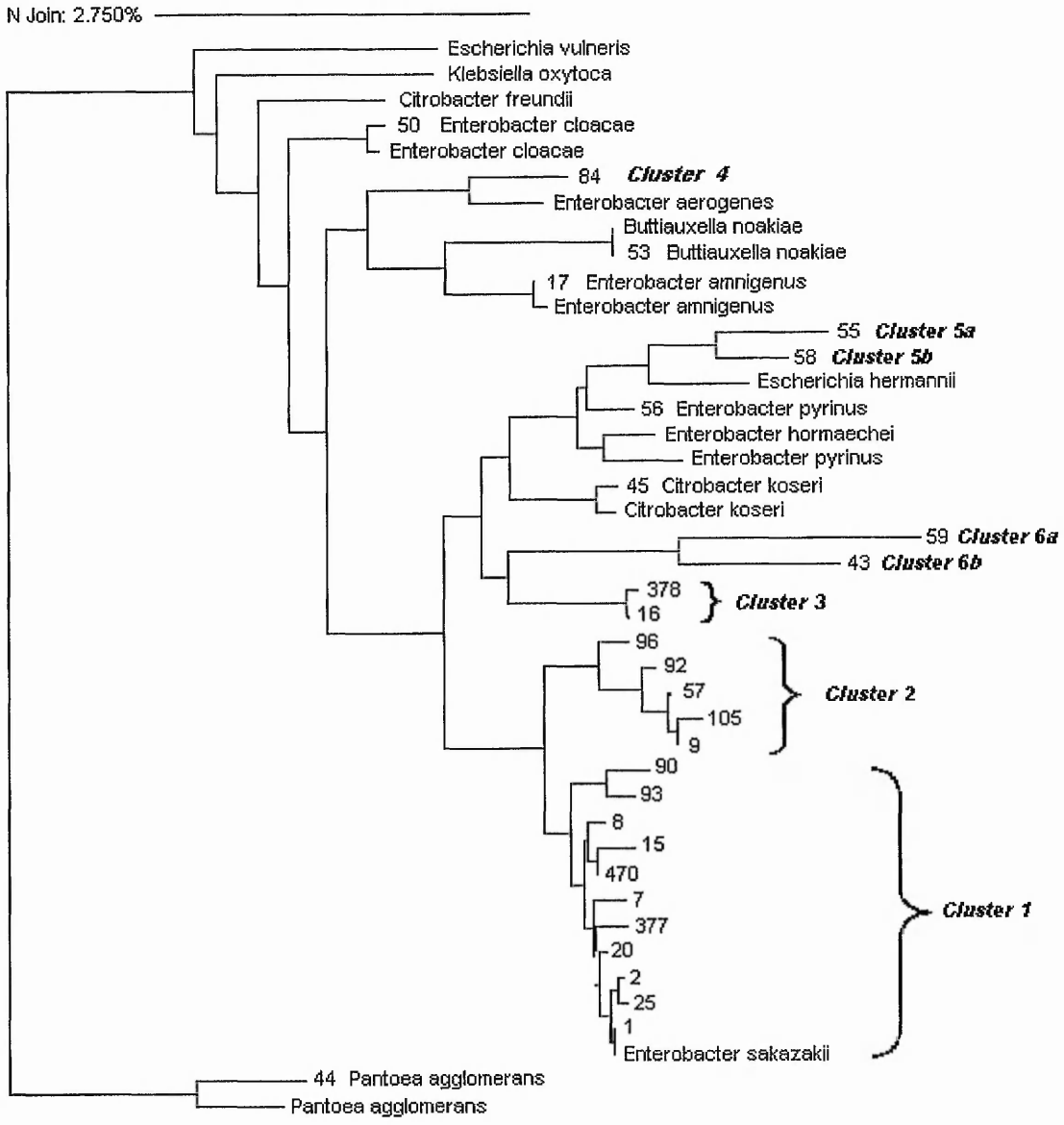
3.2.1 Genotypic identification

3.2.1.1 Partial16S rDNA gene sequencing

Partial 16S rDNA sequences (528 bp) containing less than 1% undetermined positions were obtained for all strains in this study. The initial 16S rDNA-based analysis divided 119 presumptive *E. sakazakii* strains into 3 distinct clusters and one strain which represented a fourth cluster. The majority (106/119) of the presumptive *E. sakazakii* were in one cluster with <1% difference from the type strain and represent *E. sakazakii* genomic group 1 (Table 2.1 *E. sakazakii* 1; Fig. 3.1 cluster 1). Eight strains formed a second cluster, representing *E. sakazakii* genomic group 2, that was between 1.23 - 1.89% different from the type strain and the nearest match for these strains was to *E. sakazakii* (Table 2.1 *E. sakazakii* 2; Fig. 3.1 cluster 2). The third cluster contained four strains, representing *E. sakazakii* genomic group 3, which despite being identified as *E. sakazakii* by both API20E and ID32E were a nearest match (98% homology) to *E. hormaechei* (Table 2.1 *E. sakazakii* 3; Fig. 3.1 cluster 3). The *E. sakazakii* type strain was not among the ten closest matches for strains in cluster 3. This cluster included *E. sakazakii* strain NTU 3 (ATCC 51329) which is a Preceptrol® strain used in quality control procedures. Strain NTU 84 was also identified by both biochemical profiles as *E. sakazakii* but the closest match was to *E. aerogenes* (98.76% homology). Strain NTU 84 was 3.8% divergent from the *E. sakazakii* type strain and this is a representative of *E. sakazakii* genomic group 4 (Table 2.1 *E. sakazakii* 4; Fig. 3.1 cluster 4).

Seven neonatal isolates, which were originally identified at a U.S. hospital as *E. sakazakii*, were found to be *E. pyrinus* (Table 2.1, NTU 611) and *E. hormaechei* (Table 2.1, NTU 612-617). Also, two clinical isolates from cases of neonatal sepsis supplied as *E. cloacae* by a European hospital were *E. hormaechei* (Table 2.1, NTU 450) and *E. pyrinus* (Table 2.1, NTU 451).

Figure 3.1 Representative Neighbor-Joining dendogram of *E. sakazakii* and related organisms based on partial 16S sequence.



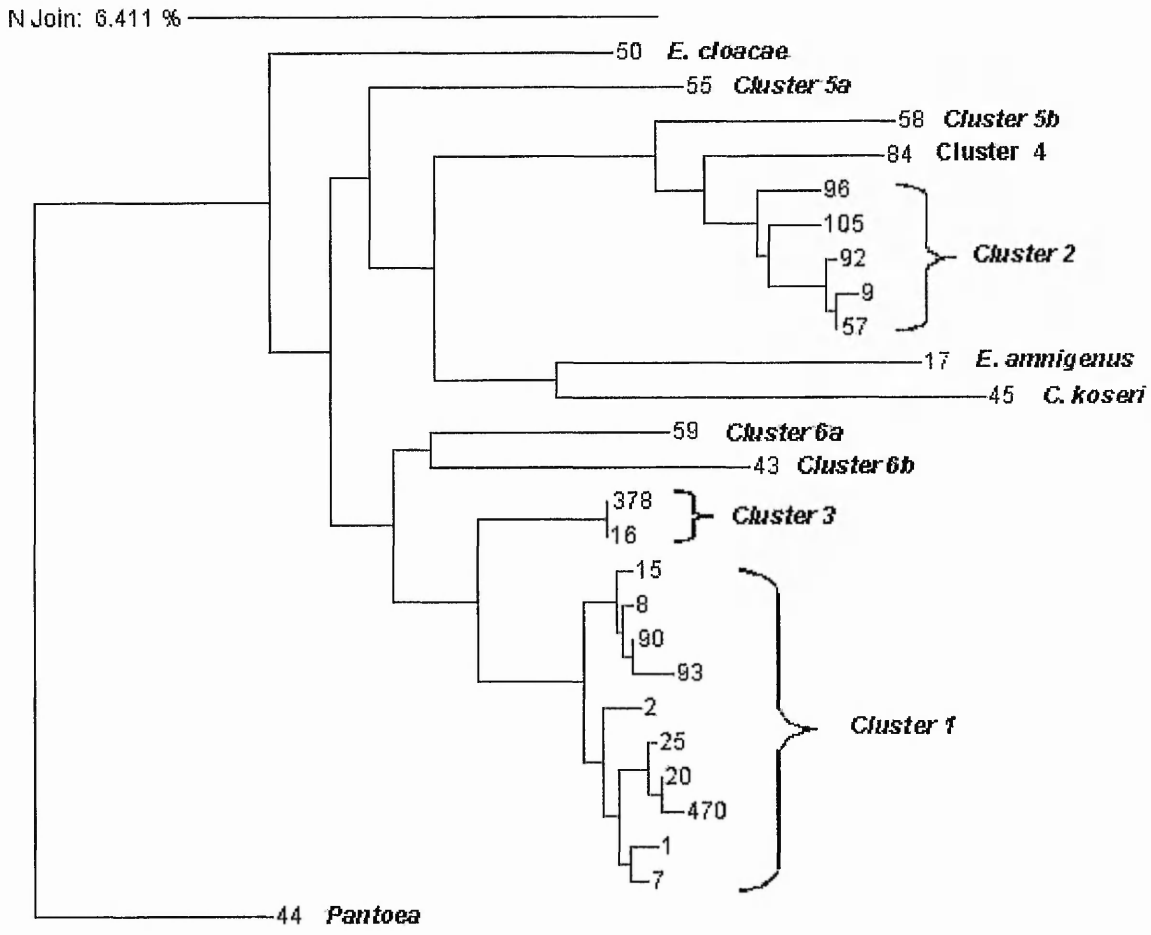
Strains presented are representatives of the different cluster groups from clinical and food sources and the numbers refer to NTU strain number (Table 2.1). Scale line is 2.750% sequence similarity. Clusters I, 2, 3 and 4 contain isolates identified biochemcially as *E. sakazakii*. Clusters 5 and 6 contain environmental isolates that are false positives on *E. sakazakii* isolation media.

Among the other Enterobacteriaceae studied, the 16S rDNA gene sequences of thirty-eight strains remained unidentified with no match (≥ 99% similarity) to any species in the 16S rDNA database. Like *E. sakazakii*, these Enterobacteriaceae strains are yellow pigmented and positive for α-glucosidase and therefore can present as false presumptive positives in tests for *E. sakazakii*. These strains included fourteen strains that returned a nearest match to *Es. hermannii*, nine with ca. 98% similarity representing genomic cluster 5a (Table 2.1 unidentified 5a; Fig. 3.1 cluster 5a) and five with ca. 98.5% similarity representing genomic cluster 5b (Table 2.1 unidentified 5b; Fig. 3.1 cluster 5b). The remaining strains had no consistent nearest match even to genus and were 2-4% different from all nearest taxa. However coherent clusters were formed with eight strains representing genomic cluster 6a (Table 2.1 unidentified 6a; Fig. 3.1 cluster 6a) and sixteen strains representing genomic cluster 6b (Table 2.1 unidentified 6b; Fig. 3.1 cluster 6b). There was no consistency in the identifications of these strains between the biochemical profiles and, although the biochemical profile nearest matches were mainly *Esch. vulneris*, *E. sakazakii*, *Pantoea* and *Buttiauxella* species, 16S Neighbor joining trees did not show these strains to cluster closely with any of these taxa. There was also no correlation between the 16S sequence clusters and the identifications from the different biochemical test systems for any of these strains. Therefore it is likely these strains represent as yet unidentified species.

3.2.1.2 hsp60 gene sequencing

*hsp*60 (*groEL* homologue) sequence was obtained for fifty-one strains. The presumptive *E. sakazakii* strains formed four distinct groups equivalent to the four *E. sakazakii* 16S rDNA genomic clusters (Fig. 3.2). The majority of strains (n=32) were from 16S rDNA genomic cluster 1 which contained the *E. sakazakii* type strain. Strains in 16S rDNA genomic cluster 2 exhibited a greater distance from the type strain by *hsp60* comparison (ca. 11.13%) than they had by 16S sequence comparison. Strains from 16S rDNA genomic cluster 3 were again also distinct from the type strain being 4.66% divergent by *hsp60* comparison. Strain NTU 84 was 12.24% divergent from *E. sakazakii* by *hsp60* comparison and although this strain grouped closest to *E. sakazakii* genomic cluster 2 there was still a >4% difference to these strains. A distinct difference was also noted between the unidentified species in genomic clusters 5a and 5b when the *hsp*60 sequences were compared.

Figure 3.2 Neighbor joining tree of *E. sakazakii* and related organisms based on partial *hsp*60 sequence.



Strains presented are representatives of the different cluster groups from clinical and food sources and the numbers refer to NTU strain number (Table 2.1). Scale line is 6.411% sequence similarity. Clusters I, 2, 3 and 4 contain isolates identified biochemcially as *E. sakazakii.* Clusters 5 and 6 contain environmental isolates that are false positives on *E. sakazakii* isolation media.

3.2.2 Phenotypic Identifications.

The biochemical profiles obtained for 189 strains of *E. sakazakii*, comprising isolates in clusters 1-4 as described in Figure 3.1, were compared to the biogroups originally proposed by Farmer *et al.* in 1980. The defining tests were motility, Voges-Proskauer, Methyl Red, indole, ornithine decarboxylase, reduction of nitrate, production of gas from glucose, malonate utilization and production of acid from inositol and dulcitol. Where strains could not be assigned to an original biogroup, a new biogroup or subgroup was designated (Table 3.1).

The majority of isolates (60/189) were in Biogroup 1 with the *E. sakazakii* type strain. These strains were motile, produced gas from glucose, produced acid from inositol, reduced nitrate and were positive for Voges-Proskauer and ornithine decarboxylase, but negative for methyl red, indole, malonate utilization and acid production from dulcitol. Biogroup 2 (n=42) contained isolates negative for acid production from inositol; four of these were also non-motile. Biogroup 3 (non-motile) contained six strains and Biogroup 4 (ornithine negative) contained nine strains three of which were non-motile. Biogroup 5 (n=16) was positive for malonate utilization and six of these were non-motile. Biogroup 6 (n=2) was positive for indole and Biogroup 7 (n=4) was negative for gas production from glucose. Biogroup 8 (n=7) was defined by the inability to reduce nitrate. Two of these 7 strains were positive in the malonate test, three were negative for the inositol test and two were inositol negative but malonate positive. Biogroup 9 contained 13 strains that were inositol negative and malonate positive. Biogroup 10 contained one strain that was inositol negative and indole positive, while Biogroup 11 contained one strain that was inositol negative and dulcitol positive. Biogroup 12 was also represented by only one strain, which was indole and malonate positive. The seven isolates in Biogroup 13 were negative for the Voges-Proskauer reaction, three were non-motile, one was negative for methyl red and one was negative for ornithine. Biogroup 14 (n=5) was negative for ornithine decarboxylase and inositol, with four of these strains being positive for malonate. Biogroup 15 was positive for all the tests performed except methyl red.

Table 3.1 Assignment of strains to biogroups.

Biogroup		Phenotype[a]										Total strains	16s cluster
Farmer	new	VP	MR	Nit	Orn	Mot	Ino	Dul	Ind	Malo	Gas		
1		+	-	+	+	+	+	-	-	-	+	60	1
2		+	-	+	+	+	-	-	-	-	+	38	1
	2a	+	-	+	+	-	-	-	-	-	+	4	1
3		+	-	+	+	-	+	-	-	-	+	6	1
4		+	-	+	-	+	+	-	-	-	+	6	1
	4a	+	-	+	-	-	+	-	-	-	+	3	1
5		+	-	+	+	+	+	-	-	+	+	10	1
	5a	+	-	+	+	-	+	-	-	+	+	6	1
6		+	-	+	+	+	+	-	+	-	+	2	4
7		+	-	+	+	+	+	-	-	-	-	4	1
8		+	-	-	+	+	+	-	-	-	+	0	1
	8a	+	-	-	+	+	-	-	-	-	+	3	1
	8b	+	-	-	+	+	+	-	-	+	+	2	1
	8c	+	-	-	+	+	-	-	-	+	+	2	1
9		+	-	+	+	+	-	-	-	+	+	12	1
	9a	-	-	+	+	+	-	-	-	+	+	1	1
10		+	-	+	+	+	-	-	+	-	+	1	4
11		+	-	+	+	+	-	+	-	-	+	1	1
12		+	-	+	+	+	+	-	+	+	+	1	4
13		-	+	+	+	+	+	-	-	-	+	2	1
	13a	-	+	+	+	-	+	-	-	-	+	3	1
	13b	-	+	+	-	+	+	-	-	-	+	1	1
	13c	-	-	+	+	+	+	-	-	-	+	1	1
14		+	-	+	-	+	-	-	-	+	+	4	1
	14a	+	-	+	-	+	-	-	-	-	+	1	1
15		+	-	+	+	+	+	+	+	+	+	6	3
	16	+	-	+	+	+	+	+	-	+	+	6	2
	16a	+	-	+	+	-	+	+	-	+	+	1	2
	16b	+	-	+	+	+	+	+	-	-	+	1	2
	16c	+	-	+	-	-	+	+	-	+	+	1	2

[a] VP, Voges-Proskauer; MR, methyl red; Nit, nitrate reduction; Orn, ornithine utilization; Mot, motility at 37°C; Ino, acid production from inositol; Dul, acid production from dulcitol; Ind, indole production; Malo, malonate utilization; Gas, gas production from glucose. All isolates positively reacted with α-methyl-D-glucosidase, with the exception of biogroup 15 isolates.

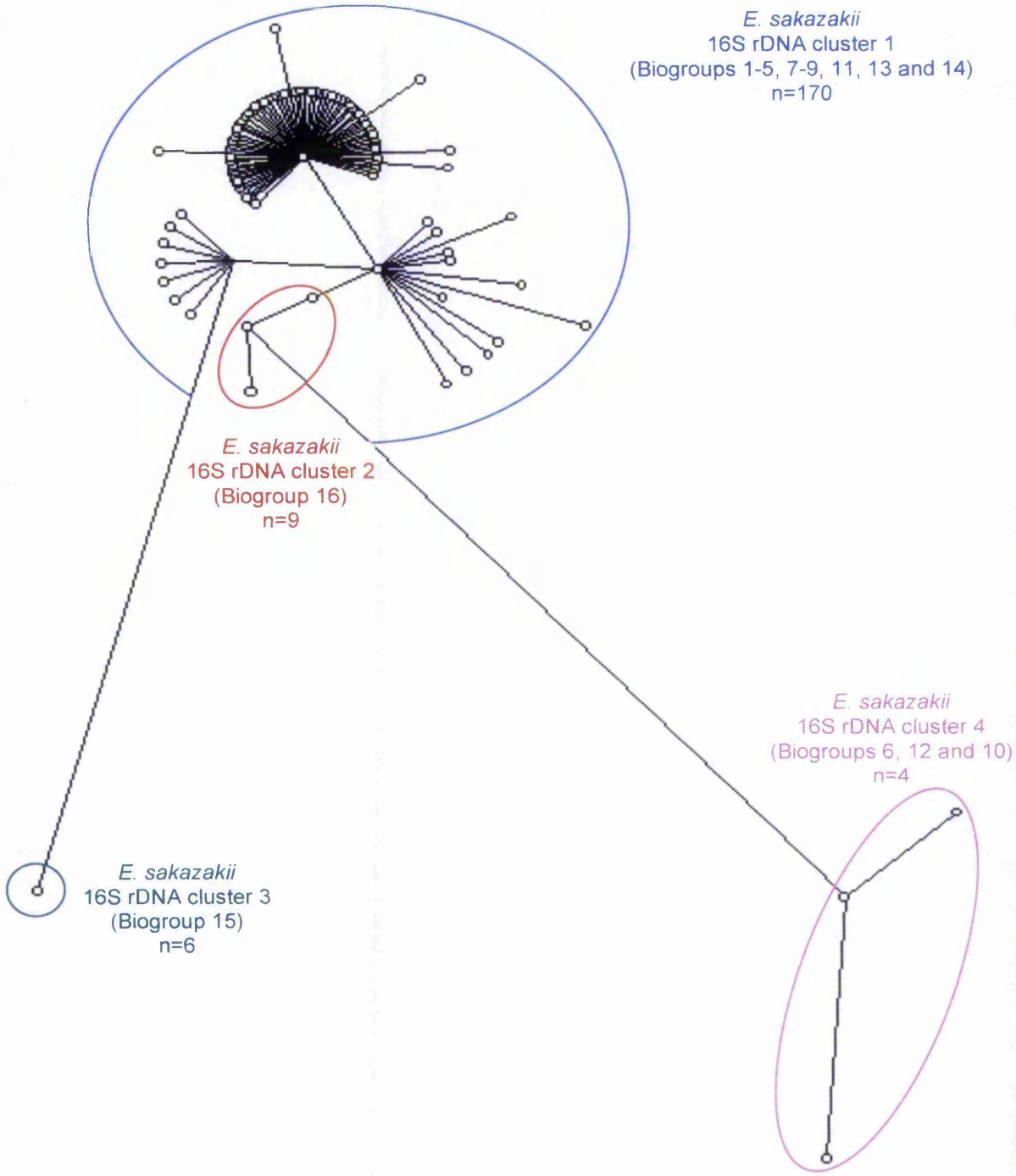
A new group (Biogroup 16) had to be defined to accommodate nine strains which were inositol and dulcitol positive, but indole negative. They were malonate positive, with the exception of one strain. Two strains were non-motile and one of these was also ornithine decarboxylase negative. Acid production from α-methyl-D-glucoside was included in the original study (Farmer *et al.* 1980) and all biogroups were reported positive for this trait with the exception of Biogroup 15. As Biogroup 15 could be distinguished from the other biogroups without the α-methyl-D-glucoside test, this was not repeated for all strains in this study.

3.2.2.1 Phylogenetic comparison of Biogroups.

The partial 16S rDNA-based analyses divided presumptive *E. sakazakii* isolates into four clusters. The relationship between these clusters is further illustrated using the Maximum parsimony method to construct an evolutionary tree (Figure 3.3). The majority (170/189) of the presumptive *E. sakazakii* isolates in this study clustered with the type strain (Figure 3.3, *E. sakazakii* cluster 1). Cluster 1 was composed of the majority of biotypes, Biogroups 1-5, 7-9, 11, 13 and 14 .

The nine isolates forming the new proposed Biogroup 16 corresponded with *E. sakazakii* cluster 2 (Figure 3.3, cluster 2). No strains representative of this biochemical profile were included in the original study by Farmer *et al.* 1980. The six isolates in Biogroup 15 corresponded to *E. sakazakii* cluster 3 (Figure 3.3, cluster 3). The four strains described as *E. sakazakii* cluster 4 (Figure 3.3, cluster 4) represent Biogroup 6, Biogroup 10 and Biogroup 12. For this dataset, the four partial 16S rDNA cluster groups can be distinguished biochemically using the indole, dulcitol and inositol tests (Table 3.2).

Figure 3.3 Maximum parsimony tree (unrooted) of the four genomic clusters of *E. sakazakii*.



The maximum parsimony tree topology was optimised using simulated annealing (3.1.1.3) and gaps were not considered an extra state. This analysis revealed no consistent grouping of different biogroups within the different genetic clusters.

Table 3.2 Biochemical differentiation of *E. sakazakii* 16S rDNA clusters.

Farmer Biogroup	Phenotype [a]			No. of strains	Genomic cluster
	Ino	Dul	Ind		
1-5, 7-9, 13, 14	(+/-)	-	-	169	1
11	-	+	-	1	1
16	+	+	-	9	2
15	+	+	+	6	3
6, 10, 12	(+/-)	-	+	4	4

[a] Ino, acid production from inositol; Dul, acid production from dulcitol; Ind, indole production.

Cluster 1 strains are variable for inositol, negative for indole and dulcitol; with the exception of Biogroup 11, which is dulcitol positive and inositol negative. Cluster 2 - Biogroup 16 strains are positive for inositol and dulcitol but negative for indole. Cluster 3 - Biogroup 15 is positive for inositol, dulcitol and indole. Cluster 4 strains were also positive for indole but can be distinguished from Cluster 3 as they are negative for dulcitol. One of the strains in Cluster 4 was inositol negative (Biogroup 10) and one was malonate positive (Biogroup 12). There were insufficient isolates in Cluster 4 biogroups to determine whether these biogroups could be further genomically divided.

3.2.3 Sensitivity and specificity of identification methods.

A total of 191 strains were phenotypically identified as presumptive *E. sakazakii* using API20E and ID32E (bioMérieux UK Ltd., Basingstoke, UK) and Biolog GN2 MicroPlates (Oxoid Ltd., Basingstoke, UK). Identification of strains based on biochemical profiles resulted in a number of false identifications and are summarised in Table 3.3. Two strains that were identified as *E. sakazakii* by API 20E were identified as *E. cloacae* (strain 50) and *E. amnigenus* (strain 17) by 16S rDNA sequence comparison. The ID32E and Biolog identifications of these strains agreed with the 16S rDNA sequencing. Also, two strains that were *E. sakazakii* by 16S rDNA sequencing, ID32E, and Biolog were identified as *E. cloacae* (strain 112) and *Pantoea* sp. (strain 21) by API20E.

Table 3.3 Identification of *E. sakazakii* strains by 16S sequencing and biochemical test kits.

Organism	16S rDNA	API20E	ID32E	Biolog GN2
E. sakazakii 1[a]	*E. sakazakii* (110)[b]	*E. sakazakii* (108) *Pantoea* (1) *E. cloacae* (1)	*E. sakazakii* (110)	*E. sakazakii* (110)
E. sakazakii 2	[c]nm *E. sakazakii* (9)	*E. sakazakii* (9)	*E. sakazakii* (9)	*E. sakazakii* (9)
E. sakazakii 3	nm *E. hormaechei* (4)	*E. sakazakii* (4)	*E. sakazakii* (4)	*E. sakazakii* (4)
E. sakazakii 4	nm *E. aerogenes* (1)	*E. sakazakii* (1)	*E. sakazakii* (1)	*E. sakazakii* (1)

[a]Number refers to genomic cluster group determined by partial 16S rDNA sequence analysis in Figure 3.1. [b]Number of strains. [c]nm indicates nearest matching partial 16S rDNA sequence in the database. The identification of some strains varied according to the method used.

The presumptive identities from the biochemical identification systems were compared with 16S rDNA sequence identity to determine the sensitivity and specificity of each method for the identification of *E. sakazakii* (Table 2.3). As the isolates in 16S rDNA cluster groups 2, 3 and 4 could not be positively identified as *E. sakazakii* by partial 16S sequence analysis they are counted as 'false positive' in this calculation (Table 3.4). Although ID32E and Biolog GN2 had the same sensitivity (100%), Biolog GN2 had a higher specificity (79% compared with 61.7%) which was equal to API20E. Statistical differences between the biochemical kits were determined using the McNemar test. Continuity correction was used, and a significance level of $\alpha = 0.01$ was applied as this is a public health issue. There was no significant difference in the number of false negatives and false positives obtained using the API20E compared to the Biolog GN2 Microplates (P=0.7237). However, there was a difference between the number of false negatives and false positives between ID32E in comparison to the API20E (P=0.0014) and the Biolog GN2 Microplate (P=0.0022). For this dataset the Biolog GN2 Microplate gave the most accurate results for identification of *E. sakazakii.*

Table 3.4 Sensitivity and specificity for identification of *E. sakazakii* by API20E, ID32E and Biolog GN2.

	Biochemical test kit		
	API20E	ID32E	Biolog GN2
True positives	108	110	110
True negatives	64	50	64
False positives	17	31	17
False negatives	2	0	0
Sensitivity (%)	98.2	100	100
Specificity (%)	79	61.7	79

The Biolog GN2 was the most specific and sensitive of the biochemical test kits.

No *E. sakazakii* strains were negative for the α-glucosidase test and this is a good predictor of the species with NPV 100% and PPV 82% for this dataset (Table 3.5). Also, all *E. sakazakii* strains were positive for sucrose and raffinose fermentation and, in combination with the α-glucosidase test, these tests further improve presumptive identification of *E. sakazakii* with NPV 100% for both tests and PPV 96 and 97% for sucrose and raffinose respectively.

Table 3.5 Biochemical tests for the differentiation of *E. sakazakii*.

	E. sakazakii	Other Enterobacteriaceae					
		X-α-Glc positive	X-α-Glc negative	All	X-α-Glc positive	X-α-Glc negative	All
	(189)	(42)	(51)	(93)	(42)	(51)	(93)
	Number of isolates positive for test				% Predictability		
X-α-glucosidase	189	42	0	42	82[a] 0[b]	100 100	82 100
Arginine dihydrolase	183	5	34	40	97 87	84 75	82 90
Citrate	187	6	41	47	97 95	82 84	80 96
D-saccharic acid	0	10	17	26	0 15	0 15	0 26
Dulcitol	15	34	14	48	31 5	51 17	24 21
glucose-1-phosphate	0	25	42	67	0 8	0 4	0 12
glucose-6-phosphate	0	19	42	61	0 11	0 5	0 14
Lipase	181	18	2	21	91 76	99 87	90 91
Methyl Red	9	40	29	69	19 1	25 11	12 12
Ornithine decarboxylase	172	0	38	38	100 71	82 44	82 76
Pyruvate	6	39	26	64	13 2	18 12	8 14
Raffinose	189	6	32	38	97 100	85 100	83 100
Sucrose	189	9	24	33	96 100	89 100	85 100
Voges-Proskauer	181	0	22	22	100 85	89 79	89 90
Yellow pigment	185	38	14	52	83 53	93 91	78 92

X-α-Glc positive refers to strains that constitutively metabolise the chromogen X-α-glucoside; conversely X-α-Glc negative refers to strains negative for this test.

[a] top row (blue text) = PPV; [b] bottom row (red text) = NPV

3.3 DISCUSSION AND CONCLUSION

When the species *E. sakazakii* was first designated in 1980, Farmer *et al.* described 15 biogroups based on 57 strains of *E. sakazakii*, five strains were used to genetically define the species and the remaining strain definitions were phenotypic. Using DNA-DNA hybridization *E. sakazakii* was shown to be 41-54% related to *Enterobacter* and *Citrobacter* species. Farmer *et al.* 1980 suggested that the organism might form a new genus containing distinct species or be transferred to a new genus if closer evolutionary relatives were found. In this study, although the majority of presumptive *E. sakazakii* strains clustered with the type strain of this species using both 16S and *hsp*60 gene sequences (cluster 1), a second cluster of closely related strains was also identified which were biochemically distinct from cluster 1 and formed a biogroup that had not been previously described by Farmer *et al.* 1980 (cluster 2). These strains were not assigned a species match using the MicroSeq 500 or supplemental bacterial databases, but the nearest match was *E. sakazakii* (1.23-1.89%). Also the nearest match for ten strains (cluster 3 and 4), identified as *E. sakazakii* by API20E, ID32E and Biolog GN2, was not *E. sakazakii* when their 16S sequences were compared to the type strain and no other species identity could be determined. These strains may represent one or more different species that are as yet uncharacterised but are phenotypically similar to *E. sakazakii*. The acknowledged standard for species delineation is DNA-DNA hybridization which was unfortunately beyond the scope of this study.

The 16S sequence clusters were confirmed by the cluster analysis of the second housekeeping gene, *hsp*60. The *hsp*60 analysis highlighted the difference between 16S clusters 1 and 2. These were approximately 1.6% divergent using 16S comparison and approximately 11.13% divergent using hsp60 comparison. Cluster 3 was ca. 2.9% divergent from the *E. sakazakii* type strain by 16S comparison and 4.66% divergent using *hsp*60 data. The divergence of strain 84 from the type strain was 3.8% and 12.24% using the 16S and *hsp*60 sequence data respectively.

The comparison of biotype and genotype for 189 strains showed that there was no clear genomic division corresponding to biogroup within the main cluster of *E. sakazakii* isolates (cluster 1) when the 16S rDNA sequences were analysed. Biogroup 15 corresponds to *E. sakazakii* cluster 3, while Biogroups 6, 10 and 12 correspond to *E. sakazakii* cluster 4. As well as defining a new biogroup (Biogroup 16), this study found 10 subgroups within the original biogroups. The malonate and motility tests account for the majority of the subdivisions of the original biogroups.

All biogroups except 10 and 16 contained at least one strain from a clinical source; also there is as yet no published data on the relative virulence potential of the different biogroups. Therefore it is desirable that all biogroups are targeted as potential health

risks to neonates and that methods designed for isolation of *E. sakazakii* are able to recover all these genomic groups. The greatest number of clinical strains (ten isolates) was found to belong to Biogroup 9. However, this included nine isolates out of the ten from the same hospital. Therefore this may not be an indication of increased pathogenicity of this biogroup but be due to an over representation of one clonal type from a single source. Most of the food isolates (including infant foods) belonged to Biogroups 1 and 2.

The difference in identification of certain strains based on the results of different sets of biochemical tests reflects the remaining taxonomic uncertainty of the *Enterobacter* and *Pantoea* organisms (Janda and Abbott, 1998). The biochemical test kits are designed to identify the maximum number of organisms and in some cases species identification is only separated by one or two tests. Strains that were identified differently by all biochemical tests were identified either as *E. sakazakii, E. cloacae, E. amnigenus, Buttiauxella* spp., *Pantoea* spp. or *Esch. vulneris* by the different methods. Identification of strains using API20E biochemical profiles, with specific reference to *E. sakazakii*, resulted in both false negative and false positive identifications. Although no false negatives were obtained using ID32E a higher number of false positives results were returned. The incorrect identification of *E. sakazakii* has implications for the safety of infant formula milk. False negatives may lead to contaminated formula passing inspections and being released for consumption, whereas false positives may lead to unnecessary product recalls. Based on the sensitivity and specificity determinations, the Biolog GN2 Microplate was the most accurate of the biochemical test systems used for the identification of *E. sakazakii.*

While there is no single test that can be used to differentiate *E. sakazakii* from other species biochemical profiles were identified that may help to improve the likelihood of correct species identification. A combination of the α-glucosidase, sucrose and/or raffinose tests gives good predictability of presumptive *E. sakazakii* isolates, however more extensive biochemical or genetic tests are need to confirm the identification.

Further work is required to determine the genomic and biochemical characteristics which accurately define *E. sakazakii* and to improve the sensitivity and specificity of biochemical identification systems for this organism.

CHAPTER 4 - DEVELOPMENT OF MEDIA FOR THE ISOLATION OF *ENTEROBACTER SAKAZAKII*

The presence of *Enterobacter sakazakii* in powdered infant formula milk for use with newborn babies is of particular concern (Codex Alimentarius Commission, 2003a and 2003b). The first reported association of *E. sakazakii* with contaminated infant formula powder was by Muytjens *et al.* (1983) studying eight cases of neonatal meningitis and sepsis. Two other groups (Simmons *et al.* 1989; Clark *et al.* 1990) reported on four neonates with *E. sakazakii* infections in Tennessee. *E. sakazakii* was isolated from faeces from all four neonates, a used can of infant formula milk and the blender used to prepare the formula. In this outbreak there was evidence of prolonged incubation in bottle heaters between 35-37°C before use. Van Acker *et al.* (2001) described 12 cases of NEC in neonates. Eleven strains of *E. sakazakii* were isolated from stomach aspirate, anal swabs and blood samples and 14 strains were isolated from milk preparations. In 2001 another outbreak occurred in a Tennessee neonatal intensive care unit in which 10 cases were identified and linked to infant formula contaminated with *E. sakazakii* (Himelright *et al.* 2002). More recently, in July 2004 in New Zealand, a premature infant died of meningitis caused by *Enterobacter sakazakii* sourced to powdered infant formula (www.news-medical.net/?id=9016). Also, in December 2004, two infants died in at separate hospitals in France after being fed with a product especially formulated for neonates with milk protein intolerance (Institute de Veille Sanitaire, 2006).

The International Commission for Microbiological Specifications for Foods (ICMSF 2002) has ranked *E. sakazakii* as 'Severe hazard for restricted populations, life threatening or substantial chronic sequelae or long duration'. Due to the raised awareness of the organism, FAO/WHO requested a review of the organism be undertaken. A full risk assessment requires a greater knowledge of its presence in food, especially those consumed by neonates and infants. However the only detailed survey of *E. sakazakii* and other Enterobacteriaceae in infant formula was published by Muytjens *et al.* (1988). One hundred and forty-one 141 powdered infant formula samples from 36 countries were examined. *E. sakazakii* was isolated at low levels (0.36 cells/100g) from 20 out of 141 infant formula products. A smaller survey by Nazarowec-White and Farber (1997) involved 5 manufacturers (each supplying 24 samples) and reported the prevalence of the organism to be between 0-12% of the samples per manufacturer.

The method used by Muytjens *et al.* (1988) and Nazarowec-White and Farber (1997b) involved pre-enrichment in Buffered Peptone Water (BPW), enrichment in EE broth, plating on Violet Red Bile Glucose Agar (VRBGA), picking five Enterobacteriaceae

colonies on to TSA plates and incubating at 25°C for 48-72 h to observe yellow pigment production followed by confirmation with biochemical profile according to API20E (bioMérieux UK Ltd.). This method has been adopted by the FDA for isolation of *E. sakazakii* from infant formula and milk powders with the modification of pre-enrichment in distilled water rather than BPW (URL:vm.cfsan.fda.gov/~comm/mmesakaz.html). Unfortunately *E. sakazakii* could be outgrown by other Enterobacteriaceae during the pre-enrichment and enrichment stages, leading to relatively few *E. sakazakii* colonies on VRBGA and subsequently a reduced chance of picking the organism onto TSA. Therefore a specific, sensitive isolation method is needed to allow detection of the organism in the finished product and also to enable monitoring of the production environment for the presence of this potential contaminant. Development of selective media entails ensuring the correct identity of the target organism and gaining an understanding of the organism's growth characteristics. During the past decade there has been a significant increase in the use of chromogenic substrates in isolation media (Manafi, 2000). In particular the indolyl-substrates have been used to differentiate a number of important organisms such as *Esch. coli* from their competitors (van Poucke and Nelis, 2000). A major advantage of these substrates is that strong colours are produced that do not diffuse out from colonies and therefore even small positive colonies are visible in the presence of more numerous competitors. It has been reported that 100% of *E. sakazakii* (n=129) were positive for α-glucosidase and that 100% of other *Enterobacter* species (n=97) were negative for this enzyme (Muytjens *et al.* 1984). Based on this observation the indolyl substrate 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside (X-α-Glc) is a potential chromogen for use in media to differentiate *E. sakazakii* colonies from other members of the Enterobacteriaceae. The enzyme α-glucosidase hydrolyses X-α-Glc liberating the aglycone, 5-bromo-4-chloro-indolol, which dimerizes in the presence of oxygen to form the pigment bromo-chloro-indigo.

This study was based on a collection of strains that were analyzed using partial 16S rDNA sequencing to determine their correct identity. The growth rate over a range of temperatures in various selective and non-selective media was measured for *E. sakazakii* strains, and typical D and z values in liquid infant formula were determined. A selective, differential agar medium was developed to improve detection of presumptive *E. sakazakii* and an enrichment broth, based on fermentation of niche carbohdrates and desiccation tolerance, was investigated to promote the recovery of *E. sakazakii* from samples contaminated with multiple microorganisms. The performances of the new media were compared against other media currently proposed for the growth of *E. sakazakii* and the ability to recover desiccation stressed cells from spike infant formula assessed. Finally a survey was conducted to determine the prevalence of *E. sakazakii* and other Enterobacteriaceae in food products.

4.1 MATERIALS AND METHODS

4.1.1 Growth characteristics

4.1.1.1 Temperature growth range

Specific growth rates were measured in non-selective (BHI, TSB, WIB), selective (EE) microbiological media and IFM using the Rapid Automated Bacterial Impedance Technique (RABIT™, Don Whitley Scientific Ltd., UK). To determine specific growth rates, 0.1 ml decimal dilutions of overnight *E. sakazakii* cultures were inoculated into 2 ml media using the direct impedance technique (2.4.1.3) and incubated at temperatures ranging from 6 to 49°C. Upper temperature growth range was determined by inoculating (10^4 cfu) 10 ml TSB and incubating at 37, 42, 44, 45 and 47°C. Fecal coliform status was determined by inoculating (10^4 cfu) 10 ml LSB and BBGB broths, containing Durham tubes for gas collection, followed by incubation at 37 and 44°C; growth and gas production were observed at 24 and 48 h.

4.1.1.2 pH growth range

To determine the pH growth range, 0.1 ml decimal dilutions were inoculated into 2 ml WIB, which had been adjusted to varying pH values, and incubated using the indirect impedance technique (2.4.1.3). The pH of the adjusted media was recorded both after autoclaving and after microbial growth.

4.1.1.3 D and z values

The decimal reduction time (D value) is the time needed at a certain temperature to reduce the numbers of a particular bacterium by 90%, i.e. a 1 Log reduction. The z-value of an organism is the change in temperature required to reduce the D value by 90%. D and z-values were determined by suspending 1 ml overnight culture in 20 ml TSB and IFM equilibrated to temperatures between 54 and 62°C in water baths. The number of survivors was enumerated at timed intervals using direct impedance by taking a 100 µl aliquot from the inoculated suspension directly into 2 ml of BPW in impedance tubes.

4.1.2 *Enterobacter sakazakii* chromogenic agar, (DFI) medium

The original formula for the DFI medium was based on non-selective agar (TSA) with addition of 1.0 g l^{-1} sodium deoxycholate to inhibit Gram positive organisms and a chromogen, 0.1 g l^{-1} 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside (X-α-Glc), which is cleaved by organisms with α-glucosidase activity resulting in blue colonies. The formulation was further improved by addition of ammonium ferric citrate and sodium thiosulphate to distinguish hydrogen sulphide producing organisms such as *Citrobacter* spp., *Proteus* spp. and *Salmonella* sv.

The final formulation of *Enterobacter sakazakii* chromogenic agar, Druggan-Forsythe-Iversen medium, was sodium deoxycholate 1 g, X-α-Glc 0.1 g, sodium thiosulphate 1 g, ammonium iron(III) citrate 1 g, TSA 40 g and distilled H_2O 1000 ml. The complete medium (pH 7.3) was autoclaved at 121°C for 15 minutes.

Inducible versus constitutive metabolism of the α-glucosidase substrate was investigated by addition of maltose 1 g l^{-1} as an inducer.

4.1.2.1 Sensitivity and specificity determination of DFI medium

The sensitivity and specificity (Table 2.3) of the DFI agar was determined by comparison with the FDA recommendations for the identification of presumptive *E. sakazakii*; yellow pigmentation after incubation on TSA at 25°C for 48-72 h. DFI medium was inoculated (10 µl quadrant streak) from cultures grown in BHI at 37°C for 18 h, and the DFI plates were then incubated at 37°C for 24 h.

4.1.3 *Enterobacter sakazakii* enrichment (ESE) medium

The enrichment broth was based on the functional recipe of Enterobacteriaceae Enrichment broth minus sugars and selective agents (disodium hydrogen phosphate, 6.5 g; potassium dihydrogen phosphate 2.0 g; yeast extract 1.5 g; neutralized peptone 4.0 g; base tryptone 12.0 g; fine salt 4.0 g and 1000 ml H_2O). Initial experiments focused on the selection of an appropriate carbohydrate followed by investigation of effective Gram positive inhibitors.

4.1.3.1 Reduction of available water using niche carbohydrates

The possible use of sugars to both increase the osmolarity and provide a niche carbohydrate source for the positive selection of *E. sakazakii* was investigated using 67 *E. sakazakii* strains and 31 other Enterobacteriaceae in broth base containing 0-25% carbohydrate solutions at 37°C for 18-24 h. Sucrose and raffinose were identified from biochemical profiles as potential niche carbohydrates (Table 3.5). Growth was analyzed by kinetic measurement of optical density at 600 nm in a Bioscreen C plate reader using 400 µl of broth per well and an inoculum of approximately 1 x 10^4 cfu ml^{-1} from cultures grown in BHI for 18 h at 37°C. Five replicates per strain were performed for each condition.

4.1.3.2 Selective agents

The minimum inhibitory concentrations of potential selective agents were determined in broth base containing 10% sucrose to evaluate useful concentrations for the inhibition of competitors. Stock solutions of potential inhibitors at maximal working concentrations (sodium deoxycholate 1 g l^{-1}, brilliant green 0.01 g l^{-1}, crystal violet 0.002 g l^{-1}, novobiocin 1 g l^{-1}, vancomycin 1 g l^{-1}, lincomycin 1 g l^{-1} and the antimicrobial peptides, lactoferrin 0.01 g l^{-1} and cecrepin 0.01 g l^{-1}) were diluted two-fold in 96 well microtitre plates, final volume 100 µl per well. Plates were inoculated using pick sticks from fresh

cultures grown 18h at 37°C on TSA. Growth was analyzed as change in optical density 590 nm after 24 h at 37°C using a TECAN SPECTRA Fluor (TECAN UK Ltd, Reading, UK).

4.1.4 Comparison of media for the isolation of *E. sakazakii*

Proposed media for the isolation of *E. sakazakii* were assessed using 177 *E. sakazakii* isolates and 74 other Enterobacteriaceae. The following media were prepared according to the manufacturers' instructions; Buffered Peptone Water (BPW), Enterobacteriaceae Enrichment broth (EE), modified Lauryl Sulphate broth (mLST), Violet Red Bile Glucose Agar (VRBGA), Violet Red Bile Lactose agar (VRBL), *Enterobacter sakazakii* chromogenic agar (DFI), and Tryptone Soya Agar (TSA). *Enterobacter sakazakii* isolation agar (ESIA) and *Enterobacter sakazakii* selective broth (ESSB) were purchased as ready prepared media.

4.1.4.1 Enrichment broth evaluation

Enrichment broths were inoculated ($1x10^4$ cfu ml^{-1}) with overnight cultures diluted in sterile saline. Initially growth was determined by change in OD 590 nm at 37°C and 44°C using a TECAN SPECTRA Fluor (TECAN UK Ltd, Reading, UK). Due to the inability to detect growth for some *E. sakazakii* strains in selective media using the optical density measurements, 10 ml of EE, ESSB and mLST were inoculated ($1x10^7$ cfu ml^{-1}) from overnight cultures in BPW (*E. sakazakii* n=18; other Enterobacteriaceae n=21). After 24 h incubation, the viable counts were determined using decimal dilutions on TSA incubated at 37°C.

4.1.4.2 Selective agar assessment.

Plates were inoculated (10 µl quadrant streak) from cultures grown in BHI at 37°C for 24 h. After inoculation, replicate plates were incubated at 37, 42 and 44°C for 24 h. TSA plates were also incubated at 25°C for 72 h to assess the formation of yellow pigment. The different media were assessed for growth and for the formation of typical presumptive *E. sakazakii* colonies (blue-green colonies on DFI and ESIA, and red colonies on VRBG and VRBL).

4.1.4.3 Recovery of *E. sakazakii* from powdered infant formula.

Appropriate quantities of desiccated cells (2.4.1.4) were used to inoculate triplicate 25 g quantities of commercial milk-based powdered infant formula (Cow & Gate Premium Stage 1) with *E. sakazakii* strains NTU 1, NTU 2 and NTU 4 at 0.2 to 2000 cfu $25\ g^{-1}$. Direct Enterobacteriaceae and aerobic plate counts (4.1.5.2) were performed after rehydration. The infant formula contained endogenous *Bacillus* spp. and one aliquot contained endogenous *Ra. terrigena*. All aliquots were additionally inoculated with yellow-pigmented Enterobacteriaceae comprising an α-glucosidase positive strain and an α-glucosidase negative *Pantoea* strain at 0.4 cfu g^{-1}.

Table 4.1. Methods for the recovery of desiccated E. sakazakii from powdered infant formula.

Method	Pre-enrichment	Enrichment	Primary isolation	Presumptive identification
FDA	BPW (37°C)	EE broth (37°C)	VRBGA (37°C)	Yellow pigment on TSA at 25°C for 48-72 h
DFI	BPW (37°C)	ESE broth (37°C)	DFI (37°C)	Blue-green colonies
mLST	BPW (37°C)	mLST (45°C)	TSA (37°C plus exposure to light)	Yellow, α-glucosidase positive colonies
AES	ESSB (37°C)		ESIA (44°C)	Blue-green colonies

Four recovery methods were compared;

FDA (http://www.cfsan.fda.gov/~comm/mmesakaz.html), DFI (this study), mLST (Guillaume-Gentil *et al.* 2002) and AES (http://www.aeslaboratoire.com/cgi-bin/go_produits.pl). The pre-enrichment, enrichment, primary isolation and presumptive identification for each of these methods are summarized in Table 4.1. Up to five presumptive isolates were selected and identified using biochemical profiles API20E (bioMérieux UK Ltd.) according to the manufacturer's instructions.

4.1.5 Isolation of *E. sakazakii* from infant formula and other foods.

4.1.5.1 Food sample preparation

The survey was composed of 82 powdered infant formula, 49 dried infant foods, 72 milk powders, 8 lactose powders, 62 cheese products, 25 fresh foods (7 meats, 18 salads), 122 herbs and spices and 66 non-herb or spice dry food ingredients. The infant formula, dried infant foods and milk powders were manufactured in South Africa, South Korea, Holland, Spain, Switzerland, USA, Belgium, Ireland, Slovenia and UK. Samples were collected in the period March to October 2003.

Twenty-five grams of each sample were homogenized for 1 minute at medium speed in a Seward Stomacher® 400 Laboratory Blender, (Seward, Thetford, UK) in 225 ml BPW. An aliquot (1 ml) of the homogenate was removed for aerobic plate count and Enterobacteriaceae enumeration (described below). The remaining material was incubated overnight at 37°C.

4.1.5.2 Aerobic plate count (APC) and Enterobacteriaceae enumeration.

Decimal dilutions (0.1 ml) of the food homogenate in BPW were used to inoculate plate count agar (PCA) and Violet Red Bile Glucose agar (VRBGA) using the surface spread method. The plates were incubated at 37°C for 24 h. On VRBGA Enterobacteriaceae form small red colonies (~1 mm diameter), with or without a precipitate; some strains produce exopolysaccharide resulting in mucoid colonies.

4.1.5.3 Conventional *E. sakazakii* isolation procedure

The conventional *E. sakazakii* isolation method was as according to Muytjens *et al.* (1988). A 10 ml aliquot of the BPW pre-enrichment was added to 90 ml EE broth and further incubated for 24 h. The incubated EE broth was streaked on VRBGA and colony morphology observed after incubation at 37°C for 24 h. Five colonies were picked and streaked on TSA. Colonies that produced yellow pigment after incubation at 25°C for 48-72 h were termed presumptive *E. sakazakii* and their identity was confirmed using API20E (bioMérieux UK Ltd.). Non-yellow colony types were also picked and identified using API20E to determine the presence of false negatives and the identities of other Enterobacteriaceae present in the sample.

4.1.5.4 Isolation of *E. sakazakii* using (DFI) medium

The incubated EE broth samples were streaked on DFI and incubated at 37°C. Presumptive *E. sakazakii* colonies appear entirely blue-green after 24 h incubation on DFI agar. The identification of presumptive *E. sakazakii* isolates was determined using API20E. *E. sakazakii* strain NTU 1 was used as the positive control organism. To determine which other Enterobacteriaceae were present in the sample after enrichment, negative colonies were also picked from DFI and identified using API20E.

4.2 RESULTS

4.2.1 Growth characteristics

4.2.1.1 Temperature growth range

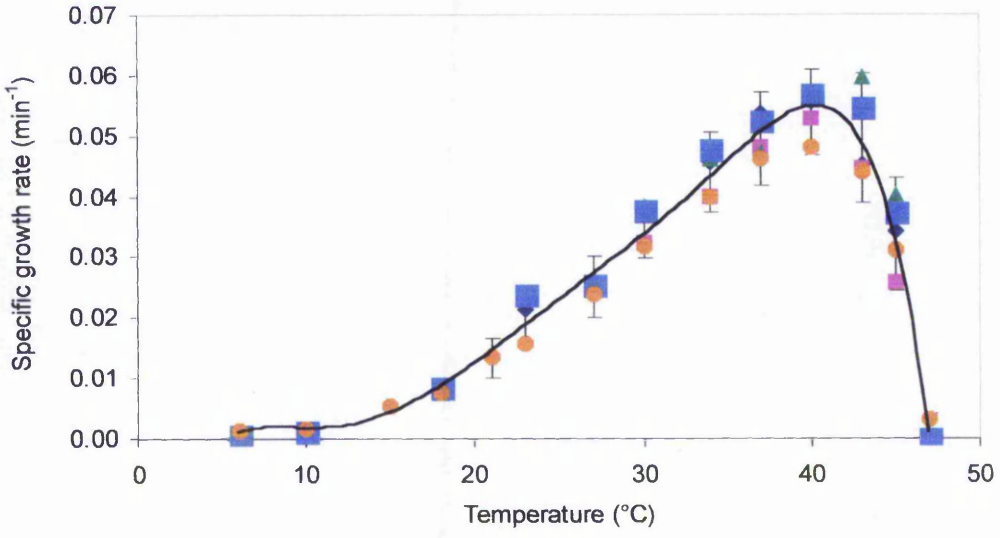
All strains (n=70) grew to approximately 10^9 cfu ml^{-1} overnight in TSB at 37°C, and at 45°C growth was evident after 24 h for all strains, however there was no visible growth for any strain after 24 h at 47°C. Growth was observed for 24/70 strains (34%) after 48 h at 47°C in TSB. Three of the seventy *E. sakazakii* strains tested in this study did not grow in LSB or BGBB at any of the temperatures, though viability was confirmed by growth in TSB at 37 and 45°C. At 37°C, 56 strains (80%) produced gas from LSB and 53 strains (76%) produced gas from BGBB after 48 h, and at 44°C, gas was produced from 11% and 23% of strains in BGBB and LSB respectively.

The growth range of six clinical strains was studied in detail. Five out of six strains grew between 6 - 45°C in all media with the optimum being 37-43°C. (Figure 4.1), the sixth strain failed to grow in EE broth however its growth profile in all other media was comparable to the other strains used.

The type strain, NTU 1, gave consistently lower doubling times in all media with the exceptions that at 6°C the type strain grew faster in IFM than the other strains and at 15°C its doubling time was the same as the other strains. Also, in IFM below 40°C the capsulated strain, NTU 2, grew slower than all other strains including the type strain, however above 40°C its growth rate was comparable to that of the other strains and it was the only strain for which growth was detectable at 47°C in IFM. No growth was detectable at 47°C for any of the strains in EE, BHI, TSB or WIB. The mean doubling time for the strains at 37°C was 21 min and ranged between 14-29 min with growth of strain NTU 6 in WIB being the fastest and NTU 2 in IFM being the slowest. In IFM the mean doubling time at 37°C was 22 minutes, with the high value (29 minutes) for NTU 2 included; the range for the other strains was 20-21 min (Figure 4.1b). A 6°C the mean doubling time was 20 h, at 10°C it was 16 h, at 15°C it was 3 h and at room temperature (18°C) the doubling time was 2 h.

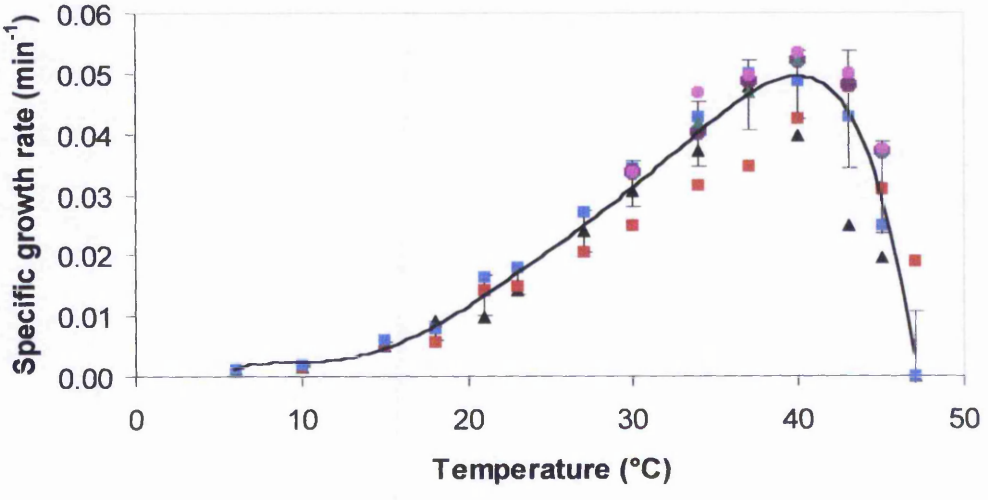
Figure 4.1 Mean specific growth rate of *E. sakazakii* strains.

a) in five media



♦ WIB, ■ EE, ■ BHI, ● IFM, ▲ TSB. Trendline is the mean specific growth rate in all media (R^2 = 0.996); error bars represent ±1 standard deviation of the mean specific growth rate in all media. Strains used were NTU 1, 2, 3, 4, 5 and 6 (strain NTU 4 did not grow in EE).

b) in IFM

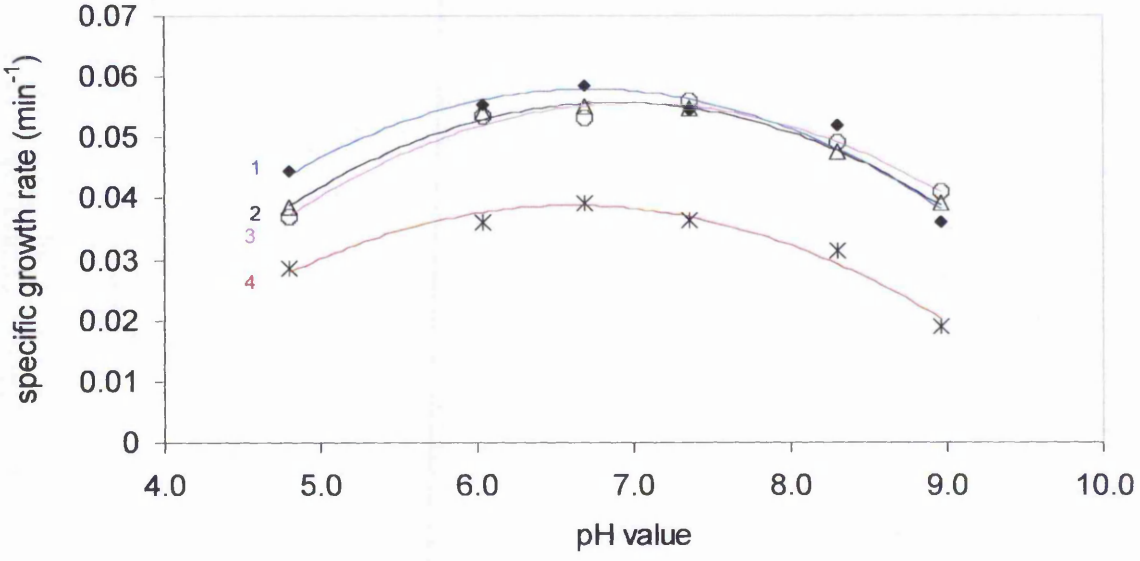


▲ NTU 1, ■ NTU 2, ■ NTU 3, ● NTU 4, ● NTU 5, ▲ NTU 6. Trendline is the mean specific growth rate for all strains (R^2 = 0.998); error bars represent ±1 standard deviation of the mean specific growth rate for all strains.

4.2.1.2 pH growth range

Optimum growth in WIB occurred between pH 6.5 – 7.0 (Figure 4.2). The growth rate of the type strain, NTU 1, was again notably slower than that of the other strains tested.

Figure 4.2 Specific growth rates of Enterobacter sakazakii non-capsulated strains (NTU 1[4] and NTU 5[1]) and capsulated strains (NTU 2[2] and NTU 6[3]).



4.2.1.3 D and z values

The D-values for *E. sakazakii* NTU 1 and NTU 2 were similar (Table 4.2).

Table 4.2 D and z-values (± 1 standard deviation) for *E. sakazakii* NTU 1 and NTU 2 in TSB and IFM

		D-value (min)					Z-value
Medium	Strain	54°C	56°C	58°C	60°C	62°C	(°C)
TSB	NTU 1	14.9 ± 0.65	2.7 ± 0.08	1.3 ± 0.28	0.9 ± 0.17	0.4 ± 0.08	5.6± 0.13
	NTU 2	10.2 ± 3.56	1.2 ± 0.01	1.7 ± 0.38	0.2 ± 0.06	0.2 ± 0.13	5.6± 0.50
IFM	NTU 1	16.4 ± 0.67	5.1 ± 0.27	2.6 ± 0.48	1.1 ± 0.11	0.3 ± 0.12	5.8± 0.40
	NTU 2	11.7 ± 5.80	3.9 ± 0.06	3.8 ± 1.95	1.8 ± 0.82	0.2 ± 0.11	5.7± 0.12

The z–values are within the range (4-6°C) determined for other Enterobacteriaceae (Nazarowec-White and Farber, 1997c).

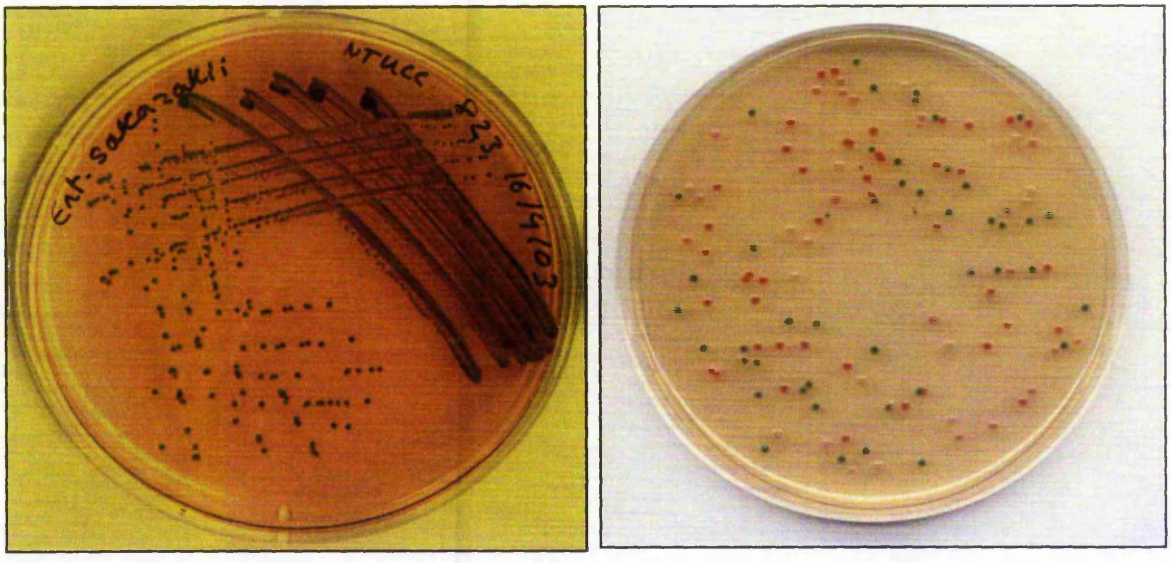
4.2.2 *Enterobacter sakazakii* chromogenic agar, (DFI) medium

In initial tests all *E. sakazakii* strains produced colonies that were an entirely blue-green colour on DFI after incubation at 37°C for 24 h (Figure 4.3).

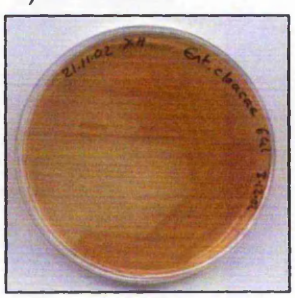
Figure 4.3 Colony morphologies on DFI medium.

a) *E. sakazakii*

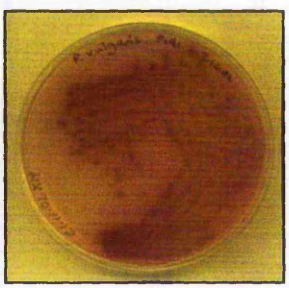
b) *E. sakazakii* (blue-green); *E. cloacae* (white); *Ser. marcescens* (red)



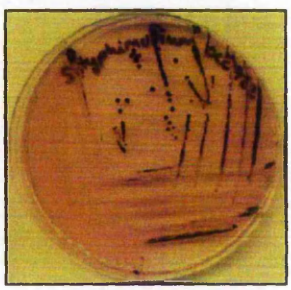
c) *E. cloacae*



d) *P. vulgaris*



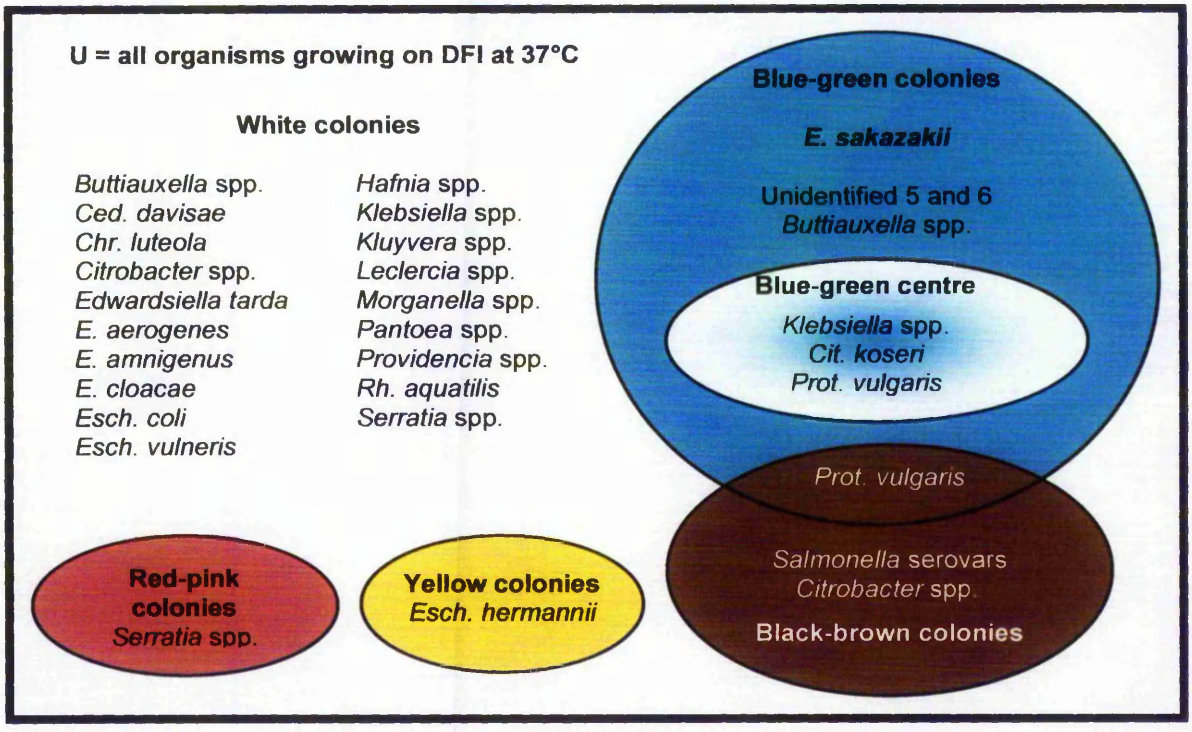
e) *S.* Typhimurium



Other strains that were blue-green were identified as a nearest match to *Esch. vulneris*, *Buttiauxella* and *Pantoea* species by API 20E, however these were found to be as yet unidentified species by 16S rDNA sequence analysis (3.2). A schematic of the colony pigmentation for different species is given in Figure 4.4. Some strains that did not utilize the substrate developed their usually pigmentation. Also, *Salmonella enterica* serovars and some *Proteus* and *Citrobacter* strains produced grey to black colonies due to hydrogen sulphide production. Some non-*E. sakazakii* organisms produced white colonies on DFI, but developed blue-green centres, this was more evident on prolonged incubation (>30 hours). The addition of maltose as an inducer resulted in entirely blue-green colonies within 24h for *Cit. koseri*, *E. aerogenes* and *Kleb.*

pneumoniae strains indicating that metabolism of the chromogenic substrate is constitutive for *E. sakazakii* but not for these other organisms.

Figure 4.4 Typical colony appearance of Enterobacteriaceae on DFI medium



4.2.2.1 Sensitivity and Specificity of DFI medium

The sensitivity and specificity of the medium was tested using 142 strains of *E. sakazakii* and 337 strains of other Enterobacteriaceae which represented 17 genera (Table 2.1). Only colonies that were entirely blue-green after 24 h incubation at 37°C were considered presumptive positives.

Table 4.3 Specificity of conventional and DFI methods for the identification of *E. sakazakii*.

		Conventional method (TSA 25°C, 48-72h)	
		Positive	Negative
DFI agar (37°C, 24h)	Positive	142	72
	Negative	0	337

Sensitivity	= 142/(142+0)*100	= 100%
Specificity	= 337/(337+72)*100	= 82.4%
Positive predictive value	= 142/(142+72)*100	= 66.4%
Negative predictive value	= 337/(337+0)*100	= 100%

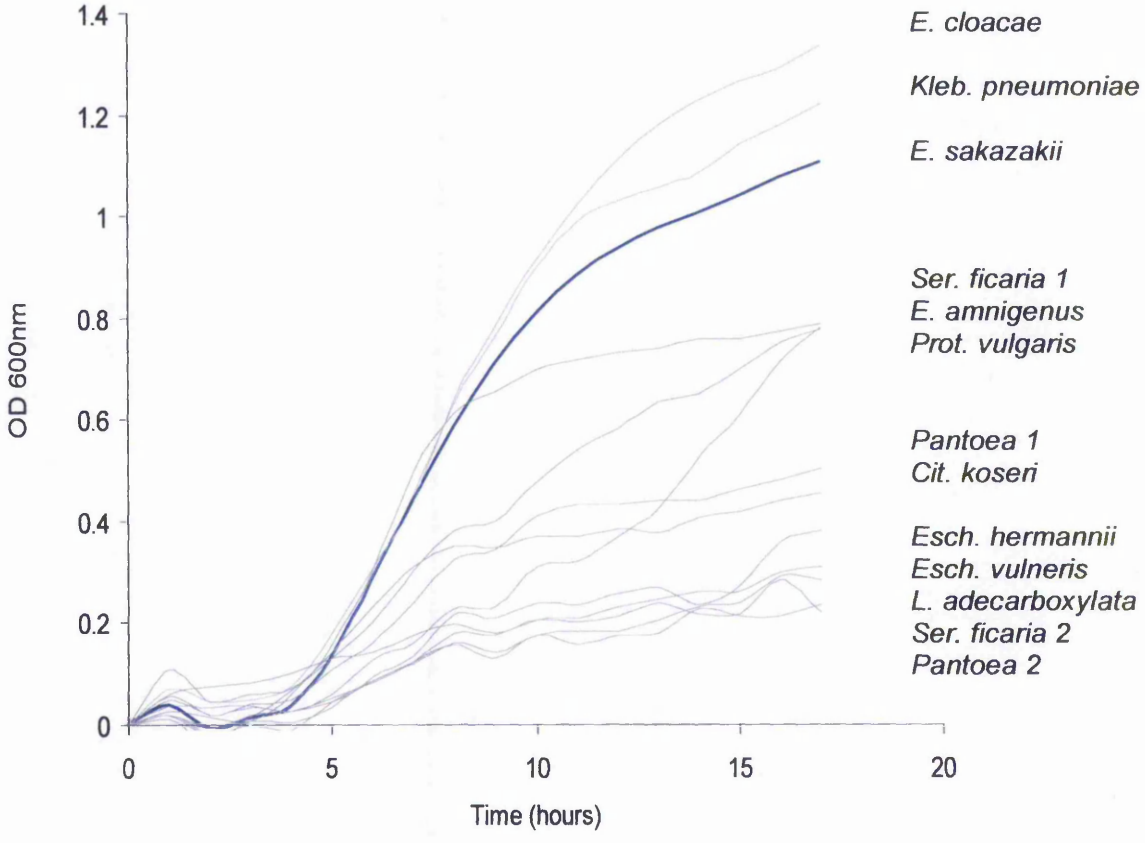
The DFI medium had 100% sensitivity (Table 4.3) for *E. sakazakii*. Using the given range of Enterobacteriaceae, the specificity of the medium was 82.4%; the positive predictive value was 66.4% and the negative predictive value was 100%.

4.2.3 *Enterobacter sakazakii* enrichment broth, (ESE) medium

4.2.3.1 Reduction of available water using niche carbohydrates

No growth was detected for *E. sakazakii* strains at carbohydrate concentrations above 20%. The best combination of *E. sakazakii* growth and competitor inhibition was seen with 10% sucrose (Figure 4.5). At this concentration the growth of *E. sakazakii* was promoted over that of competitors with the exception of *E. cloacae* and *Kleb. pneumoniae*, however these strains do not give positive colonies on DFI agar.

Figure 4.5 Growth of *E. sakazakii* and other Enterobacteriaceae in 10% sucrose broth.



E. sakazakii, *E. cloacae* and *Kleb. pneumoniae* grow better than the other Enterobacteriaceae, however *E. sakazakii* produced blue-green colonies on DFI agar.

4.2.3.2 Selective agents

The highest concentrations of the selective agents that did not inhibit any *E. sakazakii* strains are given in Table 4.4. The potentially useful agents were sodium deoxycholate 0.5 g l^{-1}, vancomycin 0.125 g l^{-1}, and lincomycin 0.01 g l^{-1}. At these concentrations 100% of the Gram positive strains tested were inhibited. The addition of sodium deoxycholate during milling of powdered media is cheaper and more convenient than adding antibiotics as sterile solutions after autoclaving.

Table 4.4 Useful working concentrations of selective agents.

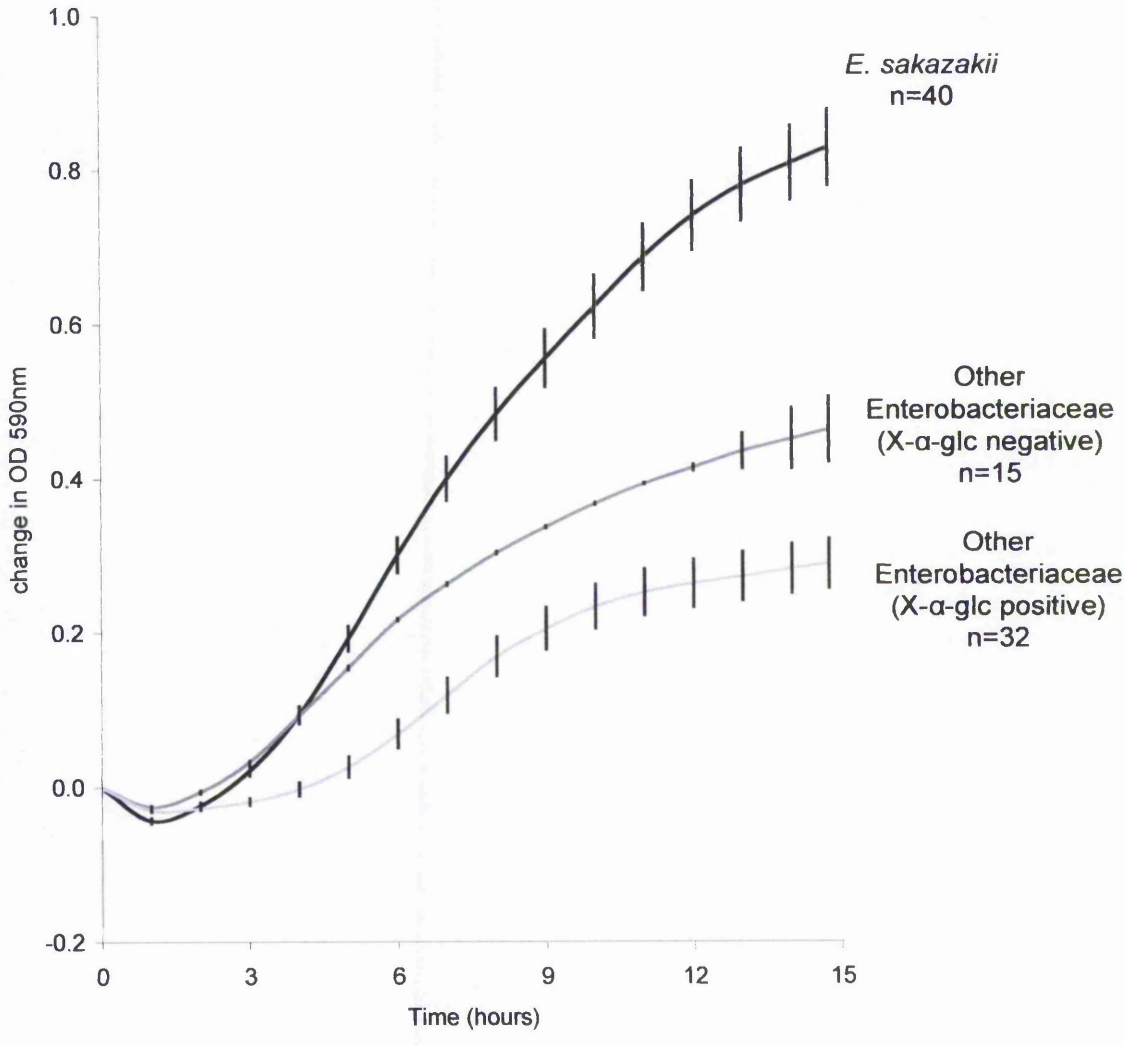
Selective agent	Highest concentration non-inhibitory to *E. sakazakii* (g l^{-1})	% sensitive strains			
		E. sakazakii (n=40)	Other Enterobacteriaceae		Gram positive (n=8)
			X-α-Glc negative (n=37)	X-α-Glc positive (n=11)	
Lactoferrin	0.0078	0	0	0	0
Cecropin	0.001	0	0	0	0
Sodium deoxycholate	0.25	0	0	0	100
Brilliant green	0.0003	0	0	0	43
Crystal violet	0.00006	0	3	0	50
Ampicillin	0.0001	0	0	0	25
Novobiocin	0.0001	0	0	0	75
Vancomycin	0.125	0	0	27	100
Lincomycin	0.01	0	0	0	100

X-α-Glc positive refers to strains that constitutively metabolise the chromogen X-α-glucoside; conversely X-α-Glc negative refers to strains negative for this test.

4.2.3.3 Formulation of ESE broth

The final formulation of the ESE broth was disodium hydrogen phosphate 6.5 g, potassium dihydrogen phosphate 2 g, yeast extract 1.5 g, neutralized peptone 4 g, base tryptone 12 g, fine salt 4 g, fine sucrose 100 g, sodium deoxycholate 0.5 g, distilled H_2O 1000 ml. The growth of *E. sakazakii* in comparison to competing microorganims was determined (Figure 4.6).

Figure 4.6 Growth of *E. sakazakii* and other Enterobacteriaceae in ESE broth.



Error bars represent ± the standard error of the sample mean, calculated from the standard deviation of the sample mean divided by √n. A difference in OD 590nm of 0.5 is equivalent to ca. 0.5 $\log_{10}$ cfu ml^{-1}.

4.2.4 Comparison of media for the isolation of *E. sakazakii*

4.2.4.1 Enrichment broth evaluation

All 177 *E. sakazakii* strains grew well in ESE broth at 37°C (Table 4.5). In contrast, growth was not detected for 5-13% (n=177) of *E. sakazakii* strains in EE, mLST or ESSB at 37°C and 44°C.

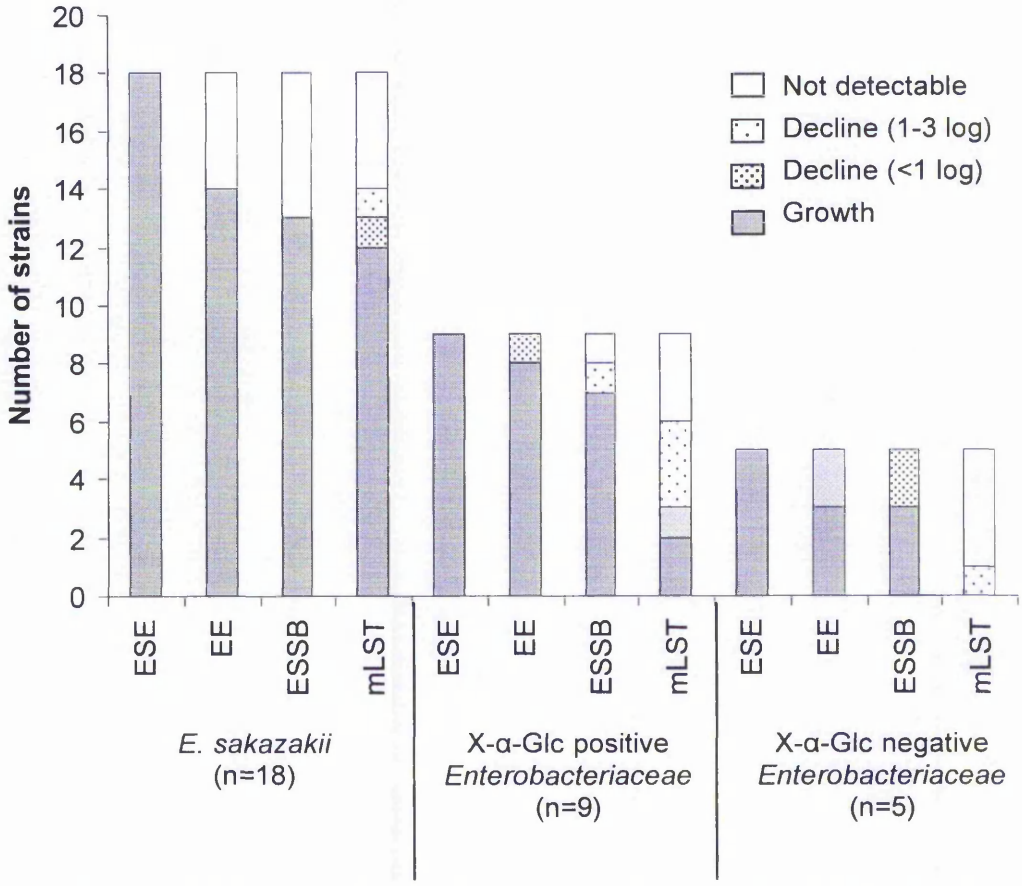
Table 4.5. Percentage of strains showing increase in optical density after 24 h incubation in enrichment media.

	Incubation temperature							
	37°C				44°C			
Organism (n)	ESE	EE	mLST	ESSB	ESE	EE	mLST	ESSB
E. sakazakii (177)	100	97	96	96	99	95	94	87
α–glucosidase positive Enterobacteriaceae (40)	100	100	95	93	98	95	78	65
α-glucosidase negative Enterobacteriaceae (34)	100	100	100	97	100	100	91	88

ESE - *E. sakazakii* enrichment broth; EE - Enterobacteriaceae Enrichment broth; mLST - modified Lauryl Sulphate broth (LST + 0.5M NaCl and 10mg l^{-1} vancomycin); ESSB - *Enterobacter sakazakii* selective broth.

The viable counts after 24 h incubation in selective broths are presented in Figure 4.7. All Enterobacteriaceae strains grew in ESE broth. In the three selective enrichment broths EE, mLST and ESSB, the viability of four to six *E. sakazakii* strains decreased and some were unrecoverable (>6 log decline). The mLST was the most selective broth with only two non-*E. sakazakii* strains able to grow (*Kleb. pneumoniae* and *Kleb. oxytoca*). *Bacillus cereus* (n=1), *Bacillus subtilis* (n=2), *Staphylococcus aureus* (n=2) and *Lactobacillus* spp. (n=2) were not recoverable from any broths (data not shown).

Figure 4.7 Changes in viable counts of *E. sakazakii* and competitive organisms in various enrichment broths.



The number of strains that either increase in numbers (grow) or decrease in numbers (decline <1 Log, decline 1-3 Log, or are undetectable) are shown for each of the test broths. The data is divided by type of organism; X-α-Glc positive refers to strains that constitutively metabolise the chromogen X-α-glucoside; conversely X-α-Glc negative refers to strains negative for this test.

4.2.4.2 Selective agar assessment.

Table 4.6 shows that 2% (n=177) of *E. sakazakii* strains did not produce yellow pigmentation on TSA after 3 days incubation at 25°C, a criterion which has been recommended for the presumptive identification of *E. sakazakii*. All of the *E. sakazakii* strains grew at 37°C and produce characteristic colonies on the Enterobacteriaceae (VRBGA) and coliform (VRBL) agars, however one strain (NTU 531) grew very poorly on these media. This isolate was also the only *E. sakazakii* in this study that did not grow and produced characteristic (blue-green) colonies on DFI after 24 h incubation at 37°C. NTU 531 appears to be more sensitive to sodium deoxycholate than the other isolates tested and decreasing the concentration of this compound in the media to 0.3g l^{-1} improved growth of this strain. At 44°C, 1% of *E. sakazakii* did not grow on TSA

whereas on ESIA and DFI, 3 and 5 *E. sakazakii* strains (respectively) were unable to grow. In addition, rather than blue-green colonies, 8 strains produced white or partially coloured colonies on DFI and 4 strains produced mauve, rather than blue-green, colonies on ESIA at this temperature.

Table 4.6. Growth of *E. sakazakii* and other Enterobacteriaceae on various selective and differential agars.

Organism (n)	Yellow pigment production 25°C (TSA, 48-72h)	Incubation temperature for 24 hours						
		37°C				44°C		
		TSA[b]	DFI[c]	VRBGA[d]	VRBL[d]	TSA	DFI	ESIA
E. sakazakii (177)	98[a]	100	99	100	100	99	93	96
Enterobacteriaceae:								
X-α-Glc positive (40)	93	100	100	100	92	95	45	63
X-α-Glc negative (34)	32	100	0	100	96	100	0	0

[a] Percentage of strains. [b] Growth. [c] Blue-green colonies. [d] Red colonies.

4.2.4.3 Recovery of *E. sakazakii* from powdered infant formula.

The numbers of samples positive for recovery of three desiccated *E. sakazakii* strains (NTU 1, NTU 2 and NTU 4) from powdered infant formula are presented in Table 4.7. There were three replicates per strain at each inoculation level (2000 to 0.2 cfu 25 g^{-1}). The direct Enterobacteriaceae and APC counts were negative for all the infant formula samples indicating the need for pre-enrichment to enable recovery of desiccated cells.

The AES method only recovered the *E. sakazakii* type strain (NTU 1). Endogenous *Raoultella terrigena* was the only organism recovered by the U.S. FDA method for one of the samples inoculated with 2000 cfu 25 g^{-1} *E. sakazakii*, therefore *Ra. terrigena* had overgrown *E. sakazakii*. The mLST method recovered both *Ra. terrigena* and *E. sakazakii* in the corresponding sample, whereas the DFI method recovered only *E. sakazakii*. Fewer presumptive positive isolates were found to be false-positive with the mLST method than with the other methods.

Table 4.7 Recovery of *E. sakazakii* from powdered infant formula in the presence of competing flora using four isolation methods.

	Number of samples positive for *E. sakazakii* recovery (n=9)			
E. sakazakii (cfu 25 g^{-1})	DFI	FDA	mLST	AES
2000	9	7	9	3
200	8	6	8	1
20	6	3	3	1
2	3	1	0	0
0.2	0	0	0	0

4.2.5 Isolation of *E. sakazakii* from infant formula and other foods.

4.2.5.1 General flora and direct Enterobacteriaceae enumeration.

The general microbial flora (APC) and Enterobacteriaceae counts for the 486 food samples are given in Table 4.8. In general the microbial loads for powdered infant formula, milk powder and dried infant foods were lower than that for the other products. The APC for the majority (149/203) of infant formula, milk powder and dried infant food samples was 10^2 cfu g^{-1} or less, and no Enterobacteriaceae were enumerated from milk powders, lactose or dried infant foods. One powdered infant formula sample had Enterobacteriaceae (*Kleb. ozaenae*) at 200 cfu g^{-1}. In contrast the majority of APC and Enterobacteriaceae count of unprocessed foods (meat, salads, herbs and spices) was $>10^3$ cfu g^{-1}.

Table 4.8 Summary of aerobic plate counts and direct Enterobacteriaceae counts

Sample	Aerobic plate count (cfu g^{-1})					Enterobacteriaceae (cfu g^{-1})				
	$<10^2$	10^2	$>10^2$-10^3	$>10^3$-10^4	$>10^4$	$<10^2$	10^2	$>10^2$-10^3	$>10^3$-10^4	$>10^4$
Infant formula Powder (82)[a]	56[b]	22	14	6	2	99	0	1	0	0
Dried infant food (49)	74	14	6	6	0	100	0	0	0	0
Milk powders (72)	40	18	22	20	0	100	0	0	0	0
Lactose powders (8)	100	0	0	0	0	100	0	0	0	0
Cheese products (62)	68	2	18	11	1	92	5	0	3	0
Fresh foods (25)	0	0	8	8	84	0	0	8	4	88
Herbs & spices (122)	22	4	7	26	41	65	4	12	6	13

[a] Number of samples analyzed are given in parenthesis. [b] Percentage of samples

4.2.5.2 Isolation of Enterobacteriaceae.

The Enterobacteriaceae isolated after enrichment in EE broth of 203 powdered infant formula, dried infant foods and milk powder samples are listed in Table 4.9. The most frequently isolated organisms were *E. cloacae* (15 samples), *Pantoea* spp (11 samples) and *E. sakazakii* (10 samples). *E. sakazakii* and *Pantoea* were the most common organisms in the infant nutritional products, the majority of the *E. cloacae* being found in milk powder samples. *Salmonella* serovars were not present in any of the samples.

Table 4.9 Enterobacteriaceae isolated from powdered infant formula milk, milk powder and dried baby foods after enrichment in EE broth.

Organism	Infant formula (82)[a]	Dried infant food (49)	Milk powder (72)	Total (203)
E. cloacae	1	1	13	15
E. sakazakii	2	5	3	10
E. amnigenus	0	0	4	4
Pantoea spp.	2	5	4	11
Esch. hermannii	0	0	3	3
Esch. vulneris	0	2	1	2
Lec. adecarboxylata	0	0	2	3
Kleb. ozaenae	1	0	1	2
Kleb. pneumoniae	0	0	1	1
Ra. terrigena	0	0	2	2
Ser. ficaria	1	0	2	3
Ra. aquatilis	1	0	0	1
Cit. freundii	1	0	0	1

[a] Number of samples analyzed is given in parenthesis.

4.2.5.3 Isolation of *E. sakazakii*.

The presumptive and confirmed identification of *E. sakazakii* for the 486 food samples is summarized in Table 4.10. Sixty-seven samples were found to be positive for *E. sakazakii* using the DFI medium compared with only 19 using the conventional method. No samples that were positive by the conventional method were negative using DFI medium. In addition, as it does not require a two-day incubation period on TSA to observe pigment production, results using the chromogenic DFI medium were obtained 2 days before the conventional method. Eleven samples contained more than one *E. sakazakii* strain, as shown by different API20E profiles.

Both detection methods isolated *E. sakazakii* from the same two powdered infant formula samples. No Enterobacteriaceae were isolated from either of these samples prior to enrichment in EE broth. Both methods isolated *E. sakazakii* from the same two cheese products. The organism was isolated from five dried infant foods by the DFI method compared to three for the conventional method. Only the DFI medium isolated *E. sakazakii* from three of the milk powders and although 40 herb and spice samples

were positive for *E. sakazakii* using the DFI medium, only 11 were positive by the conventional method. *E. sakazakii* was isolated from 15/66 dry ingredients including soy protein, almonds, coconut powder, pistachio, sunflower seeds, sesame seeds, lentils, ground maize, ground rice, sponge mix, soup, Beanfeast™, and vegetable suet.

Table 4.10 Presumptive and confirmed positive samples for *E. sakazakii* detection

Sample	no.	Presumptive positive		*E. sakazakii* positive	
		DFI[a]	VRBGA[b]	DFI[c]	VRBGA[c]
Infant formula	82	2	6	2	2
Dried infant food	49	5	9	5	3
Milk powders	72	4	10	3	0
Lactose	8	0	0	0	0
Cheese products	62	2	2	2	2
Fresh foods	25	3	1	0	0
Herbs & spices	122	69	24	40	11
Other Dry food ingredients	66	18	18	15	1
Total	486	104	70	67	19

[a] Entire blue-green colonies on DFI agar after 24 h incubation. [b] Red colonies on VRGBA and yellow pigmentation on TSA. [c] API 20E profile of presumptive isolate matched *E. sakazakii*

4.3 DISCUSSION AND CONCLUSION

4.3.1 Growth characteristics

E. sakazakii grew between 6 and 45°C in all media, with one strain being able to grow at 47°C but only in IFM. Farmer *et al.* (1980) reported that 70-90% of *E. sakazakii* strains produced gas from lactose in EC medium at 35°C compared with 58% of strains at 44.5°C. Similarly in our study, although 76% of strains produced gas from lactose at 37°C only 23% of strains produced gas at 44°C. Therefore carbohydrate metabolism in a large number of *E. sakazakii* strains may be sensitive to temperatures of 44°C and above.

The D and z values reported here (Table 4.2) are similar to those derived by other researchers. Nazarowec-White and Farber (1997) described *D* values of $D_{52\,°C}$=54.8 min, $D_{54\,°C}$=23.7 min, $D_{56\,°C}$=10.3 min, $D_{58\,°C}$=4.20 min, $D_{60\,°C}$=2.50 min, with a pooled *z* value of 5.82 °C, for a pool of ten *E. sakazakii* strains (five food isolates and five clinical isolates) in rehydrated powdered infant formula. A submerged vessel method (Buchanan and Edelson, 1999, Edelson-Mammel and Buchanan, 2004) has been used to determine the thermal resistance of 12 strains of *E. sakazakii* in rehydrated infant formula. Approximately 50% of the strains had $D_{58\,°C}$ values of <0.83 min, whereas 50% had *D*-values of >5 min and it was concluded that two distinct thermal resistance phenotypes exist. However the highest *z* value obtained was 5.6 °C which is similar to the values of 5.7 and 5.8°C determined herein as well as the pooled z value (5.82°C) determined by Nazarowec-White and Farber. However the composition of suspending media appears to influence observed D and z values as z values of only 3.1 and 3.6 °C were determined by Breeuwer *et al.* (2003) for two strains of *E. sakazakii* suspended in disodium hydrogen phosphate/potassium dehydrogenate phosphate buffer. When suspended in TSB rather than IFM the z values for the strains used in this present study were reduced to 5.6°C. This may be explained by the increased concentration of fats, proteins and carbohydrates in infant formula which could protect *E. sakazakii* from thermal inactivation. Despite these possible protective effects, using the decimal reduction time at 60°C of 1.1 minute and z-value of 5.6 degrees (Table 4.2), it is predictable that the decimal reduction time at 71.2°C is 0.7 seconds. Therefore the standard high temperature short treatment pasteurization process of 15 seconds at 71.7°C will result in approximately a 21 log reduction in the viable count of the organism. Hence any contamination of infant formula powder will probably be due to failure in hygiene standards after pasteurization.

The organism was able to grow in IFM at 6°C with a doubling time of approximately 13 hours. Although the infectious dose of the organism is unknown, unless the infant formula is highly contaminated at source it is unlikely that sufficient multiplication will occur under correct refrigeration conditions to cause an infection. However studies

have shown that up to 20% of household refrigerator temperatures are kept at 10°C or higher (Daniels, 1991; Harris, 1989, Rhodehamel, 1992, Van Garde and Woodburn, 1987) increasing the risk of *E. sakazakii* growth.

4.3.2 Development and comparison of Isolation media

It is essential that detection methods for *E. sakazakii* are established that are robust and reliable to ensure the safety of infant formula milk. The current method for the detection of *E. sakazakii* is based on pioneering work of Muytjens *et al.* (1988). The method uses sample enrichment in EE broth, followed by plating on VRBGA to isolate Enterobacteriaceae. Five colonies are selected and streaked on TSA for pigment production. This method however does not select for *E. sakazakii* and the combined use of EE broth and VRBGA could allow other Enterobacteriaceae to outgrow *E. sakazakii* and give false negative results. It is not possible to select for *E. sakazakii* colonies from VRBGA plates on the basis of colony morphology. In this study all Enterobacteriaceae produced similar colonies.

DFI agar differs from the previous media in that the chromogenic indolyl substrate X-α-Glc is incorporated into the medium to detect α-glucosidase activity. All 95 strains of *E. sakazakii* produced entirely blue-green colonies on DFI, confirming the constitutive production of α-glucosidase by this organism (Muytjens *et al.* 1988). Other members of the Enterobacteriaceae are also α-glucosidase positive. However many of these organisms (such as *Prot. vulgaris*) are differentiated from *E. sakazakii* due to their production of hydrogen sulphide and consequent blackening of the colony.

Organisms giving false positive results (entirely blue-green colonies) on DFI were mainly as yet unnamed species from environmental sources (Table 2.1). Although *Prot. vulgaris* is α-glucosidase positive (60%), only a small percentage produce false positive colonies on DFI as 95% of strains are H_2S positive (Farmer, 1999). In contrast 40% of *Cit. koseri* strains have the enzyme α-glucosidase and all are H_2S negative, therefore these strains would be expected to produce entirely blue-green colonies. However most *Cit. koseri* strains (7/8) were easily distinguished from *E. sakazakii* as the chromogenic substrate did not appear to be preferentially utilised. *Cit. koseri* (NTU 45), *E. aerogenes* (NTU 219) and *Kleb. pneumoniae* (NTU 273) produced entirely blue-green colonies only on DFI supplemented with maltose. This suggests that in these organisms α-glucoside permease and hydrolase are not constitutively expressed but are inducible and therefore they would not present as false positives on DFI medium.

The false positive result with *Esch. vulneris* was expected since this species was reported to be 25% positive for α-glucosidase and 50% positive for yellow pigment production (Farmer, 1999). It is currently unclear how significant this organism would be in screening infant formula as Muytjens *et al.* (1988) only isolated one *Esch. vulneris* strain from 141 batches of milk powder.

The ESE broth was developed to try and reduce the occurrence of false positives on the chromogenic medium. Preliminary experiments had shown that all *E. sakazakii* strains were able to ferment sucrose, whereas the majority of non-*E. sakazakii* α-glucosidase positive Enterobacteriaceae did not. Therefore, the ESE broth was formulated to support good growth of *E. sakazakii* as compared with competing organisms (Figure 4.7). Although other Enterobacteriaceae also utilize sucrose, these are mainly α-glucosidase-negative organisms and so can be differentiated on current chromogenic media. As *E. sakazakii* has been shown to have greater desiccation tolerance in comparison to most other Enterobacteriaceae (Breeuwer *et al.* 2003), a high concentration of sucrose was incorporated in the broth to act as a humectant lowering the available water. Sodium deoxycholate was incorporated to suppress the growth of Gram-positive bacteria. Unfortunately the ESE broth was not selective enough to be considered for use as an enrichment medium. Selective enrichment media ideally achieve at least a 2 log difference between the target and non-target organisms (personal communication, P. Druggan, Oxoid UK Ltd.) whereas the ESE only achieved 0.5 log as determined by OD 600 nm measurement (Figure 4.6).

All *E. sakazakii* isolates grew at 37°C in ESE broth, but 2 to 4% of the strains were undetected in EE, mLST or ESSB (Table 4.5). Assessment of the viability of these strains using standard plate counts showed that, for five strains, one or more of the selective media were bactericidal. Farmer *et al.* (1980) indicated that reduced growth occurred for some *E. sakazakii* strains on common Enterobacteriaceae and coliform isolation media. It has also been previously reported that an *E. sakazakii* strain failed to grow in mLST (Lehner *et al.* 2006). Other α-glucosidase organisms lost their viability in mLST to a greater extent than *E. sakazakii.* Therefore, the selectively of this medium is not necessarily the increased growth of *E. sakazakii*, but the greater die off of non-target cells.

All strains produced characteristic red colonies on VRBGA and VRBLA. However, these media are only selective for Enterobacteriaceae and coliforms, respectively and are not specific for *E. sakazakii*. They are therefore of use with respect to general hygiene monitoring, but not for specific pathogen detection such as *Salmonella* and *E. sakazakii.* At their recommended incubation temperatures of 37 and 44°C, respectively, 99 % of *E. sakazakii* strains grew on DFI agar, but only 96% on ESIA (Table 4.6). Incubation of DFI at 44°C (above the manufacturer's recommendation of 37°C) resulted in 7% of strains not showing the characteristic blue-green colony morphology. As one percent of *E. sakazakii* were unable to grow on non-selective media (TSA) at 44°C incubation at this temperature may not assure the recovery of *E. sakazakii.*

Detection methods should be evaluated using desiccated *E. sakazakii* cells in the presence of competing flora to mimic environmental samples from manufacturing facilities. Comparison of four methods for the recovery of desiccated *E. sakazakii* from

infant formula indicated that the most sensitive method was pre-enrichment in BPW, followed by enrichment in ESE and plating on DFI agar. However, at the lower inoculum levels, this method produced a high number of false-positive colonies on DFI. The presence of competing organisms reduced the sensitivity of the FDA method, and the AES method only recovered the *E. sakazakii* type strain. The mLST method was not as sensitive as the DFI method at low inoculum levels, but produced fewer false presumptive positives. As one of the strains (NTU 4) used in this experiment was sensitive to lauryl sulphate, the ability of the mLST method to recover it at the higher inoculum levels suggests that this method works better in the presence of the sample matrix (infant formula) than when used for pure cultures. This may be due to the divalent cations in the infant formula counteracting the effects of the lauryl sulphate. Therefore the performance of the mLST method may be reduced if used for other sample matrices.

This study has used a large number (177) of *E. sakazakii* strains to demonstrate that the levels of selective agents such as crystal violet, sodium lauryl sulphate, brilliant green and sodium deoxycholate in media need to be reassessed to ensure the recovery of the organism especially from mixed cultures. The ESE broth showed good sensitivity for *E. sakazakii.* However this broth is not selective enough to be considered as a viable enrichment method and further development of effective media for the isolation of *E. sakazakii* is needed.

4.3.3 Isolation of Enterobacteriaceae from infant formula and other foods.

No Enterobacteriaceae were detected following direct plating of rehydrated milk powder, lactose or infant foods on to VRGBA (Table 4.8). Sixteen out of one hundred and twenty-two herbs and spices contained Enterobacteriaceae $>10^4$ cfu g^{-1} which exceeded the maximum acceptable during the shelf-life of the product (IFST 1999). In contrast, most other dry food ingredients (54/66) did not contain Enterobacteriaceae, although five samples had $>10^3$ cfu g^{-1} and two of which were greater than 10^4 cfu g^{-1}. Microbiological criteria for good manufacturing practice of these ingredients would be <10 with a maximum of 10^3 Enterobacteriaceae/g (IFST 1999). As would be expected for uncooked foods such as raw meat and salads, the Enterobacteriaceae count was predominantly $>10^4$ cfu g^{-1}.

The APC of 75/82 powdered infant formula samples was less than 10^3 cfu g^{-1} (Table 4.8). Sixteen were between 10^3 and 10^4 cfu g^{-1} and two were $>10^4$ cfu g^{-1} which is the maximum limit set by CODEX for dried and instant products (Codex Alimentarius Commission 1979). The milk powder, lactose and dried infant food samples all had APC lower than the CODEX maximum (10^4 cfu g^{-1}), although 14/72 milk powders had APC between 10^3 and 10^4 cfu g^{-1} which is close to the maximum set criteria.

Only one powdered infant formula sample contained detectable Enterobacteriaceae without enrichment in EE broth (Table 4.8). This sample had a corresponding APC of 1.6×10^3 cfu g^{-1} which is higher than most of the other samples although *E. sakazakii* was not isolated from it. In contrast a wide variety of Enterobacteriaceae were recovered from 33.5% (68/203) powdered infant formula, dried infant foods and milk powders after enrichment in EE broth (Table 4.9). These Enterobacteriaceae could have been below the initial plate count detection limit (100 cfu g^{-1}) or were sublethally injured and unable to form colonies on VRGBA without resuscitation. The Enterobacteriaceae isolated included *Enterobacter* species, *Pantoea* species, *Esch. coli* and *Klebsiella* species (Table 2.1). The most frequently isolated organisms being *E. cloacae* and *Pantoea* spp. Muytjens *et al.* (1988) reported that 52.5% of powdered infant formula contained Enterobacteriaceae and the two species most frequently isolated were *E. agglomerans* (35/141) and *E. cloacae* (30/141). However the identity of *E. agglomerans* in the Muytjens paper is uncertain since the *Enterobacter-Pantoea-Citrobacter* group has been revised and the former *E. agglomerans* description now encompasses both *Pantoea* spp. and *Esch. vulneris* (Janda and Abbott 1998). The FAO/WHO (Codex Alimentarius Commission 2003b) are evaluating the organisms of concern in powdered infant formula and it is important to recognize that various members of the Enterobacteriaceae may be present in high risk foods despite the absence of recovery by direct enumeration.

E. sakazakii was isolated from 2/82 powdered infant formula samples (Table 4.10) by both detection methods. Results using the chromogenic DFI medium were obtained two days before the conventional procedure, since a two day incubation period on TSA is not required to observe pigment production. The two positive samples had APC of >100 and $>10^4$ cfu g^{-1}. *E. sakazakii* was isolated from five out of forty-nine dried infant foods. Two of the positive samples contained herbs, one was fruit-based, one was rice-based and one contained both rice and fruit. Although these products have not been associated with outbreaks of *E. sakazakii* infection, they are nevertheless a potential source of the pathogen. Although no Enterobacteriaceae were enumerated from seventy-two milk powder samples using the direct method, *E. sakazakii* was isolated from three samples (Tables 4.8 and 4.10). No bacteria were isolated by direct or enrichment procedures from lactose powder, which is an ingredient of infant formula.

E. sakazakii was isolated from 40/122 herbs and spices and 15/66 other dried food ingredients. Although many of these ingredients would be heat treated prior to ingestion, the high prevalence of the organism and the potential for cross-contamination means that these could be a source of *E. sakazakii* in a food manufacturing process. It is notable that *E. sakazakii* was frequently isolated from plant related material and this may reflect the ecology of the organism in common with *Pantoea* and other *Enterobacter* species (Janda and Abbott 1998).

The conventional method only detected nineteen samples containing *E. sakazakii* whereas the DFI method detected sixty-seven. For the conventional method five colonies are selected from the Enterobacteriaceae medium VRGBA and streaked on TSA (25°C, 48-72 h) for pigment production. This method however does not select for *E. sakazakii* and the combined use of EE broth and VRBGA could allow other Enterobacteriaceae to outgrow *E. sakazakii* leading to false negative results. It is not possible to select for *E. sakazakii* colonies from VRBGA plates on the basis of colony morphology. Therefore the greater the number of competitors the less chance that one of the 5 colonies picked will be *E. sakazakii*.

It is essential that detection methods for *E. sakazakii* are established that are robust and reliable to ensure the safety of infant formula milk. These results show that *E. sakazakii* is present in a wide range of food products, and may only be recoverable using the chromogenic DFI medium and not the conventional method. The DFI medium requires a two day shorter incubation period than the standard protocol, can differentiate the organism in the presence of other Enterobacteriaceae, and has a higher probability of detecting *E. sakazakii* amongst competing Enterobacteriaceae from enrichment media.

This survey shows that *E. sakazakii* is found in high risk products such as powdered infant formula, dried infant food and milk powder as well as various plant related ingredients. The organism was isolated frequently from dried material, indicating an ability to survive desiccation and persist for long periods of time. Other Enterobacteriaceae were also present in these high risk products. They were only detectable after enrichment in EE broth and not by direct enumeration on VRGBA. It is therefore timely that the FAO/WHO is revising the Recommended International Code of Hygienic Practice for Foods for Infants and Children (Codex Alimentarius Commission 2003).

The definition of a bacterial virulence factor can include expression of genes that are required for colonization of the host as well as genes and expression regulators that are directly involved in disease progression (Domingue and Woody, 1997; Finlay and Falkow, 1997; Wassenaar and Gaastra, 2001). In order to cause infection via ingestion of infant formula powder, an organism has to persist in the desiccated state in the dry formula prior to rehydration. After surviving rehydration the ingested organism has to survive the acidic pH of the stomach, however the pH of neonate stomachs varies greatly between individuals and is generally not as acidic as an adult stomach until the age of 2 years (Barbero *et al.* 1952). The next barrier encountered by pathogens is usually the mucosal surface of the gut epithelium and, as the turnover of epithelial cells is approximately 48 hours, the bacteria must attach and replicate to remain *in situ.* Motility likely plays a role in the ability of microorganisms to gain access to the gut epithelium through the mucosal layer. Motility has been linked to virulence in *Listeria monocytogenes* with motile deficient mutants showing the greatest attachment impairment (Gorski *et al.* 2003). Non-motile *Salmonella* (*S.* Pullorum and *S.* Gallinarum) are host adapted to poultry and have very low virulence for humans.

The adhesive properties of Enterobacteriaceae are usually mediated by pili/fimbriae which are nonflagellar, filamentous projections consisting of polymeric globular protein subunits (pilin) on the bacterial surface. (Ofek and Doyle, 1994; Jones and Isaacson, 1983). Basic attachment can be achieved through interaction of a host cell surface receptor and fimbrial tip proteins that bind D-Mannose and sialic acidic on host cells (Mouricout, 1997). Adherence and colonization factors, including flagella and fimbriae, may also enable the bacterial cell to cross the epithelial barrier and invade the host (Sauer *et al.* 2000).

Invasins are mainly enzymes, such as hyaluronidase, lecithinases and phospholipases that act extracellularly to break down tissue matrices and cell membranes. Other invasins include bacterial proteins that induce engulfment by acting on host cell actin filaments (Bliska, 2000). Exotoxins secreted by viable pathogenic cells are strongly antigenic. Some act on host cell surfaces binding to target cell molecules, such as diphtheria toxin, or form membrane pores through which cell lysis occurs, such as *Clostridium perfringens.* Most Gram negative bacterial pathogens use four secretion systems to transport protein toxins into the host or extracellular matrix. Type 1 proteins are secreted via a pore through inner and outer membranes and many of these exotoxin genes are clustered on pathogenicity islands next to their respective secretion apparatus genes. A/B toxins bind to the target membrane with a receptor (B subunit) and deliver a second moiety (A subunit) via the type II system. These toxins are

transported through the *sec* machinery, the amino terminus is cleaved forming a periplasmic intermediate which then passes through a second set of transmembrane proteins. Toxins of this type include cholera, pertussis and shiga toxin, and heat-labile enterotoxin from *E. coli*. Type IV secreted exotoxins also use the inner membrane *sec* proteins, but are passed through the outer membrane via their carboxyl terminus. *Helicobacter pylori* uses a type IV secretion system to inject Cag A into gastric epithelial cells. Some Gram-negative enteropathogens, such as *Shigella* and *Yersinia*, exhibit type III secretion systems which secretes effectors through a specialized macromolecular "needle" that injects exotoxins directly into the host cell cytoplasm. The structural moieties and effectors are encoded together on large pathogenicity islands on the bacterial chromosome (Alouf and Popoff, 2006).

To cause systemic disease bacteria must translocate across the epithelium into the vascular system, usually initially to mesenteric lymph nodes. The first line of defence by the host against invading microorganisms includes phagocytosis by polymorphonuclear granulocytes and the bactericidal effect of serum. Serum bactericidal activity is mediated primarily by complement proteins activated in a cascade. The terminal complement proteins C5b-C9 accumulate as a membrane attack complex on the surface of Gram-negative bacteria producing a transmembranous pore in the outer membrane which leads to an influx of Na^+ and subsequent osmotic lysis of the bacteria (Taylor, 1983; Ramm *et al.* 1983; Taylor and Kroll, 1985). The complement cascade can be activated by two different mechanisms; the classic complement pathway which requires specific antibodies to be activated, and the alternative complement pathway. The alternative pathway is regarded as an early defence system of innate immunity, which enables a reaction to invading microorganisms before specific antibodies are formed (Joiner, 1988.). Both complement pathways lead, via the activation of C3, to the formation of the opsonin C3b, ultimately resulting in formation of the terminal C5b-C9 complex. Most commensal gram-negative bacteria are sensitive to complement mediated cytotoxity , however in response to this host defence, pathogenic microorganisms have developed strategies to counter the serum bactericidal effect (Vosti and Randall, 1970).

The mechanisms behind bacterial serum resistance are thought to include various components of the outer membrane such as lipopolysaccharide (LPS) (Albertí *et al.* 1993.). LPS is a cell surface component anchored in the outer membrane by Lipid A, which is a strong biological enhancer that becomes toxic at higher concentrations. The polysaccharide core of LPS is immunogenic and contains genus specific antigens, while a series of O-linked oligosaccharides at the extracellular surface contains species and intra species specific antigens. Release of LPS into the host circulation promotes binding by the 'LPS-binding complex', which interacts with monocyte and macrophage CD14 receptors to trigger inflammatory cytokine release and activation of the

complement and coagulation cascades (el-Samalouti *et al.* 2000). Surface components, such as membrane bound proteins and polysaccharide capsules, can prevent phagocytocis of the bacterial cell by inhibiting opsonization via IgG antibodies and the C3b, C4b, and iC3b components of the complement system (Williams and Tomas, 1990; Bliss and Silver, 1996). Capsule polysaccharides may mask underlying surface molecules thus inhibiting complement activation and preventing deposition of C3b at the bacterial cell membrane. Serum-sensitive strains may activate both the classical and alternative complement pathways leading to a higher level of C3b deposition, resulting in a higher bactericidal effect than is seen with serum-resistant strains expressing smooth LPS which activates only the alternative pathway (Alberti *et al.* 1996). Serum resistance however may not be a stable characteristic with the composition and effect of LPS subject to environmental factors such as osmolarity (Aguilar *et al.* 1997). *Aeromonas hydrophila* serotype O:34 cells grown at high osmolarity show smooth LPS and are resistant to normal human serum, whereas growth at low osmolarity results in rough LPS causing serum sensitivity. If phagocytosis does occur some pathogens may persist and even replicate within host cells. *Cit. koseri* has been found to survive phagolysosomal fusion and to replicate within macrophage phagolysosomes in vitro, with bacterial uptake increasing in the presence of human serum (Townsend *et al.* 2003).

The prevalence and concentration of *E. sakazakii* in powdered infant formula is equivalent to that of some other Enterobacteriaceae, such as *Pantoea* spp. (formerly *Enterobacter agglomerans*) (Muytjens 1988, this study 4.2). However there has as yet been no link between outbreaks of neonatal infection and ingestion of infant formula for these other organisms. Also there are few reported cases of *E. sakazakii* causing serious infection in adults and older children (Lai, 2001) . Therefore it is likely that, in association with infant formula, *E. sakazakii* possesses characteristics that make it a greater risk specifically to neonatal health. In this study a preliminary investigation of risk factors was undertaken including desiccation persistence, biofilm formation, attachment to and invasion of human epithelial cells, survival in human macrophages and resistance to complement-mediated cytotoxicity for *E. sakazakii* and related Enterobacteriaceae.

5.1 MATERIALS AND METHODS

5.1.1 Desiccation survival

E. sakazakii strains NTU 1, 2, 3, 4, 5, 6, 7, 12, 15 and 23, along with Enterobacteriaceae strains NTU 44 (*Pantoea*) , 45 (*Cit. koseri*), 49 (*E. cloacae*), 153 (*Cit. freundi*), 178 (*Ser. marcescens*), 273 (*Kleb. pneumoniae*), 358 (Salmonella Enteritidis) 373 (*Esch. coli*), 379 (*Kleb. oxytoca*) (Table 2.1) were desiccated as described (2.4.1.4). Bacterial strains were grown 48 h on milk agar (2.3) at 37°C, harvested and resuspended in IFM. The cell suspensions were freeze dried in 1 ml aliquots. Duplicate aliquots for each strain were rehydrated in 1 ml BPW and enumerated using the MPN method (n=8) at 0, 3, 5, 6, 9, 11, 13, 18, 25 and 200 days after desiccation. Rehydrated aliquots were decimally diluted to 10^{-11} (100 µl in 900 µl BPW) and 100 µl of each dilution dispensed into the 8 wells of one column of a 96 well plate. The plates were incubated at 37°C for 48 h, and the positive wells used to estimate MPN g^{-1} powder.

5.1.2 Exopolysaccharide (EPS) capsule and motility

High CHO:N media, comprising XLD and IFM agar plates, were inoculated from cultures grown 24 h at 37°C in BHI. Inoculated plates were incubated 24 h at 37°C. Capsule production was assessed in comparison to NTU 1 (negative capsule) and NTU 2 (positive capsule). The amount of exopolysaccharide (EPS) was graded subjectively on a scale of 0-3 with 0 referring to dry colonies and 3 referring to strains which produced large mucoid colonies (>5mm). Motility was determined at 37°C after 24 h and 48 h by inoculating the centre of a tube of soft agar (5 cm depth) and observing for diffuse growth.

5.1.3 Biofilm formation

To assess adherence to infant feeding materials, pieces of latex, silicon and polycarbonate (10 x 10 x 0.5-1.0mm) were cut from infant feeding bottles, also stainless steel squares were cut to the same dimensions. The pieces were sterilized by autoclaving at 121°C for 15 min. Pieces were placed individually into universals containing 10ml BHI, IFM or SCH (2.3.4); 10 squares per media per material. The media were inoculated with NTU 1 and NTU 2 (10^4 cfu ml^{-1} from cultures grown 18 h in BHI at 37°C). The squares were incubated for 24 h at 37°C and were then washed individually by shaking gently for 1 minute in 10 ml sterile distilled water. The squares were dried for 3 h in a Class 1 airflow cabinet, being turned once. The microbial load remaining on each square was enumerated by direct impedance (2.4.1).

To prevent contact with the electrodes a 10 x 10 x 0.25 mm square of synthetic mesh (Scotch Brite™) was placed between the electrodes prior to sterilization of the RABIT electrode assembly tubes. Prior experiments with/without the mesh insert in

comparison to established calibration curves for NTU 1 indicated that the use of this material did not adversely affect the impedance measurement and accurate enumeration of inocula. Also prior experiments with sterile latex, silicon, polycarbonate and stainless steel squares were performed to ensure these materials in conjunction with the mesh did not affect the impedance measurements.

The viable counts were analyzed using the Box-whisker plot option of SlideWrite Plus for Windows Version 2.10 (Advanced Graphics Software Inc., C.A.).

5.1.3.1 Electron microscopy

Stainless steel, latex, silicon and polycarbonate squares were inoculated, washed and dried as for biofilm formation before mounting on 12.5 x 10 mm aluminium stubs (JEOL SEM, TAAB UK) using 12 mm self-adhesive carbon discs (TAAB UK). The samples were sputter-coated with gold and scanned on a JEOL-840A scanning electron microscope. Representative photographs were taken at various magnifications.

5.1.4 Attachment to Caco2 human colorectal epithelial cells

Preliminary experiments determined the Triton X-100 concentration and length of incubation that would effectively lyse Caco2 cells without affecting the viability of the bacterial strains. Caco2 cells were grown to confluence in 96 well plates and a two-fold serial dilution of Triton X-100 was applied. The plates were incubated at 37°C in 5% CO_2/95% air and observed microscopically for lysis after 5, 10, 15 and 30 min. Bacterial strains were grown in BHI overnight to ~10^9 cfu ml^{-1}, 1 ml removed and centrifuged at 13,000 rpm for 5 min. The pellet was washed x 1 in 1 ml DMEM, resuspended in 1 ml DMEM and incubated for 1h at 37°C prior to infection of Caco2 cells. Bacterial strains were decimally diluted and 100 µl aliquots of each dilution dispensed into triplicate wells of 96 well plates. Two-fold serial dilutions of Triton X-100 and a negative control comprising PBS were applied to separate plates (100 µl per well) and the plates incubated at 37°C. After 0, 5, 10, 15 and 30 min the wells were spotted onto TSA using a 96 pin blotter. The TSA plates were incubated at 37°C for 24 h and the positive spots used to assess any reduction in bacterial viability. A final Triton X-100 concentration of 0.1% and incubation for 30 min was determined to effectively lyse Caco2 cells without affecting the viability of any of the bacterial strains.

Bacterial attachment to human epithelial cells was investigated by growing Caco2 cells as a confluent monolayer in 24 well plates (2.4.2.1) and infecting with bacterial suspensions (10^8 cfu ml^{-1}). Control wells for each bacterial strain (T_0) were immediately aspirated and washed x 3 in 2 ml sterile PBS. Test wells were incubated for 3 h at 37°C in 5% CO_2/95% air before aspiration and washing. Cells were harvested, resuspend in total 1ml with PBS and counted using trypan blue exclusion. Caco2 cells were lysed by adding an equal volume of 0.2% Triton X-100 in PBS to the remaining cell suspension and incubating for 30 min at 37°C. The lysed suspension was decimally diluted to 10^{-8}

and viable bacteria counted on TSA. The attachment efficiency was calculated as the % of the inoculum that was associated with the Caco2 cells. All assays were preformed in triplicate.

5.1.4.1 Giemsa stain

Caco2 cells were grown as confluent monolayers on sterile coverslips in 6 well plates seeded with 4 ml cell suspension per well at $2x10^5$ cells ml^{-1} and infecting with bacterial suspensions (10^8 cfu ml^{-1}). Control wells for each bacterial strain (T_0) were immediately aspirated and the coverslip washed x 3 in 5 ml sterile PBS. Test wells were incubated for 0.5 – 3 h at 37°C in 5% CO_2/95% air before aspiration and washing. Coverslips were fixed in 95% methanol at -20°C for 10 min and air dried. The coverslips were stained with Giemsa as per supplier's instructions and examined under a light microscope using oil immersion.

5.1.5 Invasion of Caco2 human colorectal epithelial cells

Preliminary experiments determined the effective concentration of gentamicin that would kill bacterial strains without affecting Caco2 viability. Caco2 cells were grown to confluence in 24 well plates and a two-fold serial dilution of gentamicin was applied. The plates were incubated at 37°C in 5% CO_2/95% air for 1 h, washed x 3 2 ml PBS, harvested and the viable count determined by trypan blue exclusion. Bacterial strains were aliquoted (100 µl per well) in triplicate columns of a 96 well plate. Two-fold serial dilutions of gentamicin were applied across the rows (100 µl per well) and the plates incubated at 37°C for 1 h. The wells were spotted onto TSA using a 96 pin blotter. The TSA plates were incubated at 37°C for 24 h and the positive spots used to assess the reduction in bacterial viability. A gentamicin concentration of 0.5 mg ml^{-1} for 1 h was determined to effectively kill the bacterial strains without affecting the viability of Caco2 cells.

For invasion assays, Caco2 cells were grown as a confluent monolayer in 24 well plates and infected as above (5.1.4). Control wells were immediately treated with gentamicin (0.5 mg ml^{-1}) and further incubated for 1 h to kill planktonic and cell-surface attached bacteria (T_0). Test wells were incubated for 3 h at 37°C in 5% CO_2/95% air before addition of 0.5 mg ml^{-1} gentamicin then incubated as for the control wells. Cells were washed x 3 with 2 ml PBS to remove residual gentamicin, harvested, counted using trypan blue exclusion, and lysed in Triton X 100 as above (5.1.4), before serial dilution and plate count of viable bacteria. The invasion efficiency was calculated as the % of the inoculum that survived internally in the Caco2 cells. All assays were preformed in triplicate.

5.1.6 Resistance to complement mediated cytotoxicity

Bacterial survival in human serum was determined by comparing microbial growth in fresh-frozen versus heat-inactivated serum. Heat inactivation was carried out by heating fresh-frozen serum in a water bath at 56°C for 30 min. Bacterial cultures were grown 18 h at 37°C in BHI, 1 ml centrifuged at 13,000 rpm, the pellet washed x 1 in 1 ml PBS and resupended in 1ml PBS. Equal volumes (200 µl) of bacterial suspension and serum were dispensed into wells of a Bioscreen C honeycomb plate (n=5). The plates were incubated at 37°C for 4 h and the OD 600 nm read at 15 min intervals. The OD 600 nm mean value for the heat-inactivated wells was subtracted from the OD 600 nm mean value for the fresh-frozen wells and plotted against time (Figure 5.5).

5.1.7 Persistence within U-937 human macrophage cells

The ability of bacterial strains to survive internally in macrophages was investigated by infecting U-937 cells with bacterial suspensions.

PMA (0.1 µg ml^{-1}) was added to U-937 macrophages seeded in 24 well tissue culture plates (1ml ~$1x10^5$ cells per well). Cells were incubated 24 h at 37°C, 5% CO_2/95% air to differentiate and adhere before washing gently x 3 with 2 ml RPMI, to remove residual PMA, and addition of 1 ml fresh media prior to infection.

Bacterial strains were grown in BHI overnight to ~10^9 mg ml^{-1}, 1 ml removed and centrifuged at 13,000 rpm for 5 minutes. The pellet was washed x 1 in 1 ml RPMI, resuspended in 1 ml RPMI and incubated for 1 h at 37°C; 100 µl per well was used to infect the U-937 human macrophages. Infected wells were incubated at 37°C in 5% CO_2/95% air for 1 h after which 0.5 mg ml^{-1} gentamicin was added to each well and the plates incubated for an additional 1 h at 37°C in 5% CO_2/95% air. Control cells (T_0) were harvested by aspirating the media and washing the macrophages gently x 3 with 2 ml PBS to remove residual gentamicin. The macrophages were lysed by addition of 200 µl 0.1% Triton X-100 in PBS to each well and incubating at 37°C for 30 min. The lysate was serially diluted, and plate counts performed to determine the number of intracellular bacteria.

For extended incubation assays (intracellular replication assays), after the 1 h incubation with 0.5 mg ml^{-1} gentamicin, test cells were aspirated and replenished with fresh media containing 10 µg ml^{-1} gentamicin. Test wells were harvested at 24, 48 and 72 h; for each time point results are presented as percent survival of the initial intracellular population recovered at T_0. All assays were preformed in triplicate.

5.1.8 Cytotoxicity of bacterial fractions

5.1.8.1 Preparing bacterial cell fractions

Bacterial strains grown as lawns on infant formula milk (IFM) agar (2.3.3) for 24 h at 37°C, were harvested into sterile endotoxin free H_2O (1:100 w/v cell suspension), and

centrifuged at 10000 rpm 30 min at 4°C. The washed cell pellet was resuspended 1:10 w/v in sterile endotoxin free H_2O, frozen at -20°C for 24 h and sonicated in an ice bath (6 x 30 seconds). Cell debris was removed by centrifugation at 13,000 rpm for 5 min and the supernatant filtered (0.2 µm, Millipore™). Approximately 50% of this cell free extract (CFE) was passed through a polymixin B column (Detoxi-Gel™, Pierce, IL) to remove endotoxin (void volume 0.3-0.5 ml). The CFE (0.4 ml aliquots) was applied to the column, the run-off discarded and the protein extract (P) eluted with 0.5 ml endotoxin free H_2O. Protein extracts were divided into two equal volumes and one volume heated at 56°C for 30 min to denature proteins (Ph). The protein concentrations of the fractions were determined using the *DC* Protein Assay (Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK) according to manufacturer instructions. The fractions were standardize to a protein concentration of 1 mg ml^{-1} by diluting in N2a growth medium (2.4.2.2) and the cytotoxicity measured according to the reduction of methyl blue tetrazolium (MTT) by N2a cells.

5.1.8.2 MTT assay

The MTT assay is a standard colorimetric assay for measuring cellular proliferation (Mossman, 1983). Active mitochondrial reductase enzymes reduce yellow methylthiazolyldiphenyl-tetrazolium bromide (MTT) to purple formazan. Spectrophotometric measurement of formazan is directly related to the number of viable cells. Therefore the production of purple formazan in cells treated with an agent is measured relative to the production in control cells to indicate increases or decreases in mitochondrial activity due to the agent.

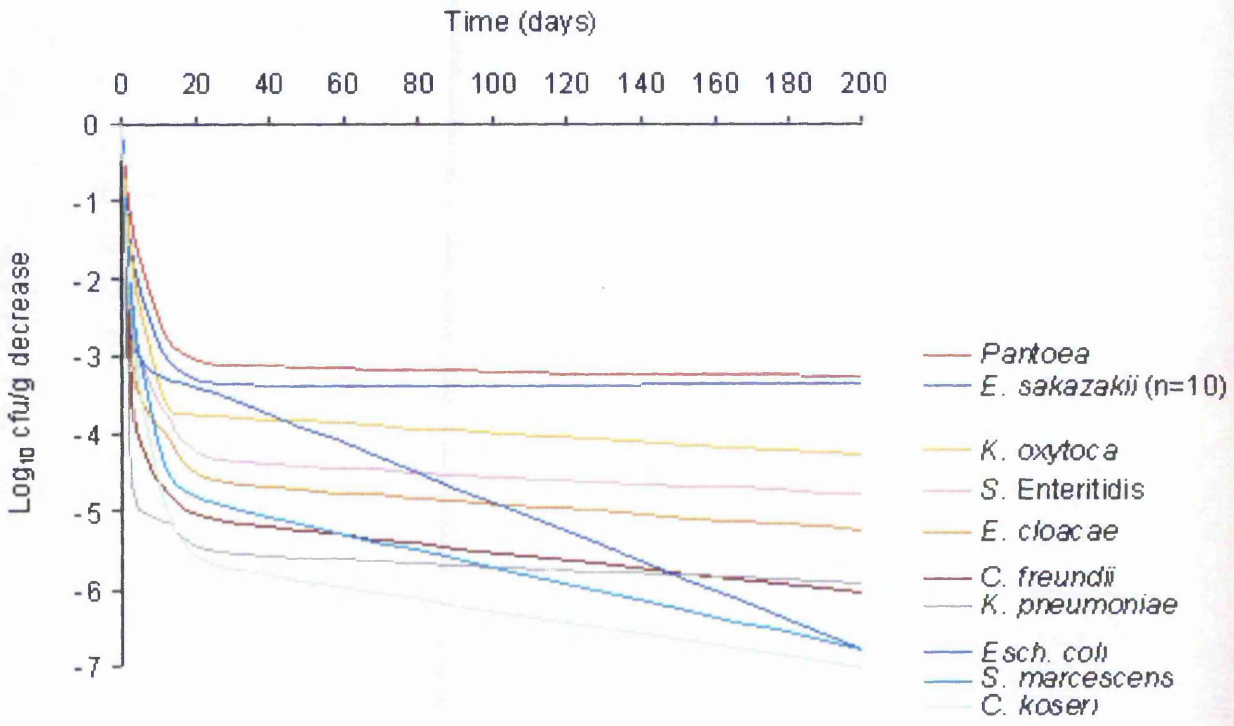
The middle 60 wells of 96 well plates were seeded with N2a cells ml^{-1} (2.4.2.2) with 200 µl DMEM in the outer wells to limit evaporation and edge effects. Cells were incubated 24 h at 37°C 5% CO_2/95% air and checked by light microscopy to ensure normal cell morphology. Fresh growth medium (100 µl per well) was added to three wells each of the first and last column of the 60 test wells (positive control 0% killed cells) and 100 µl of 1% Triton X-100 added to the remaining three wells of the first and last column (negative control). Bacterial cell fractions were added to triplicate test wells at 100 µl per well (to give a final concentration 0.5 mg ml^{-1} protein in CFE fractions) and the cells were incubated for 48h at 37°C 5% CO_2/95% air. MTT was prepared at 5.5 mg ml^{-1} in PBS, sterilized by filtration and 20µl MTT solution added to each well (final concentration of 0.5 mg ml^{-1}). Cells were incubated for 4h at 37°C 5% CO_2/95% air, the medium removed and 200 µl DMSO added to each well. The plates were shaken for 20 min at room temperature, the OD 550 nm measured and the % MTT reduction calculated as (OD 550 treated/OD 550 positive control) x 100 after correction for negative control background. The % decrease in MTT reduction in comparison to positive control wells was calculated for each extract.

5.2 RESULTS

5.2.1 Desiccation survival

In this initial study spanning approximately 6 months, *Pantoea* and *E. sakazakii* showed the greatest resistance to freeze-drying and storage in a desiccated state with an initial decrease in viability of approximately 3 logs (Figure 5.1) during the first two weeks after which the viable counts remained stable. Intermediate persistence was seen with *K. oxytoca*, *Salmonella* Enteritidis and *E. cloacae* which showed 4-5 log decreases, the greatest decrease occurring in the two weeks post desiccation followed by a gradual decline. *Cit. freundii* and *Kleb. pneumoniae* decreased approximately 5 log within the first two weeks and a further 0.5 log during the following 6 months of storage. The viability of *Esch. coli*, *Ser. marcescens* and Cit. koseri decreased approximately 6.5 log over the test period.

Figure 5.1 Desiccation survival of Enterobacteriaceae in infant formula.



Pantoea and *E. sakazakii* isolates were the most persistent organisms in the desiccated infant formula.

5.2.2 Exopolysaccharide (EPS) capsule and motility

A total of 178 strains confirmed as *E. sakazakii* by partial 16S rDNA sequencing were also tested for production of EPS and for motility. Greater than 80% of strains produced capsule with 19% producing relatively large amounts of EPS (grade 3, Table 5.1). Only 11% of strains were non-motile with 5% of these also non- capsulated and 5% producing the lowest amount of EPS (grade 1, Table 5.1). Two strains that produced large amounts of EPS were non-motile.

Table 5.1 Production of EPS and motility for *E. sakazakii* strains.

		MOTILITY			
	Grade	+	(%)	-	(%)
EPS	0	34	(19)	9	(5)
	1	96	(54)	9	(5)
	2	29	(16)	0	(0)
	3	19	(11)	2	(1)

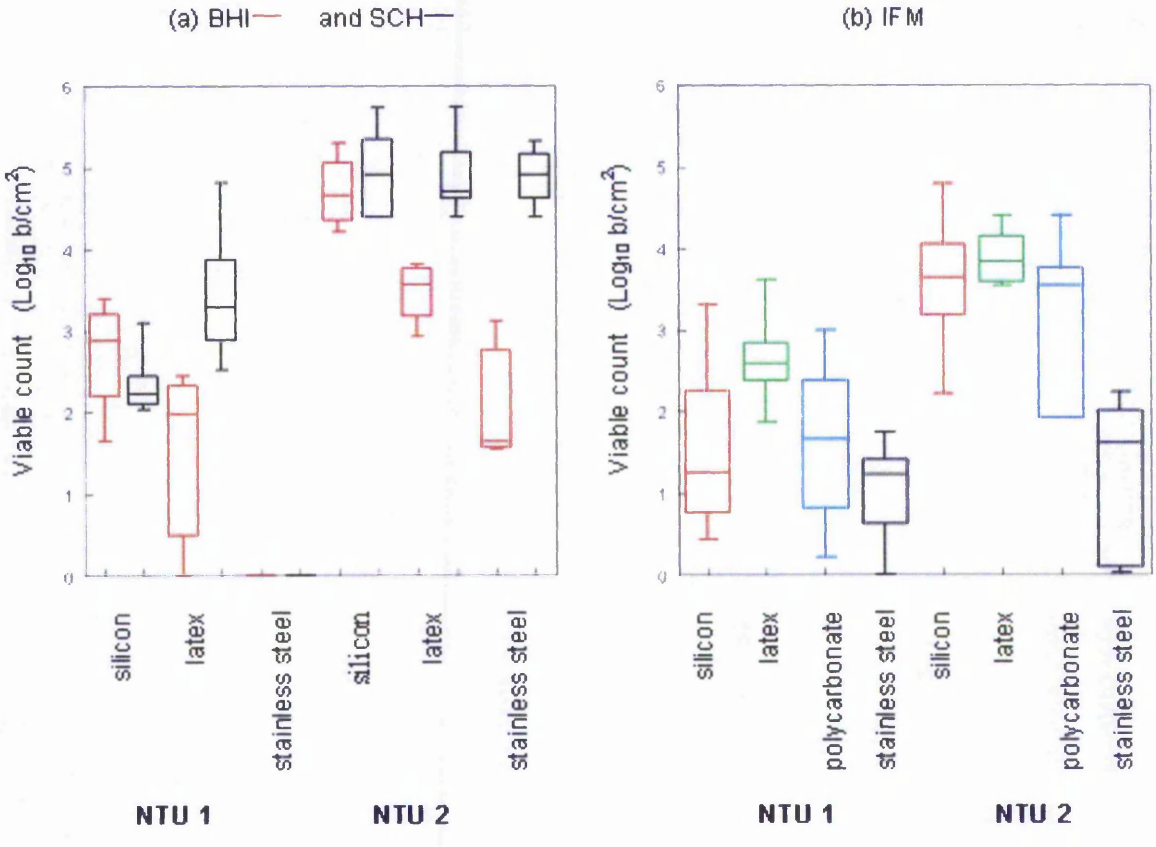
All the *E. sakazakii* strains used in the attachment and invasion assays were motile, with the exception of NTU 472. Capsule was produced by 50% of the *E. sakazakii* clinical isolates (NTU 4, 6 and 658) and all the *E. sakazakii* food isolates, with the exception of NTU 121. The unidentified species 5 and 6 also produced capsule but the other Enterobacteriaceae in the attachment and invasion studies did not. All the comparative Enterobacteriaceae in these experiments were motile with the exception of *Pantoea* sp., *But. noakiae*. and NTU 55, which is from unidentified genomic group 5 (Figures 5.3 and 5.6).

5.2.3 Biofilm formation

When grown in BHI strain NTU 1 appeared to adhere to the materials tested to a lesser extent than did the capsulated strain NTU 2 with no organisms detectable on the stainless steel for NTU 1 (Figure 5.2a). When NTU 1 was grown in SCH the mean value for latex increased but there were still no organisms detectable on stainless steel. The mean value for NTU 2 was higher for silicon than for latex when grown in BHI with the lowest value being obtained for stainless steel ($8x10^4$, $4x10^3$, and $5x10^2$ cfu/cm^2 respectively). When NTU 2 was grown in SCH the mean value for silicon adherence remained the same as for growth in BHI, however the adherence to latex and stainless steel increased and were equal to silicon. These results indicate that growth in high C:N media, promoting capsule formation, can increases the ability of *E. sakazakii* capsulated strains to adhere to stainless steel and latex surfaces.

There was no significant difference in adherence of the two strains to any of the materials when grown in IFM (Figure 5.2b), however strain NTU 1 was detectable on stainless steel when grown in this medium albeit at low cell density (20 cfu per cm^2). The mean values for silicon and latex when NTU 1 was grown in IFM were very similar to the values obtained when this strain was grown in SCH. The mean values for attachment to silicon, latex and stainless steel for NTU 2 grown in IFM were similar to those obtained when this strain was grown in BHI and did not reach the levels obtained in SCH medium.

Figure 5.2 Estimation of *E. sakazakii* NTU 1 and NTU 2 biofilm formation.

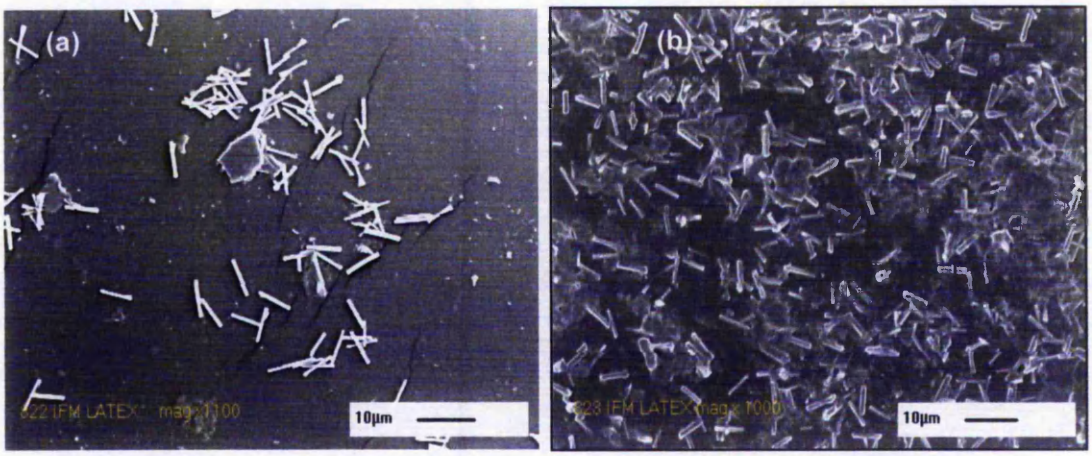


The bars represent minimum and maximum values, box represents upper and lower quartile, and the midline represents median.

5.2.3.1 Electron microscopy

Scanning electron microscopy (SEM) of silicon, latex, polycarbonate and stainless steel 1cm^2 pieces supported the results of the biofilm assays (Figure 5.3). More organisms were observed adherent to materials that had been incubated with the capsulated strain, NTU 2, than with the non-capsulated strain, NTU 1.

Figure 5.3 SEM of *E. sakazakii* strains NTU 1 (left) and NTU 2 (right) adhering to a latex teat.

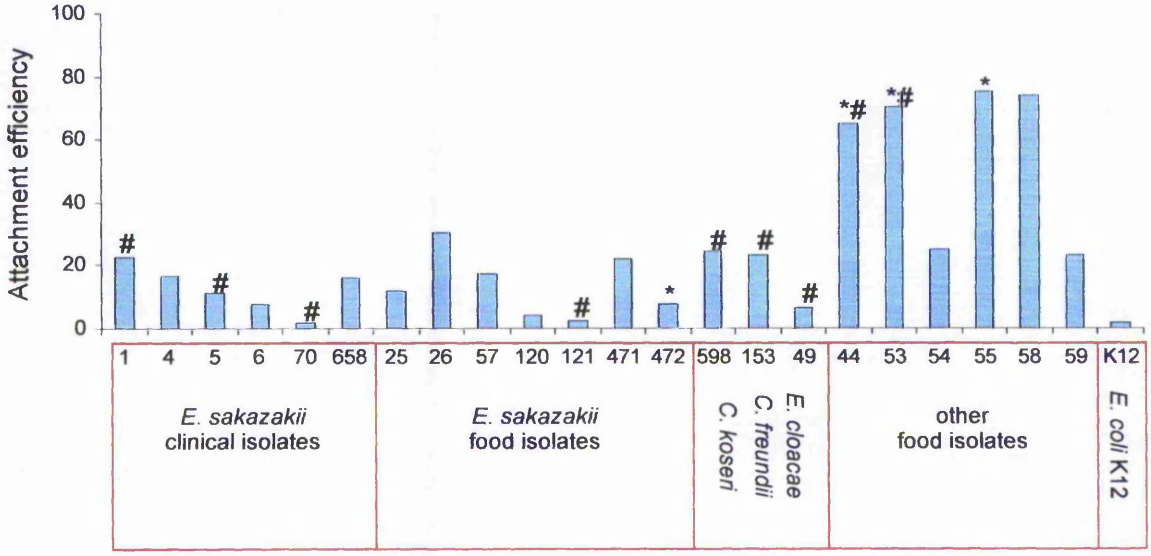


Bacterial cells grown on latex cut from an infant feding bottle teat in IFM.

5.2.4 Attachment to Caco2 human colorectal epithelial cells

The highest attachment to Caco2 cells was observed with *Pantoea* sp. (NTU 44), *But. noakiae* (NTU 53) and unidentified species from genomic group 5 (NTU 55, 58) with approximately 60-70% attachment efficiency.

Figure 5.4 Attachment to Caco2 epithelial cells



- strain does not produce capsule; * - strain is non-motile. The attachment efficiency was calculated as the % of the inoculum that was associated with the Caco2 cells.

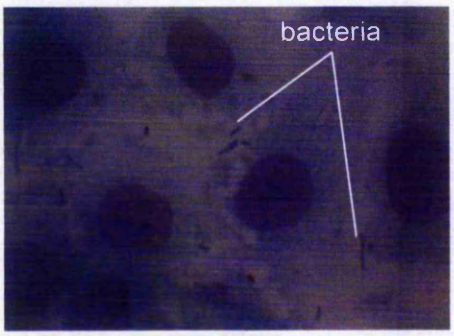
There was no difference in attachment between clinical and food isolates of *E. sakazakii*. The highest attachment efficiency (~30%) was observed for NTU 26. Clinical and food isolates, NTU 70 and 121 respectively, showed attachment efficiency similar

to the *Esch. coli* K12 negative control, while the attachment of the remaining *E. sakazakii* strains ranged between 4-23%. The *Citrobacter* strains (NTU 153 and 598) had attachment efficiencies of approximately 25%; while the *E. cloacae* type strain (NTU 49) had an attachment efficiency of 8% (Figure 5.4).

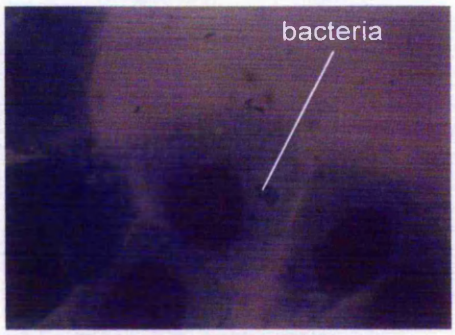
5.2.3.2 Giemsa staining

The association of bacterial cells with Caco2 epithelial cells was observed using Giemsa stains. Attachment of *Esch. coli* NTU 373 (a 0157:H7$^-$ strain) appeared to be randomly distributed across the Caco2 monolayer (Figure 5.5). Attachment of *S.* Enteritidis NTU 358 and *Pantoea* sp. NTU 44 appeared to be localised to specific areas of the cell cytoplasm. The attachment of *E. sakazakii* NTU 2 appeared to occur in large clusters around Caco2 cellular extensions and nuclei. Closer examination of infected cells after 1.5h incubation indicated that *E. sakazakii* NTU 2 appears to attach close the nucleus of Caco2 cells and replicate *in situ* (Figure 5.6).

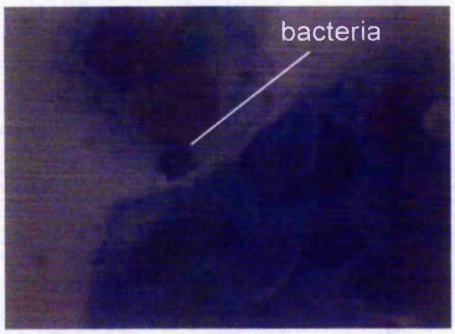
Figure 5.5 Colonization of Caco2 epithelial cells after 3h incubation



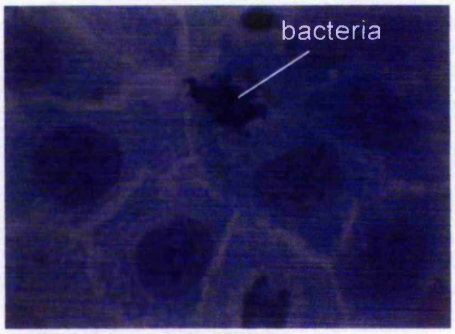
a) *Esch. coli* NTU 373



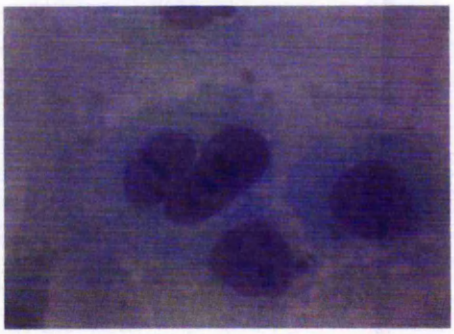
b) *S.* Enteritidis NTU 358



c) *Pantoea* sp. NTU 44



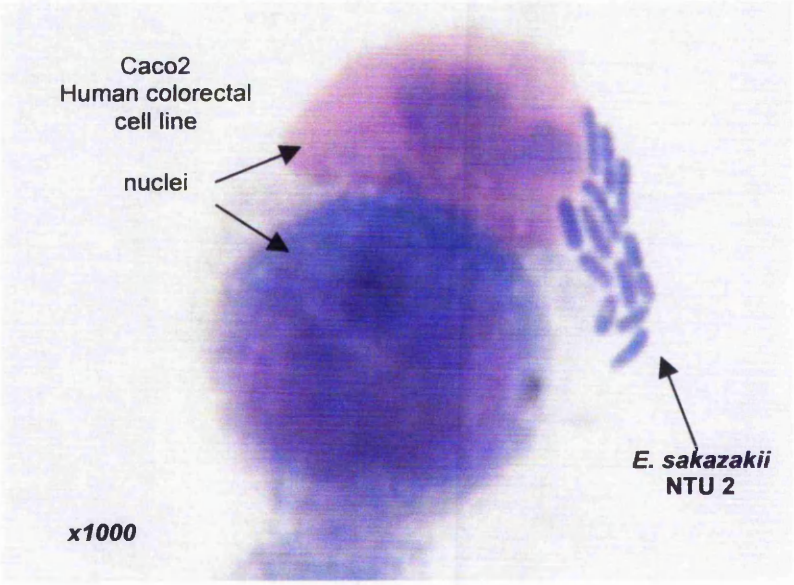
d) *E. sakazakii* NTU 2



e) Control, uninfected

magnification x1000

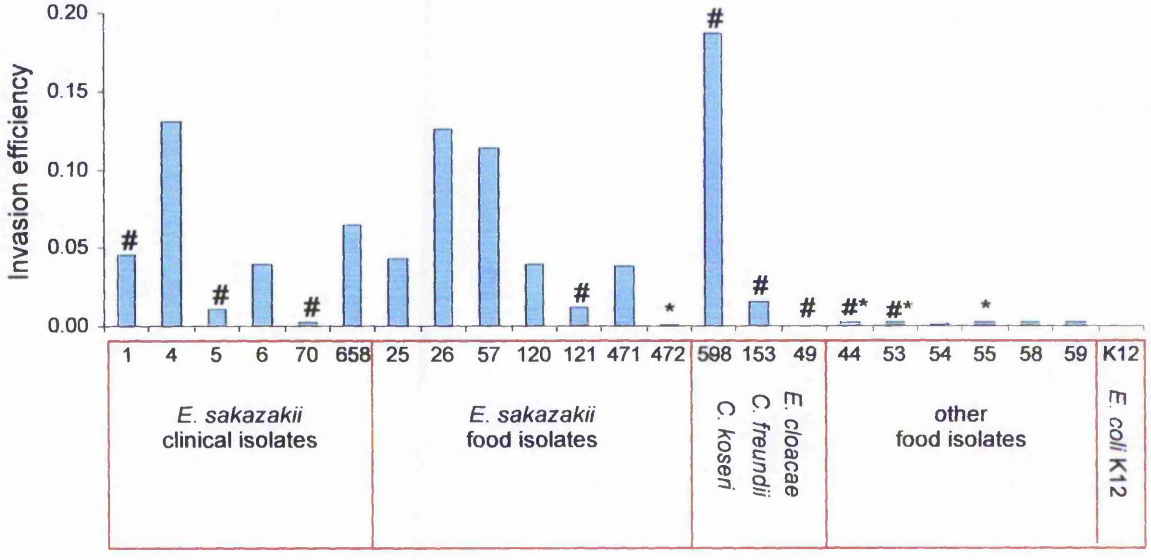
Figure 5.6 Colonization of Caco2 epithelial cells after 1.5h incubation



5.2.5 Invasion of Caco2 human colorectal epithelial cells

The highest invasion efficiencies were observed with *Cit. koseri* NTU 598 (0.18%) and with *E. sakazakii* strains NTU 4, 26 and 57 (0.13-0.17%). The invasion efficiency of other *E. sakazakii* strains ranged from 0-0.07% and there was no difference in invasion between clinical and food isolates. *Cit. freundii* had an invasion efficiency of 0.02% and no invasive cells were detected for the *E. cloacae* type strain (NTU 49). Although the *Pantoea* sp. (NTU 44), *But. noakiae* (NTU 53) and unidentified species from genomic group 5 and 6 (NTU 54, 55, 58, 59) were able to attach to Caco2 cells they appear to be unable to invade (Figure 5.7).

Figure 5.7 Invasion of Caco2 epithelial cells

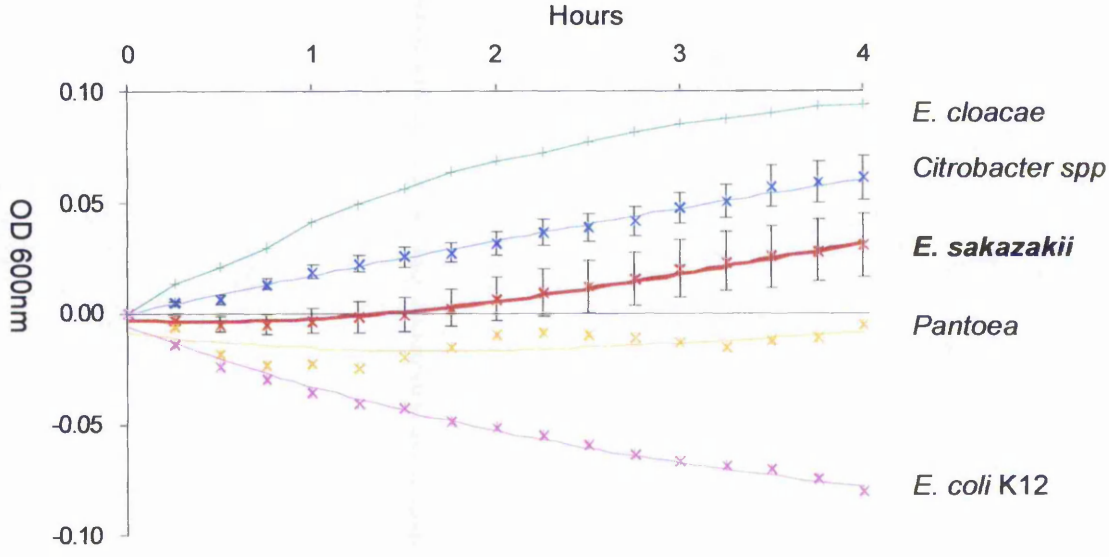


- strain does not produce capsule; * - strain is non-motile. The invasion efficiency was calculated as the % of the inoculum that survived internally in the Caco2 cells.

5.2.6 Resistance to complement mediated cytotoxicity

The *Esch. coli* K12 negative control clearly decreased in the presence of human serum (Figure 5.8). The other organisms were able to persist with *E. cloacae*, *Citrobacter* sp. and to a lesser extent *E. sakazakii* appearing to grow at a faster rate in fresh-frozen than in heat-treated serum. This is possibly due to a decrease in nutrient properties during the heat treatment.

Figure 5.8 Complement-mediated cytotoxicity survival



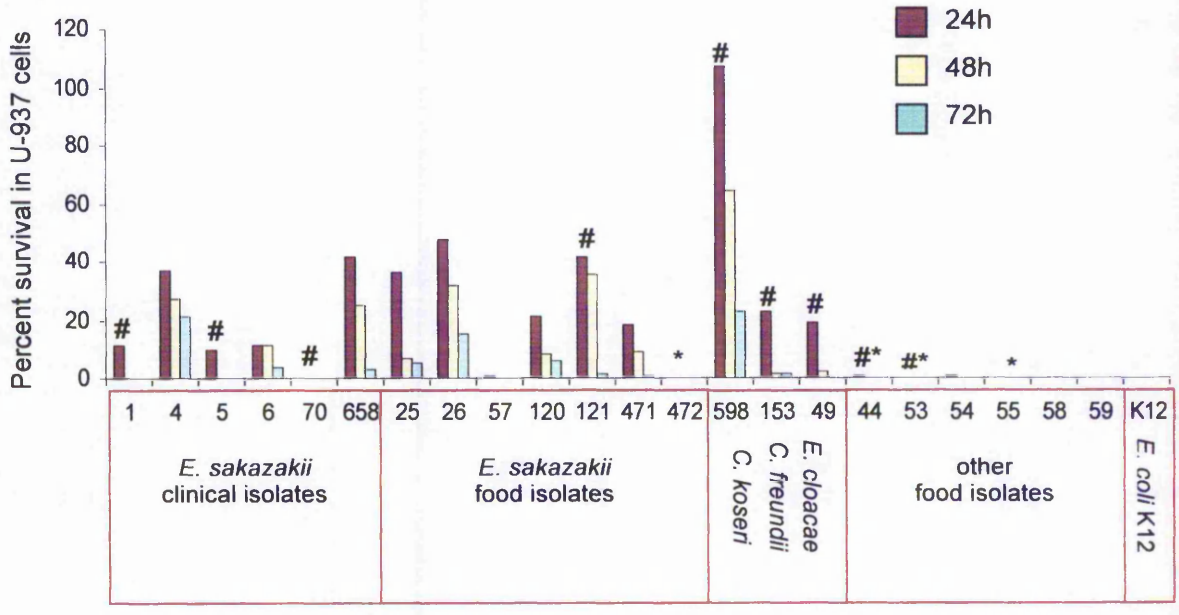
All strains, with the exception of *E. coli* K12, were able to resist complement-mediated cytolysis.

5.2.7 Persistence within U-937 human macrophage cells

The greatest persistence was observed with *Cit. koseri* NTU 598 with an apparent increase in intracellular bacteria (110% survival) within the initial 24h incubation (Figure 5.9). The numbers of *Cit. koseri* decreased during extended incubation to 63% and 22% of the initial intracellular value at 48 and 73 h respectively. *E. sakazakii* strains NTU 4 and 658 (clinical), 26, 25 and 121 (food) were among the most persistent at 39-50% survival in the initial 24h period with recovery still possible at 48 and 72 h. The survival of *E. sakazakii* strains NTU 1, 5, 6, 120 and 471 at 24 h ranged from 10-22% with NTU 6, 120, and 471 exhibiting extended persistence. *E. sakazakii* strains NTU 57, 70 and 472 as well as *Pantoea* sp. NTU 44, *But. noakiae* NTU 53 and unidentified species from genomic group 5 and 6 (NTU 54, 55, 58, 59) did not persist in

macrophages. *Cit. freundii* NTU 153 and *E. cloacae* NTU 49 exhibited approximately 20% survival in 24 h but did not persist over 48 h.

Figure 5.9 Persistence in U-937 human macrophages

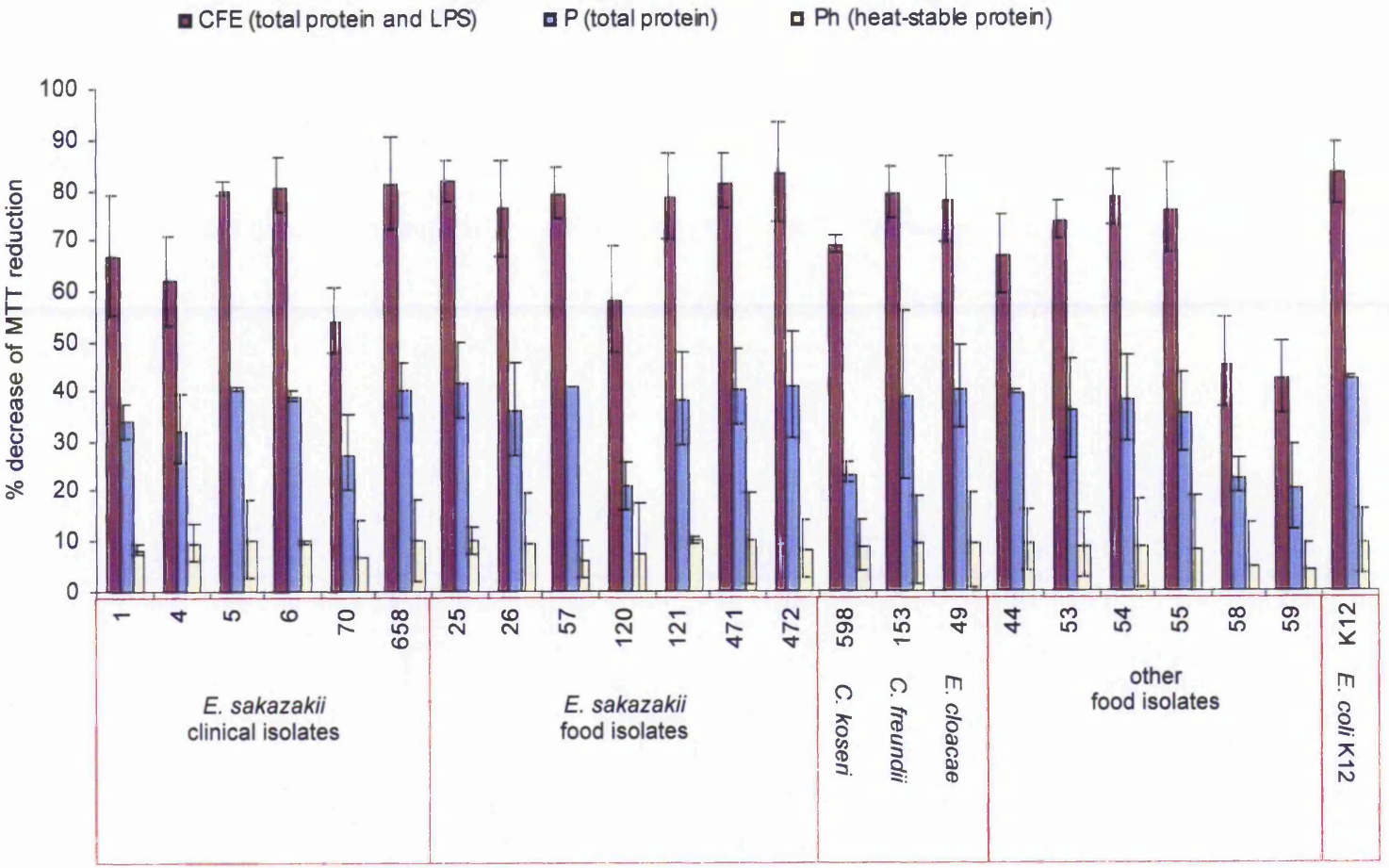


- strain does not produce capsule; * - strain is non-motile. *C. koseri* was the most persistent of the bacterial isolates in the macrophages, showing initial growth. Some *E. sakazakii* isolates were also able to persist, however other species isolated from food were not able to survive.

5.2.8 Cytotoxicity of bacterial fractions

The mean % decrease in reduction of MTT for the CFE fractions from the majority of strains tested was greater than 60%. The lowest values were seen with NTU 58 (unidentified genome group 5) and NTU 59 (unidentified genomic group 6). The CFE fraction contains LPS as well as bacterial cell proteins which may be the reason this fraction exhibited the most affect on the N2a mitochondrial activity. LPS was removed from protein extracts using polymixin B affinity columns. The cytotoxicity of these fractions reduced by approximately 50% in comparison to CFE for all bacterial strains. All extracts appeared to have some heat stable cytotoxic effects. The *Esch. coli* K12 control strain produced similar values to the test strains for all fractions (Figure 5.10).

Figure 5.10 Cytotoxicity of bacterial fractions



5.3 DISCUSSION AND CONCLUSION

This study included a basic investigation of possible factors associated with risk of disease in infants such as desiccation survival, biofilm formation, attachment and invasion to epithelial cells and survival mechanisms within the host.

Edelson-Mammel and Buchanan (2004) found the viability of *E. sakazakii* in powdered infant formula decreased by approximately 2.5 logs during an initial 5 month study with a further 0.5 log decreased over the subsequent year. The desiccation study herein covered an initial period of 6 months and showed that *Pantoea* sp and *E. sakazakii* isolates were the most resistant to initial freeze-drying and also the most persistent in the desiccated state. This may contribute to the reason these organisms were isolated the most frequently of Enterobacteriaceae from infant formula powders and dried infant food (Table 4.9). Desiccation studies at Nottingham Trent University have been extended to two years after which *E. sakazakii*, *Pantoea* sp. and *Kleb. oxytoca* were the surviving organisms (Caubilla-Barron *et al.* 2004). In addition, the frequency of isolation of the unidentified species comprising genomic groups 5 and 6 from dried food products indicates that these species may also be desiccation tolerant. *E. sakazakii* is the only one of these organisms for which a link has been recognised between infant formula contamination and neonatal infections. *Pantoea* spp. have been implicated in cases of bacteraemia resulting from thorn injuries (de Baere *et al.* 2005) and from contaminated parenteral nutrition solutions (Habsah *et al.* 2005). In the later case 7/8 infants died of respiratory failure and septic shock. These cases indicate that although *Pantoea* spp. do not appear to be capable of invading host tissue, once introduced into the vascular system some strains are capable of overcoming host defences. In this study the *Pantoea* sp. (NTU 44) was able to attach to but not invade Caco2 epithelial cells and was able to persist in human serum but did not survive macrophage phagocytosis. No difference was seen in patterns of attachment, invasion, serum survival or cytotoxicity between *E. sakazakii* isolates from clinical or food sources. One clinical isolate (NTU 70) exhibited poor attachment, invasion and macrophage survival and one food isolate (NTU 26) consistently exhibited the highest values of the *E. sakazakii* for these assays. The *Cit. koseri* strain was the most invasive isolate and also replicated as well as persisting in U-937 cells. Mange *et al.* (2006) found diffuse adhesion and formation of localized bacterial clumps for *E. sakazakii* strains to human epithelial (HEp-2 and Caco2) and human brain microvascular endothelial cells (HBMEC). Mannose, hemagglutination, trypsin digestion experiments and transmission electron microscopy suggested that the adhesion of *E. sakazakii* to the epithelial and endothelial cells was non-fimbrial based.

Pagotto *et al.* (2003) used a suckling mouse model and in vitro assays (CHO, Vero, and Y-1 lines) to investigate virulence and determined that 4/18 *E. sakazakii* strains (three clinical isolates and one food isolate) tested positive for production of

enterotoxin. The strains in this study all showed similar values for reduction in N2a mitochondrial activity due to the bacterial fractions including the negative control *Esch. coli* strain. The amount of residual LPS in the protein fractions should have been assessed using a limulus amoebocyte lysate assay to determine the efficiency of removal.

The majority of *E. sakazakii* strains tested produced a capsule (144/178) and most of the non-motile strains produced either no capsule or the lowest amount of capsule (Table 5.1). All the *E. sakazakii* strains used in the attachment and invasion assays were motile, with the exception of NTU 472. Capsule was produced by 3/6 of the *E. sakazakii* clinical isolates and 6/7 of the *E. sakazakii* food isolates. No obvious relationship between motility, capsule production, attachment or invasion could be seen from these results. Further studies are necessary to determine the attachment and invasion mechanisms involved in the interaction of *E. sakazakii* with epithelial cells.

When grown in IFM, *E. sakazakii* strains NTU 1 (non-capsulated) and NTU 2 (capsulated) formed a biofilm on surfaces such as latex, silicon and to a lesser extent stainless steel. These materials are commonly used for infant feeding equipment and in preparation areas. Attachment and adherence may be enhanced due to the production of exopolysaccharide. It has been reported that micro-organisms attach more rapidly to hydrophobic, nonpolar surfaces such as plastics than to hydrophilic materials such as metals and that surfaces exposed in aqueous media become conditioned (coated by polymers from the media) almost immediately and the resulting chemical modification affects subsequent microbial attachment (Donlan, 2002). Nutrient levels, ionic strength, pH and temperature may also contribute to the rate of microbial attachment. In this study, both strains tested appeared more adherent to silicon, latex and polycarbonate than to the stainless steel with the highest values being obtained for silicon. The uncapsulated strain was only detectable on stainless steel when grown in IFM, this may be due to a greater propensity for the components of IFM than BHI or SCH to form a conditioning layer facilitating adhesion. The capsulated strain, NTU 2, was more adherent than the uncapsulated strain to silicon, latex and stainless steel when grown in BHI and SCH media but there was no significant difference in adherence of the two strains to any of the materials when grown in IFM. The mean values for silicon and latex when NTU 1 was grown in IFM were very similar to the values obtained when this strain was grown in SCH, which were higher than when grown in BHI. Whereas the mean values for NTU 2 grown in IFM were similar to those obtained when this strain was grown in BHI, which were lower than when grown in SCH. The high C:N ratio of SCH medium promotes production of exopolysaccharide and when grown in this medium the adherence of NTU 2 to latex and stainless steel appeared to increase to values comparable to those obtained for silicon. It may be that the capsule formation was the main factor in increased adhesion for strain NTU 2 but the formation of a

conditioning layer was the main factor in adhesion of the uncapsulated strain, NTU 1. Neonatal infections have been associated with *E. sakazakii* colonisation of food preparation equipment such as brushes, blenders and spoons (Simmons *et al.* 1989; Bar-Oz *et al.* 2001). Therefore all bottles and utensils should be cleaned thoroughly as soon as possible after use to deter *E. sakazakii* biofilm formation, which could result in neonatal infections.

All *E. sakazakii* strains tested were resistant to complement-mediated cytolysis indicating an ability to survive in the bloodstream and cause bacteraemia. The mechanisms influencing development of meningitis as opposed to bacteraemia and sepsis are most likely dependent on the status of the host development than on particular properties of bacterial strains. Bektas *et al* (1990) demonstrated various abnormalities in neutrophil function from preterm neonates, which could predispose them to overwhelming bacterial infections. Langerhan's cells are present in the neonate at 18 weeks gestation and are important in local infection. Mucosal Associated Lymphoid Tissue (MALT) cells are also present by 18 weeks gestation and allow the efficient processing of antigen. At 23 weeks gestation a foetus has T and B lymphocytes, macrophages, monocytes and polymorphonuclear cells. However the ability of lymphocytes to produce cytokines is impaired in comparison to adults. Phagocytes from preterm neonates show normal activity when suspended in normal adult serum. However, neonatal serum is deficient in immunoglobulin and complement so adherence and chemotaxis is reduced and phagocytosis is limited. During pregnancy, IgG is transported across the placenta and at 40 weeks gestation neonate's IgG levels are higher than maternal levels. However neonates have no circulating IgA or IgM and the IgG has a half-life of approximately three weeks (Cole, 1998; Johnston, 1998; Cloherty and Stark, 1998; Rennie and Robertson, 1999). A retrospective study of forty-six infants indicated that meningitis is more prevalent in infants of normal gestational age and birth weight with onset of disease usually occurring within the first week following birth (Bowen and Braden, 2006). In contrast low birth weight, premature infants were more likely to develop bacteraemia with no progression to CNS disease and the age of onset was usually over one month. A higher mortality rate and adverse sequelae in survivors were associated with meningitis cases.

Further work is needed on the mechanisms of *E. sakazakii* infection and reliable *in vitro* and *in vivo* model assays need to be developed to reflect factors relevant to actual occurrence of disease in neonates. Recently Mange *et al.* (2006) have shown that attachment of *E. sakazakii* to HBMEC cells was affected by the addition of blood. A determination of the optimal MOI and incubation time for *E. sakazakii* strains would improve the assays used herein. The high inoculum (10^8) was used as this should saturate the invasion process for the given incubation period (3h). However this inoculum size and incubation time allows bacterial fermentation of the glucose in the

culture medium which may reduce the pH and result in detrimental effects on the mammalian cells. The pH before and after incubation should have been monitored. Friis *et al.* (2005) describe an Invasion Success Model which allows statistical comparison of the ability of bacterial strains to invade epithelial cells *in vitro*. This would be an improved approach to comparing attachment and invasion of *E. sakazakii* strains and other Enterobacteriaceae. Struve *et al.* (2003) found a lack of correlation between *in vivo* and *in vitro* studies when looking at *Kleb. pneumoniae* virulence. Non-capsulated mutants were able to adhere to and invade epithelial cells *in vitro*, however this was not associated with higher infectivity in an animal model. Although *in vitro* studies offer a simple means of investigating bacterial-host cell interactions, care must be taken to ensure the results reflect real infectivity. Evaluating the *in vitro* assay results of strains isolated from normally sterile body sites would give an indication of the effectiveness of a particular series of assays at predicting virulence.

CHAPTER 6 – GENERAL DISCUSSION AND FUTURE WORK

An awareness of the risks associated with powdered infant formula in respect to the possibility of *E. sakazakii* infection has grown tremendously over the past four years. In response to a request from the Codex Committee on Food Hygiene to provide scientific advice for the revision of the Recommended International Code of Hygienic Practice for Goods and Infants and Children, the World Health Organization and Food and Agriculture Organization of the United Nations held a joint conference on infant formula safety in February, 2004. An extensive list of recommendations was issued including informing caregivers that powdered formula is not sterile therefore appropriate hygiene practices should be followed, feeding high-risk infants sterile formula if they cannot breast-feed, encouraging manufacturers to develop a range of affordable sterile formula options, setting standards for *E. sakazakii* as well as *Enterobacteriaceae* in infant formula (World Health Organization; 2004). Governments have issued guidelines to medical establishments on improved hygiene practices to control the risk of outbreaks occurring amongst vulnerable infants in neonatal intensive care units (American Dietetic Association; 2004). The use of powdered breast milk substitutes is only recommended when no sterile liquid formula product is available or appropriate for the needs of the infant. Stringent temperature control of stored feeds and restriction of enteral nutrition 'hang-times' to ≤ 4h have been recommended. Manufacturers of infant formula products have investigated improved cultural and molecular methods for screening products prior to release and for monitoring contamination in production environments (Guillaume-Gentil *et al.* 2005; ISO DTS 22964, 2005). Rehydration of infant formula at≥70°C has been suggested as a practical way to reduce risk of *E. sakazakii* infection (Edelson-Mammel and Buchanan, 2004). However the increased risk of scalding injuries and the effect of high temperature rehydration on the nutritive value of formula needs thorough investigation. The 1980 Infant Formula Act, revised in 1986, regulates the production and distribution of infant formula and sets minimum standards for nutrients (Baker, 2002).

Due to implementation of control measures it is possible that the occurrence of outbreaks within neonatal units will become even rarer. However the risk factors associated with *E. sakazakii* contamination of infant formula warrant further research.

It is interesting that as yet there is no established link for infant formula as the vehicle of infection for other organisms, such as *Enterobacter cloacae*, a recognized cause of neonate nosocomial infection, as well as other yellow-pigmented desiccation resistant Enterobacteriaceae, which are isolated as frequently as *E. sakazakii* from powdered infant food products and factory environments. The presence of endogenous *E.*

sakazakii in both hospital and home environments should also be investigated to identify other possible niches that may act as reservoirs for infection.

Future work on the organism should also include a thorough investigation of the relationships between the *E. sakazakii* genomic groups 1-4 to determine their status as subspecies or separate species. DNA-DNA hybridization is the current acknowledged standard for determining species relationships; however more accessible techniques such as f-AFLP and multilocus sequence analysis (MLSA) are complementary and may have the potential to replace hybridization methods in the future. It is important to the protection of infant health that any species delineation does not result in the exclusion of potential pathogenic strains from the scope of legislation or control protocols. A clear definition of *E. sakazakii* in terms of both genotype and phenotype should prevent discrepancies which may occur if limited biochemical identification, such as API20E, is performed on presumptive isolates.

The unidentified organisms referred to as genomic groups 5 and 6 in this study are in the process of being designated as new species by researchers at the University of Zurich, Switzerland. Although these species are yellow-pigmented, desiccation resistant and positive for metabolism of X-α-glucoside, they have distinct biochemical differences to *E. sakazakii*. They display positive methyl red reactions and are negative for arginine dihydrolase and ornithine decarboxylase, with most also being negative for sucrose and raffinose fermentation. Therefore these false presumptive positives are unlikely to be mistaken as true *E. sakazakii* at the confirmation stage. The differences in phenotype coupled with the separate clustering by partial 16S rDNA analysis indicates these species are distinct from *E. sakazakii* and should not be considered as belonging to the *E. sakazakii* clade. However, these species remain a challenge to method design as their occurrence as false presumptive positives increases the risk of true positive *E. sakazakii* colonies being overlooked, as well as increasing costs and workload associated with current *E. sakazakii* isolation protocols.

The work reported herein has contributed directly to the development of methods for the detection of *E. sakazakii* with the chromogenic agar currently being commercially available as Chromogenic *Enterobacter sakazakii* agar, DFI formulation CM1055, Oxoid Ltd. However further work is needed on media development with no currently proposed method being either the most sensitive or specific for isolation of *E. sakazakii*. To improve DFI, the amount and type of protein in the base medium and the concentration of the chromogen should be optimized to promote the formation of pigmented colonies for target strains with weak X-α-glucosidase activity. Also the concentration of sodium deoxycholate could be reduced to improve growth of bile salt sensitive strains. The prevalence in both clinical and food settings of *E. sakazakii* strains sensitive to commonly used selective agents is of interest. This information would allow assessment of the relative risk of these organisms to infant health and

contribute to determination of criteria for method performance. The properties that confer this sensitivity should also be investigated to elucidate possible strategies for strain recovery, and the impact of these sensitive strains on other methods directed at Enterobacteriaceae detection and enumeration should be considered.

Since the comparisons conducted in this work further media have been developed including both an enrichment broth and chromogenic agar from R&F Laboratories, Downers Grove, Illinois, USA. The enrichment broth is of a similar composition to ESE broth described herein, although the carbohydrate concentration is reduced and sodium pyruvate incorporated to aid recovery of stressed cells. It is unlikely this broth has sufficient selectivity to be a viable commercial enrichment media. The chromogenic agar, *Enterobacter sakazakii* plating medium (ESPM), addresses the possibility of α-glucosidase negative *E. sakazakii* strains by incorporating a second chromogen, X-β-cellobioside into the plating medium along with X-α-glucoside. To reduce the number of false positives that could occur with this second chromogen, carbohydrates are also incorporated that are not fermented by *E. sakazakii* allowing the differentiation of the target organisms due to localized changes in pH as the non-target colonies metabolize the sugars. Unfortunately it is likely that this medium has been designed based on incorrectly identified strains similar to those encountered in this study, which were determined to be other *Enterobacter* species by molecular methods despite identification as *E. sakazakii* by the API 20E test gallery. These strains, along with the X-α-glucosidase positive environmental strains such as those belonging to unidentified genomic clusters 5 and 6, produce presumptive positive colonies on ESPM. However a second medium, *Enterobacter sakazakii* screening medium (ESSM), can be used prior to confirmation by biochemical or molecular methods to effectively reduce the number of false presumptive positives from the ESPM medium. The screening plate is based on sucrose and melibiose fermentation. Melibiose is a di-saccharide derivative of raffinose and appears less specific for *E. sakazakii* than the tri-saccharide, also it is necessary to observe the melibiose fermentation at 6h as well as at 24h as alkination can occur which would lead to false negative results at 24h. This screening method does not however screen out the potentially falsely identified *Enterobacter* strains and this necessitates the use of a more stringent confirmation method than API20E in combination with this media.

An ad' hoc working group has been established by the International Standards Organization to propose a horizontal method for detection of *E. sakazakii*. The principal object of the group is to develop a method for finished products prior to release. However factory environmental samples also need to be monitored and included in the scope. It is possible that separate methods may be necessary to account for the difference in background flora, level of contamination and relative health risk of environmental samples in comparison to product samples. In environmental samples,

with high levels of microbial contamination and diverse flora, the specificity of the method gains increased importance to ensure that target strains are not out-competed in the initial enrichments.

This study included a basic investigation of possible factors associating infant formula with risk of disease in infants. *E. sakazakii* and *Pantoea* sp. exhibited the greatest resistance to desiccation. Breeuwer *et al.* (2004) studied the genetic basis for survival of *E. sakazakii* under dry conditions and found that the response of *E. sakazakii* to dry stress involved expression of functionally different groups of genes from across the genome. They also tested the osmotic resistance of *E. sakazakii* in brain heart infusion broth supplemented with 75% sorbitol and observed it to be more resistant than *E. agglomerans*, *Esch. coli* and *Salmonella* sv. Over 14 days, *E. sakazakii* strains decreased 3-4 log compared to a decreased of 6 log by other Enterobacteriaceae with stationary phase cells exhibiting greater resistant than exponential phase cells.

The survey of Enterobacteriaceae in milk powders and infant foods indicated that *Pantoea* sp., *E. sakazakii* and *E. cloacae* are the most prevalent. *Klebsiella* sp. and *Escherichia* sp. were also isolated. *Esch. coli*, *Klebsiella* spp. (*Kleb. pneumoniae* and *Kleb. oxytoca*) and *E. cloacae* have been associated with neonatal infections including bacteraemia and septicaemia as well as necrotizing enterocoloitis (NEC), which is the most common important gastrointestinal illness in the newborn (Beaugerie *et al.* 2003; Yalaz *et al.* 2006, Afroza 2006). The role of *E. sakazakii* in NEC is unclear however parallels could be drawn with *Klebsiella* species which are a major factor in this condition. It is possible that the production of exopolysaccharide may disrupt effective peristalsis and the fermentation of lactose contribute to the build up of gas exacerbating ischemia and abdominal perforations. In NEC it is likely that factors such as attachment and invasion play a less important role with necrosis of intestinal cells allowing invasion of underlying tissues to occur. Westra-Meijer *et al.* (1983) reported that colonization with *Klebsiella* spp. increased the risk of NEC, while Chan *et al.* (1994) found *Enterobacter* spp were the most common organisms in neonates with NEC. Outbreaks due to multi-resistant *Klebsiella* strains have been reported (Blahova *et al.* 1998, Ben-Hamouda *et al.* 2003). The capsules of *Klebsiella* spp., thick bundles of fibrillous structures covering the bacterial surface, contribute to virulence by protecting the bacterium from phagocytosis by polymorphonuclear granulocytes and preventing killing of the bacteria by bactericidal serum factors (Highsmith and Jarvis, 1985; Williams *et al.* 1983; Podschun and Ullmann, 1992). Apart from their antiphagocytic function, *Klebsiella* capsule polysaccharides have been reported to inhibit the differentiation and functional capacity of macrophages *in vitro* (Yokochi *et al.* 1977).

The principal reservoirs for transmission of *Klebsiella* and *Enterobacter* spp. to neonates are the gastrointestinal tract and the hands of hospital personnel

(Montgomerie, 1979). The prevalence of *Klebsiella* spp. and *E. cloacae* within hospital settings and regular horizontal and vertical transmission during birth and postnatal care probably means that sporadic low level contamination of infant formula with these organisms is insignificant to the overall incidence of neonatal disease. There are no reports of infant feeds being included in epidemiological investigations of *Klebsiella* related neonatal disease, however powdered infant formula is a potential source of neonatal colonization by *Klebsiella* and other genera of Enterobacteriaceae and may therefore be a risk factor to vulnerable infants. Infant food has been implicated as the source in a nosocomial outbreak due to *Citrobacter freundii* contamination (Thurm and Gericke 1994). However it is not known if the contamination of the formula was intrinsic or due to poor preparation hygiene . It is likely that some outbreaks of *E. sakazakii* infection are due to poor hygienic practices (storage at ambient temperatures) allowing growth of low numbers of intrinsic contaminating cells, which possibly also facilitates formation of biofilms as a secondary reservoir on formula preparation equipment (Simmons *et al.* 1989, Clark *et al.* 1990; Mathus-Vliegen *et al.* 2000). Pagotto *et al.* (2003) used a suckling mouse model and determined that *E. sakazakii* strains were lethal to mice at high oral doses of 10^8 cfu indicating that a minimum lethal dose in neonates would possibly require an unusually high number of viable cells such as might occur in infant formula stored at ambient temperature for extended periods. Other routes of potential contamination, such as vertical and horizontal transfer during birth and during cohort nursing respectively, or contamination of prepared feed from personnel, are possible but as yet epidemiologically unproven. Cases of *E. sakazakii* infection have occurred in infants delivered by Caesarean section precluding vertical transmission in these instances. In some reported outbreaks in neonatal intensive care units (NICU), the numbers of colonized individuals that do not show symptoms of disease outweighs the number of infants that suffer severe morbidity so the probability of infection is less than the probability of ingestion (Forsythe, 2004). In one case in Iceland (Biering *et al.* 1989) only one of two full-term healthy neonate twins were infected by *E. sakazakii* further complicating hypotheses about the relationship between host factors and bacterial factors in the onset of disease.

Further work is needed to clarify the taxonomy of *E. sakazakii* in relation to *Enterobacter* spp. and other genera of the Enterobacteriaceae. A clearer phenotypic and genotypic description would help in the development and evaluation of detection and identification methods. Methods that are as accurate and efficient as possible need to be developed to protect the health of neonates and also to facilitate regular monitoring of the incidence of *E. sakazakii* in food products and other environments. Research is needed into the mechanisms which lead to *E. sakazakii* pathogenicity in contrast to the innate background flora found in infant formula.

CONCLUSIONS

- This work reports on the taxonomic relationships of *E. sakazakii* isolates from diverse clinical, food and environmental sources. Four genomic groups were identified using both partial 16S rDNA and hsp60 sequence analysis. These four groups could be distinguished using biochemical traits. Further work, including DNA-DNA hybridisation is needed to determine if these groups represent subspecies of *E. sakazakii* or separate novel species.
- Identification methods were compared and the most sensitive and specific commercial identification method was Biolog GN2. False positive and false negative identifications can occur with the more commonly used API20E test gallery.
- A novel chromogenic medium was developed to improve the efficiency of *E. sakazakii* isolation methods and is commercially available as Chromogenic *Enterobacter sakazakii* medium, CM1055, Oxoid Ltd.
- A novel enrichment medium to improve the recovery of *E. sakazakii* and reduce false presumptive positive colonies on the chromogenic agar was investigated. However, despite excellent sensitivity, the resulting medium was not specific enough to the target organism to be considered as a viable commercial enrichment medium.
- A survey of 486 food samples including 82 samples of infant formula determined the incidence of *E. sakazakii* in infant formula to be 2.4% for this dataset. *E. sakazakii* was also isolated from other dried food products, including dried infant foods, cheese powder and herbs and spices.
- A preliminary investigation into risk factors associated with *E. sakazakii* contamination of infant formula showed that *E. sakazakii* is able to persist in a desiccated state for over 6 months, is able to form biofilms on infant feeding equipment, can attach and invade human epithelial (CaC0-2) cells and can survive in human serum. Some strains produce exopolysaccharide capsules which enhance biofilm formation and may contribute to evasion of host immune defences.

CHAPTER 7 - REFERENCES

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APPENDIX 1 – PUBLICATIONS

Refereed Papers

Iversen C, Forsythe SJ. (2003) Risk profile of *Enterobacter sakazakii*, an emergent pathogen associated with infant milk formula. *Trends in Food Science and Technology* **14**:443-454.

Iversen C, Lane M, Forsythe SJ. (2004) The growth profile, thermotolerance and biofilm formation of *Enterobacter sakazakii* grown in infant formula milk. *Letters in Applied Microbiology* **38** 378-382.

Iversen C, Druggan P, Forsythe SJ. (2004) A Selective Differential Medium for *Enterobacter sakazakii*, a preliminary study. *International Journal of Food Microbiology* **96**:133-139.

Iversen C, Forsythe SJ. (2004) Isolation of *Enterobacter sakazakii* and other Enterobacteriaceae from powdered infant formula milk and related products. *Food Microbiology* 21 (6): 771-777.

Iversen C, Waddington M, On SL, Forsythe S. (2004) Identification and phylogeny of *Enterobacter sakazakii* relative to *Enterobacter* and *Citrobacter* species. *Journal of Clinical Microbiology* **42**:5368-5370

Iversen C, Lancashire L, Waddington M, Forsythe S, Ball G. (2006) Identification of *Enterobacter sakazakii* from closely related species: The use of Artificial Neural Networks in the analysis of biochemical and 16S rDNA data. *BMC Microbiology* **6**:28

Iversen C, Forsythe S. Comparison of media for the isolation of *Enterobacter sakazakii. Applied and Environmental Microbiology* in press January 2007

Iversen C, Waddington M, Farmer JJ III, Forsythe S: (2006) The biochemical differentiation of *Enterobacter sakazakii* genotypes. *BMC Microbiology* **6**:94

Other articles

Iversen C. *Enterobacter sakazakii*; an emergent pathogen in infant formula milk. *Microbiologist* September 2004.

APPENDIX 2 –POSTERS

103rd ASM General Conference, Washington D.C., USA (2003)

C. Iversen, A. J. Hargreaves and S. J. Forsythe. Growth rates and D-values of *Enterobacter sakazakii* in five suspending media.

104th ASM General Conference, New Orleans, LA, USA, May 2004

C Iversen, H Dale, P Druggan, M Waddington, S Forsythe. The identification of *Enterobacter sakazakii* using partial 16S rDNA sequencing and biochemical techniques.

C Iversen, N Lazar Adler, A Hargreaves, S Forsythe. Virulence factors of *Enterobacter sakazakii.*

C Iversen, J Caubilla-Barron, S Forsythe. Isolation of *Enterobacter sakazakii*, Enterobacteriaceae and other microbial contaminants from powdered infant formula milk.

P Druggan, S Forsythe, C Iversen. *The Druggan-Forsythe-Iversen agar (DFI), a chromogenic medium for the detection of Enterobacter sakazakii.* J. Caubilla-Barron, C. Iversen and S.J. Forsythe. The Desiccation Survival of *Enterobacter sakazakii* and Related Enterobacteriaceae.

105th ASM General Conference, Atlanta, GA, USA, June 2005

Carol Iversen, Lee Lancashire, Graham Ball and Stephen Forsythe. Identification of *Enterobacter sakazakii:* the Use of an Artificial Neural Network to Determine Biochemical Markers for 16S rDNA Cluster Groups.

Carol Iversen, Patrick Druggan and Stephen Forsythe. Comparison of Methods for the Isolation of *Enterobacter sakazakii.*

SGM Herriot Watt University, April 2005.

Carol Iversen; Jong Hyun Park; Juncal Caubilla Barron; Alan Hargreaves; Stephen Forsythe. Isolation, characterisation and virulence of *Enterobacter sakazakii* from Korean infant foods. .

ELSEVIER

Trends in Food Science & Technology 14 (2003) 443–454

TRENDS IN FOOD SCIENCE & TECHNOLOGY

Viewpoint

Risk profile of *Enterobacter sakazakii*, an emergent pathogen associated with infant milk formula

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Enterobacter sakazakii infections in neonates cause bacteraemia, necrotizing enterocolitis (NEC) and infant meningitis. Where the source of an outbreak was traced to infant formula milk powder the levels of *Enterobacteriacae* were below the statutary limit. In order to determine whether a full risk assessment of *E. sakazakii* in IFM is required, a risk profile is necessary summarizing our knowledge to date. The risk profile presented here includes hazard identification, exposure assessment and hazard characterisation which are parts of a microbiological risk assessment (MRA), as well as risk management. In addition current detection methods are described.

Hazard identification

Organism description

E. sakazakii is an occasional contaminant of powdered IFM that can cause a rare, but life-threatening form of neonatal meningitis, bacteraemia, necrotizing enterocolitis (NEC) and necrotizing meningoencephalitis after ingestion (Table 1, Muytjens & Kollee, 1990). Although recently brought to manufacturers and the public's attention through a number of outbreaks and product recalls, it was first associated with neonatal deaths in 1958 by Urmenyi and Franklin (1961). Recently the International Commission for Microbiological Specifications for Foods (2002) ranked the organism as 'Severe hazard for restricted populations, life threatening or substantial chronic sequelae or long duration'. Subsequently it has the same ranking as more familiar food and waterborne pathogens such as *Listeria monocytogenes*, *Clostridium botulinum* types A and B and *Cryptosporidium parvum*.

E. sakazakii is a motile, non-sporeforming, Gram-negative facultative anaerobe. It was known as 'yellow pigmented *Enterobacter cloacae*' until 1980 when it was designated as a new species by Farmer, Asbury, Hickman and Brenner in honour of the Japanese bacteriologist Riichi Sakazaki. They reported that DNA–DNA hybridization studies found no clear generic assignment for *E. sakazakii* as it was 53–54% related to *Enterobacter* and *Citrobacter* species. A comparison of the type strains of these two genera showed that *E. sakazakii* was 41% related to *C. freundii* and 51% related to *E. cloacae*. Subsequently, since it was also phenotypically closer to *E. cloacae*, Farmer, Asbury, Hickman and Brenner (1980) assigned the organism to the *Enterobacter* genus. In general *Enterobacter* organisms are responsible for around 50% of nosocomial infections, mostly in immunocompromised patients (Leclerc, Mossel, Edberg, & Struijk, 2001).

Growth and death characteristics

The organism grows on media used to isolate enteric organisms such as MacConkey, eosin methylene blue and deoxycholate agar. On agar plates it may form two colony types (glossy and matt) depending upon media and strain. Growth on tryptone soya agar at 25°C produces a non-diffusible, yellow pigment. It produces a delayed extracellular DNase reaction on toluidine blue agar (36°C, 7days). It is α-glucosidase positive that can be detected using 4-nitrophenyl-α-D-glucopyranoside after 4 h at 36°C. The organism produces D-lactic acid and is mucate negative. Most isolates do not ferment sorbitol. It can grow over a wide temperature range (6–47°C; Fig. 1). At room temperature (21°C) the

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Table 1. *E. sakazakii* infections in neonates and infants

Year of outbreak	Number of neonates and infants	Age	Number of deaths	Symptoms	Source	References
1958	2	5 and 10 days	2	Meningitis	Unknown	Urmenyi and White-Frankin (1961)
1958	1	4 days		Meningitis	Unknown	Jöker, Norholm and Siboni (1965)
1958	1	7 days	0	Bacteremia	Unknown	Monroe and Tift (1979)
1958				Meningitis + sepsis		Adamson and Rogers (1981)
1958	1	5 weeks	0	Meningitis	Unknown	Kleiman, Allen, Neal and Reynolds (1981)
1977–1981	8		6	Meningitis	IFM	Muytjens *et al.* (1983), Smeets *et al.* (1998)
1977–1981	NS	NS	NS	NS	NS	Aldová *et al.* (1983), Postupa and Aldová (1984)
1984	11	2 days–2 months	5	Colonisation	Unknown	Arseni, Malamou-Ladas, Koutsia, Xanthou, and Trikka (1987)
1984	1	21 days	0	Meningitis	Unknown	Naqvi, Maxwell and Dunkle (1985)
1984	2	8 days and 4 weeks	0	Meningitis	Unknown	Willis and Robinson (1988)
1986–1987	3	5 days	2	Meningitis	IFM	Biering *et al.* (1989); Clark *et al.* (1990)
1986–1987	4	NS	NS	Wound exudates, appendicitis, conjunctivitis	NS	Reina Parras, Gil, Salva, and Alomar (1989)
1981–1988	2	NS	2	Meningitis	Unknown	Lecour, Seara and Cordeiro (1989)
1988	4	28–34.5 weeks	0	Sepsis/bloody diarrhoea	IFM, blender	Simmons *et al.* (1989); Clark *et al.* (1990)
1988	1	6 months	0	Bacteremia	IFM	Noriega *et al.* (1990)
1988	1	2 days	0	Meningitis	NS	Gallagher and Ball (1991)
Five year period	NS	NS	NS	NEC	Unknown	Chan, Saing, Yung, Yeung, and Tsoi (1994)
Five year period	1	NS	NS	Meningitis	Unknown	Reis, Harms, and Scharf (1994)
Five year period	1	20 months	0	Wound infection	Unknown	Tekkok, Baeesa, Higgins, and Ventureyra (1996)
Five year period	1	6 days	0	Meningitis	NS	Burdette and Santos (2000)
1997	1	7 days	0	Meningitis	Unknown	Weekly Report (1997)
1998	12	4 days 2 months	0	Enterocolitis	IFM	Van Acker *et al.* (2001)
1999–2000	NS	NS	NS	NS	IFM & blender	Block *et al.* (2002)
1999–2000	2	3 and 4 days	0	Bacteraemia, meningitis	IFM & Blender	Bar-Oz *et al.* (2001)
1999–2000	1	3 years	0	Bacteraemia	NS	Lai (2001)
2001	11	11 days	1	Meningitis, enterocolitis	IFM	Himelright *et al.* (2002)
2002	1	4 days	1	Meningitis	Unknown	Safetylist (Web site)

IFM = Infant formula milk powder; NS = Not specified in paper.

organism has a doubling time of about 75 min in reconstituted IFM (Fig. 1). Skladal, Mascini, Salvadori, and Zannoni (1993) found UHT milk cartons inoculated with 10–15 *E. sakazakii* cells/500 ml and incubated at 30°C supported good bacterial growth, with acidification of the milk due to D-lactate production. The doubling time at low temperatures is approximately 10 h in IFM at 10°C and therefore may grow, albeit slowly, under refrigerated conditions (Fig. 1). Limits of growth with respect to water activity and pH are unknown.

Decimal reductions times and *z*-values for the organism in IFM were determined by Nazarowec-White and Farber (1997b, 1999). The D_{52} value was 54.8 min and D_{60} was 2.5 min. The data was extrapolated to 72°C to give a value indicating that the organism was very thermotolerant (*z* value 5.82°C) and hence a possible explanation for its survival following dehydration during

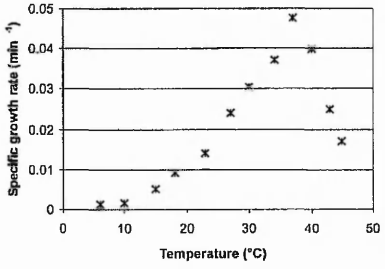

Fig. 1. Growth rate of *E. sakazakii* NCTC11467 in infant formula milk according to temperature (Iversen *et al.*, 2003).

IFM manufacture. However subsequent work (Nazarowec-White & Faber, 1999; Iversen, Hargreaves, & Forsythe, 2003) have clarified that the organism is less thermotolerant than *L. monocytogenes*.

E. sakazakii produces a novel heteropolysaccharide comprising 29–32% glucuronic acid, 23–30% D-glucose, 19–24% D-galactose, 13–22% D-fucose and 0–8% D-mannose (Harris & Oriel, 1989). Optimal production is under nitrogen-limited growth conditions (C/N ratio 20:1) (Scheepe-Leberkühne & Wagner, 1986).

The capsule could be involved in the organism's ability to survive the long IFM shelf-life (24 months). It may also enable the organism to attach to surfaces and form a biofilm that is more resistant to cleaning and disinfectant agents. The unique biophysical properties of the capsule have lead to patents being filed for the exploitation of *E. sakazakii* capsule as a thickening agent in foods to replace xanthan gum (Harris & Oriel, 1989).

Isolation and identification methods

The FDA (2002) has a recommended method for the isolation and enumeration of *E. sakazakii* from dehydrated powdered infant formula that takes 5 days (Fig. 2). This method is similar to that first used by Muytjens, Roelofs, and Jaspar (1988) except the FDA resuspend the IFM in water rather than buffered peptone water. It is based on the most probable number (MPN) approach, using a total of 333 g of product (3×100 g, 3×10 g, 3× 1 g), and follows the conventional steps of pre-enrichment, enrichment in EE broth and isolation using Violet Red Bile Glucose (VRBG) selective media. This protocol is however only selective for Enterobacteriacae and is not specific for *E. sakazakii*. Therefore 5 presumptive *E. sakazakii* colonies are chosen from VRGA and subcultured on TSA at 25°C for 48–72 h for pigment production, followed by confirmation using the API 20E biochemical identification system and the oxidase test. The reason for stipulating 36°C rather than 37°C is uncertain, though it definitely is not due to the optimum temperature for growth (see later section). Heuvelink, Kodde, Zwartkruis-Nahuis, and de Boer (2001) used a presence/absence test instead of the MPN approach by analysing 25 g sample size pre-enriched in 225 ml BPW. To date none of these methods have been validated. Nevertheless it would seem reasonable to use a detetion limit <1 cell in 25 g IFM akin to Salmonella detection in milk powder (Mansfield and Forsythe, 2000).

Farmer *et al.* (1980) found *E. sakazakii* differed from *E. cloacae* as it is D-sorbitol negative, gives a delayed

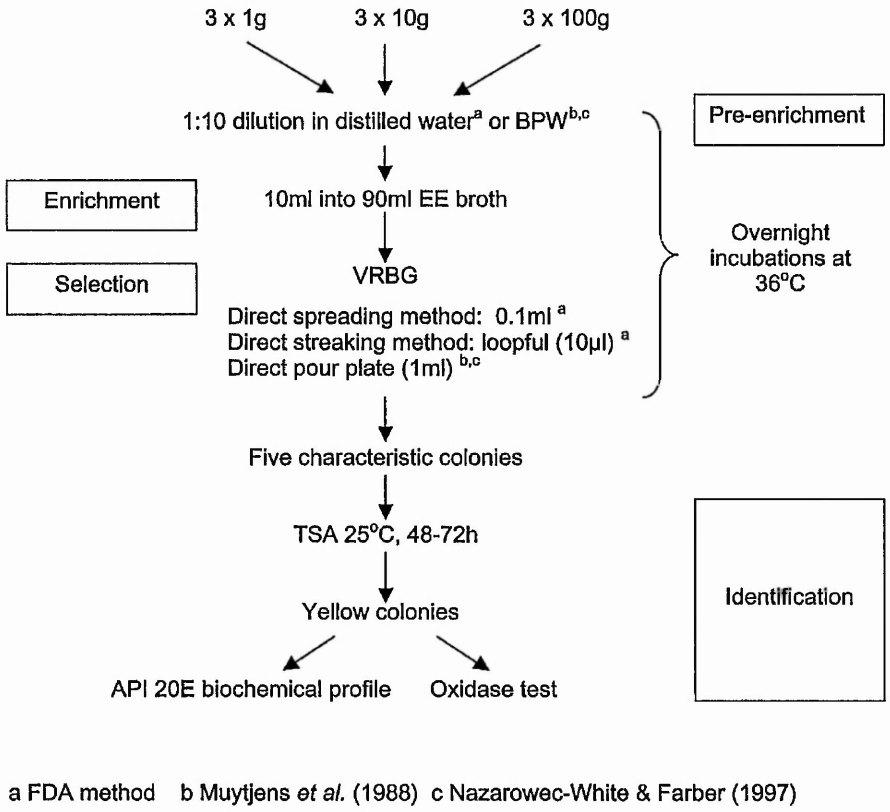

Fig. 2. Quantitative *E. sakazakii* isolation procedure. (a) FDA method, (b) Muytjens et al. (1988); (c) Nazarowec-White and Farber (1997a,b,c).

positive DNase test and produces a yellow (non-diffusible) pigment at 25°C. It should be noted that recently sorbitol-fermenting *E. sakazakii* have been isolated (Heuvelink *et al.*, 2001). Mutyjens, van der Ros-van de Repe, and van Druten (1984) found α-glucosidase activity in all 129 *E. sakazakii* strains tested but not in 97 other *Enterobacter* strains of *E. aerogenes*, *E. cloacae* and *E. agglomerans*. *E. sakazakii* also uniquely lacked phosphoamidase. Aldová, Hausner, and Postupa (1983) found 97.3% of *E. sakazakii* isolates contained Tween esterase activity. Six strains isolated from dried milks produced Tween 80 esterase after 7 days at 25 and 37°C which suggested that this enzyme could be used to distinguish *E. sakazakii* from *E. cloacae* (which is esterase negative). Inositol positive strains (approx. 70%) decrease the pH of test medium, whereas inositol negative strains make the medium alkaline. Additionally some strains of *Enterobacteriaceae* including some strains of *E. sakazakii* decolourise acid-base indicators.

Typing

The initial investigation by Farmer *et al.* (1980) found 15 biogroups of *E. sakazakii* with the wild type biogroup 1 being the most common. It was suggested that further investigation may lead to *E. sakazakii* being reorganised into a new genus with biogroups 1–14 being strains of one species and biogroup 15 being a separate species (indole, malonate and dulcitol positive, α-methyl glucosidase negative) within the genus. Goullet and Picard (1986) reported that the high degree of enzyme polymorphism in *E. sakazakii* enabled the differentiation of different strains. Clark, Hill, O'Hara, Steingrimsson, and Cooksey (1990) found that plasmid analysis, ribotyping with HindIII, chromosomal restriction endonuclease analysis and multilocus enzyme electrophoresis were equally effect as epidemiological typing methods. Nazarowec-White and Farber (1999) found RAPD and PGFE to be the most effective typing methods; food and clinical isolates with the same ribotypes had different PFGE profiles with XbaI and SpeI indicating they were different strains. These isolates were also distinguishable by RAPD (using the primer 5′-CGC GTG CCA G-3′). Clementino, de Filippis, Nascimento, Branquinho, Rocha, and Martins (2001) found tDNA-PCR resulted in profiles of *E. sakazakii* that were distinct from *E. cloacae* and *E. aerogenes*.

Environmental and food sources

Due to neonate infections *E. sakazakii* is perceived as associated with infant formula and milk powder. However it has been isolated from a diverse range of environments and foods (Table 2). Since the organism is not part of the normal animal and human gut flora it is probable that soil, water and vegetables are the principal sources of contaminated food. In addition rats and flies may be additional sources of contamination. Despite such widespread occurrence of the organism (Table 2), Mutyjens and Kollee (1990) did not isolate the organism from raw cow's milk, cattle, rodents, grain, bird dung, domestic animals, surface water, soil, mud or rotting wood.

The organism has been isolated from a range of foods including cheese, fermented bread, tofu, sour tea, cured meats, minced beef and sausage meat. *E. sakazakii* has also been found in Khamir bread due to the organism being part of the sorghum seed surface flora (Gassem, 1999). The organism has also been isolated from rice seeds (Cottyn, Regalado, Lanoot, de Cleene, Mew, & Swings, 2001). It is plausible that the organism occurs in a larger range of food ingredients, however these have not been studied in detail to date. It should be recognised that the efficiency of current isolation methods is unproven and hence studies to date probably underestimate the organism's prevalence and concentration in food.

E. sakazakii can be isolated from processing plant environments and contaminated UHT milk (Skladal *et al.*, 1993). DuPont Qualicon describe on their web site the use of the RiboPrinter (Microbial Characterisation System) to trace *E. sakazakii* contamination within three European factories producing IFM.

Clinical sources

E. sakazakii has been isolated from a wide range of clinical sources including cerebrospinal fluid, blood, bone marrow, sputum, urine, inflamed appendix, intestinal and respiratory tracts, eye, ear, wounds and faeces (Table 2). It can also be isolated from the hospital environment. Unfortunately some papers such as Aldová *et al.* (1983), Al-Hadithi and Al-Edani (1995) and Nazarowec-White and Farber (1999) do not give sufficient detail to determine the age of the patient nor the clinical source. Nevertheless, Nazarowec-White and Farber (1999) studying three isolates obtained from one hospital over 11 years showed that they had indistinguishable ribotype patterns. Smeets, Voss, Muytjens, Meis, and Melchers (1998) used PFGE to confirm the epidemiological evidence that a contaminated dish brush used for cleaning bottles was the source of three cases (in 1981) and was not related to an isolate from a 1979 case. The organism has also been isolated from a doctor's stethoscope (Farmer *et al.*, 1980) and from nursery food preparation equipment such as spoons and up to 6 weeks on a blender (Bar-Oz, Preminger, Peleg, Block, & Arad, 2001; Noriega, Kotloff, Martin, & Schwalbe, 1990; Simmons, Gelfand, Haas, Metts, & Ferguson, 1989).

Infant milk formula and dried milk powders

A number of outbreaks have been traced to contaminated infant formula milk powder (Tables 1 and 2).

Table 2. Sources of *E. sakazakii*

Source	Details	References
Neonates and infants	Meningitis	Urmenyi and Franklin (1961), Jøker *et al.* (1965), Kleiman *et al.* (1981), Muytjens *et al.* (1983), Naqvi *et al.* (1985), Arseni *et al.* (1987), Willis and Robinson (1988), Biering *et al.* (1989), Lecour *et al.* (1989), Simmons *et al.* (1989), Clark *et al.* (1990), Muytjens and Kollee (1990), Noriega *et al.* (1990), Gallagher and Ball (1991), Reis *et al.* (1994), Burdette and Santos (2000), Bar-Oz *et al.* (2001), Lai (2001)
	Bacteraemia	Monroe and Tift (1979), Clark *et al.* (1990), Bar-Oz *et al.* (2001)
	Necrotizing enterocolitis	Van Acker *et al.* (2001)
	Wound exudates, appendicitis, conjunctivitis	Reina *et al.* (1989)
Adults	Bacteraemia	Jimenez and Gimenez (1982), Pribyl *et al.* (1985), Murray, Welch, and Kuhls (1990), Hawkins *et al.* (1991), Lai (2001), Dennison and Morris (2002)
Food and drink	Infant formula milk powder	Farmer *et al.* 1980, Postupa and Aldová (1984), Block *et al.* (1988), Biering *et al.* (1989), Simmons *et al.* (1989), Muytjens *et al.* (1988), Smeets *et al.* (1998), Bar-Oz *et al.* (2001), Heuvelink *et al.* (2001), Himelright *et al.* (2002),
	Preparation equipment:(blender, spoons)	Block *et al.* (1988), Clark *et al.* (1990), Smeets *et al.* (1998), Bar-Oz *et al.* 2001
	Milk powder	Postupa and Aldová (1984), Muytjens *et al.* (1988), Heuvelink *et al.* (2001)
	Water, pipelines & biofilm	Bartolucci, Pariani, Westall, Gardini, Guerzoni (1995), Al-Hadithi and Al-Edani (1995), Oliver (1997)
	Hydrothermal springs	Mosso, de la Rosa, Vivar, and Medina (1994)
	Rice seed	Cottyn *et al.* (2001)
	Beer mugs	Schindler and Metz (1990)
	Cured meat	Watanabe and Esaki (1994)
	Fermented bread	Gassem (1999)
	Lettuce	Soriano, Rico, Molto, and Manes (2001)
	Tofu	No, Park, Lee, Hwang, and Meyers (2002)
	Sour tea	Tamura, Kato, Omori, Nanba, Miyagawa, Wang, and Zhou (1995)
	Cheese, minced beef, sausage meat, vegetables	Leclercq, Wanegue, and Baylac (2002)
Environmental	Hospital air	Masaki *et al.* (2001)
	Clinical material	Tuncer and Ozsan (1988), Janicka, Kania, Ulatowska, Krusznska, and Wojda (1999)
	Flies	Kuzina *et al.* (2001)
	Rats	Gakuya, Kyule, Gathura, and Kariuki (2001)
	Soil	Neelam, Nawaz, and Riazuddin (1987)
	Rhizosphere	Emilani, Lajmanovich, and Gonzalez (2001)
	Sediment, wetlands	Espeland and Wetzel (2001)
	Crude oil	Assadi and Mathur (1991)
	Cutting fluids	Suliman, Abubakr, and Mirghani (1988)

Unlike commercially available liquid feeds, dried milk powders are not sterile. In fact the paper by Farmer *et al.* (1980) defining *E. sakazakii* species includes a strain (NCTC 8155) which was originally isolated from dried milk by Thornley (1960). Therefore *E. sakazakii* has been present in dried milk products for many decades including the period of the first meningitis case in 1958 (Table 1; Urmenyi & Franklin, 1961). In 1988, Muytjens *et al.* found 52.2% of 141 powdered infant formula samples from 35 countries were contaminated with *Enterobacteriaceae*, with 25% containing *E. agglomerans*, 21% containing *E. cloacae* and 14% containing *E. sakazakii*. In total *E. sakazakii* was cultured from unused infant formula products from 13 countries. The level of contamination ranged from 0.36 to 66.0 cfu/100 g. This is similar to the value of 8 cells/100 g estimated by Simmons *et al.* (1989) for an open can of powdered milk formula used during the time of an outbreak on an neonatal intensive care unit. Nazarowec-White and Farber (1997c) tested 120 cans of infant formula from five different companies in Canada and found 6.7% contained *E. sakazakii*. The levels of *E. sakazakii* in positive samples were frequently 0.36 cfu/100 g. Heuvelink *et al.* (2001), using a present/absence test for 25g quantities, detected *E. sakazakii* in 1/40 infant formula powders and 7 out of 170 milk powders.

Hazard characterisation

Reported infections and outbreaks associated with *E. sakazakii* are summarised in Table 1. Infection of the newborn is probably through ingestion of contaminated IFM and not through vertical transmission from the mother during birth (Mutyjens & Kollee, 1990). The first reported association of *E. sakazakii* with

contaminated IFM powder was by Muytjens, Zanen, Sonderkamp, Kollee, Wachsmuth, and Farmer (1983) in the Netherlands studying eight cases of neonatal meningitis and sepsis. *E. sakazakii* was isolated from prepared milk formula, a dish brush and a stirring spoon. These isolates were studied in more detail later by Smeets *et al.* (1998). In Iceland three cases were reported linked to milk formula contaminated with *E. sakazakii* (Biering, Karlsson, Clark, Jonsdottir, Ludvigsson, & Steingrimsson, 1989). Two groups (Simmons *et al.*, 1989; Clark *et al.*, 1990) reported on four neonates with *E. sakazakii* infections in Tennessee. Three patients had sepsis, and three had bloody diarrhaea. *E. sakazakii* was isolated from faeces from all four patients, a used can of infant formula milk and the blender (which had heavy growth of the organism). In this outbreak identical biotypes, antibiograms and plasmid profiles were obtained for patients and environmental isolates. Although the level of contamination was probably low, there was evidence of prolonged incubation in bottle heaters between 35 and 37°C before use.

More recently, van Acker, de Smet, Muyldermans, Bougatef, Naessens, and Lauwers (2001) described 12 cases of NEC in neonates that occurred in 1998. Eleven strains of *E. sakazakii* were isolated from stomach aspirate, anal swabs and blood samples and 14 strains were isolated from Alfaré milk preparations. Arbitary primed PCR (AP-PCR) was used to type all the *E. sakazakii* isolates and determine common sources of the outbreak. Three AP-PCR profiles were obtained for patient and milk isolates with the 14 milk isolates matching the profile from three patients. It was known that 4 years earlier *E. sakazakii* had been isolated from a gastrostomy tube of a neonate fed the same type of milk. This original isolate was subsequently shown to have an AP-PCR profile almost identical to the 14 milk and patient isolates. Thus demonstrating a persistent contamination problem.

US Centers for Disease Control and Prevention (Himelright *et al.*, 2002) reported an investigation into the 2001 Tennessee outbreak of *E. sakazakii* in a neonatal intensive care unit in which 10 cases were identified. The index case was a male infant (born at 33.5 weeks) who had been admitted to the neonatal intensive care unit because of premature birth weight and respiratory distress. After 11 days the baby developed symptoms of meningitis (fever, tachycardia, decreased vascular perfusions and suspected seizure activity) and despite being given intravenous antibiotics the infant died after a further 9 days. *E. sakazakii* was cultured from the cerebrospinal fluid. Following increased surveillance a further 10 cases of *E. sakazakii* colonisation were found on the neonatal unit; 2 from 'non-sterile' site with clinical deterioration. The use of infant formula milk was the only factor associating the cases. Following the cultivation of *E. sakazakii* from a batch of the same product the company (Mead Johnson Nutritionals, USA) voluntarily recalled the batch in March 2002. Further IFM recalls in the USA due to contamination with *E. sakazakii* occurred in November 2002 (1.5 millions cans, Wyeth Nutritionals) and Jan 2003 (3,030 cases of EnfaCare LIPIL, Mead Johnson Nutritionals). No outbreaks have been reported with the withdrawn batches.

Infectious dose

Although there is no epidemiological evidence for a value of the infectious dose, it is reasonable to use 1000 *E. sakazakii* cells as a first approximation. This is similar to the infectious dose of the pathogenic bacteria *Neisseria meningitidis*, *E. coli* O157 and *L. monocytogenes* 4b. The infectious dose can vary according to the organism's history (stress response factors), host state (healthy or immunocompromised) and food matrix. In the case of infant formula milk and neonates, the organism will have been stressed during spray drying and storage, the host is immunocompromised and being a liquid the milk would pass quickly through the stomach (mild acidic conditions) into the small intestines. Taking a concentration of *E. sakazakii* in contaminated IFM as ~0.36 cells/100 g (Muytjens *et al.*, 1988; Nazarowec-White & Farber, 1997a,b,c) and that a single feed requires 18 g to be reconstituted then a prepared feed (115 ml) could contain 0.0648 *E. sakazakii* cells. The growth rate of the organism (Fig. 1) can then be used to calculate the time required for the organism to multiply through 14 generations to an infectious dose (1000 cells) at different incubation temperatures (Table 3). Reconstituted IFM would need to be kept almost 9 days at 8°C compared with 17.9 h at room temperature before an infectious dose was achieved. This simplistic model assumes there is no kill of *E. sakazakii* during preparation due to the addition of hot (rather than cooled) water and no bacterial multiplication in the stomach. In addition this calculation assumes that although babies have 4–6 feeds in a 24 h period, the infectious dose is acquired in one feed and is not cumulative (Forsythe, 2002). Despite these limitations in the calculation it is evident that IFM at the normal low levels ($\leqslant$0.36 *E. sakazakii* cells/100 g) is unlikely to cause an infection unless there is gross temperature abuse or contamination via poor hygiene preparation such contaminated blender or mixing spoon. Unfortunately such poor hygienic practice has been the probable source of outbreaks (Block *et al.* 1988; Clark *et al.* 1990; Smeets *et al.* 1998).

Pathogenicity

Necrotizing enterocoloitis (NEC) is the most common important gastrointestinal illness in the newborn and can be caused by a variety of bacterial pathogens. The

Table 3. Time required for infectious dose (1000 cells) to be reached in reconsituted infant formula milk

Temperature (°C)	Doubling time (h)	Time required to reach infectious dose (14 generations)*
10	13.6	7.9 days
18	2.9	1.7 days
21	1.3	17.9 h
37	0.5	7 h

Calculations assume an average of 0.36 *E. sakazakii* cells/100 g infant formula powder and that a single feed is 18 g powder (reconstituted to 115 ml) with no kill during preparation nor any multiplication in the stomach. Lag time at 10°C was 2 h, for all other temperatures the lag time was not significant. The infectious dose is assumed to be due to 1000 *E. sakazakii* cells being ingested as a single dose and not due to cumulative exposure. However it should be recognised that babies receive 4–6 feeds in a 24 h period.

pathogenesis is associated with neonatal intestinal ischaemia, microbial colonization of the gut and excess protein substrate in the intestinal lumen. The latter being associated with oral formula feeding. It has an incidence of 2–5% in premature infants and 13% in those weighing less that 1.5 kg at birth. NEC is 10 times as common in babies fed formula milk compared with those fed breast milk (Lucas & Cole, 1990). Both meningitis and NEC due to *E. sakazakii* have high mortality rate; NEC 10–55% and meningitis 40–80%. In a study of 125 neonates with NEC *Enterobacter* spp. were the most common organisms, being isolated from 29% of patients (Chan, Saing, Yung, Yeung, & Tsoi, 1994). Van Acker *et al.* (2001) described 12 cases of NEC in neonates that occurred in 1998. In the outbreak, a total of 11 *E. sakazakii* strains were isolated from a cohort of 50 neonates from stomach aspirate, anal swabs, and blood samples.

The first reported cases of meningitis due to 'yellow-pigmented *E. cloacae*' occurred in England (Urmenyi & Franklin, 1961). Since then cases have been reported worldwide including countries such as Denmark, Iceland, Netherlands, Greece, Canada and the US (Table 1). Table 1 should not be regarded as exhaustive since the authors are aware of cases that have not been reported in the public domain. Most reports are from hospital nurseries and neonatal intensive care units. It is probable that premature infants and those with underlying medical conditions are at highest risk for developing an *E. sakazakii* infection. However it has been reported that a healthy, full-term, newborn infant in Iceland, became ill prior to hospital discharge and suffered permanent neurological sequelae as a result of an *E. sakazakii* infection (Health Canada Food Program, 2002). In 1981, Kleiman, Allen, Neal, and Reynolds and also Adamson and Rogers reported separate cases of previously healthy 5-week-old infants admitted to hospital with meningitis due to *E. sakazakii*, showing that the infection can also be acquired in the home environment. The low incidence is predictable since healthy neonates not requiring intensive care will be slightly more resistant to *E. sakazakii* infection. Mutyjens *et al.* (1983) re-evaluated *Enterobacter* strains from blood and CSF and uncovered several cases of meningitis and bacteraemia due to *E. sakazakii* infection. This suggests that the organism had been under reported. In addition to the reported 40–80% case-fatality rate for *E. sakazakii* meningitis it should be recognised that in survivors, severe neurological sequelae such as hydrocephalus, quadriplegia and retarded neural development often occur.

E. sakazakii infections have occurred in adults. Usually these are patients with underlying fatal conditions but there are no reports of meningitis. Pribyl *et al.* (1985) described a case where the organism was one of three isolated from a foot ulcer. Other cases have included urosepsis (Jimenez & Gimenez, 1982) and bacteraemia (Hawkins, Lissner, & Sanford, 1991). More recently, Lai (2001) reported four adult cases between 1995 and 1996, and Dennison and Morris (2002) reported a multiple antibiotic resistant *E. sakazakii* wound infection.

It is probable that, like the three major pathogens that cause meningitis in children <5 years (pneumococcus, *Haemophilus* and meningococcus), *E. sakazakii* has a developmental dependence on access to the central nervous system. Cases of *E. sakazakii* meningitis have been reported in children between the ages of 3 days and 4 years, with half occurring in the first week of age and almost three-quarters during the first month (Lai, 2001). Bektas, Goetze, and Speer (1990) demonstrated various abnormalities in neutrophil function from preterm neonates, which could predispose them to overwhelming bacterial infections. *E. coli*, Group B streptococci and *Listeria* also cause meningitis in neonates.

The route by which most pathogens enter the cerebral spinal fluid to produce meningitis is not yet established, though it is thought the choroids plexus is the most likely entry site and that methods of invasion include paracellular and transcellular mechanisms. Circulating microbial products such as cell wall glycopeptides, endotoxins, proteases, collagenases, and elastases have been shown to induce permeability of the blood/brain barrier.

In order to cause meningitis an organism has to colonise mucosal surfaces, translocate into the bloodstream, avoid host defence mechanisms, cross the blood/brain barrier and survive in the cerebral spinal fluid. Adegbola and Old (1983) found 2/4 *E. sakazakii* strains tested produced no haemagglutinins while the other 2

strains produced only mannose-sensitive haemagglutinins and were coated by the type-1 fimbrial antiserum of *E. cloacae* strain 035 but not that of *Klebsiella serogenes* strain 55. Nazarowec-White and Farber (1997a) and Pagotto, Nazarowec-White, Bidawid, and Farber (2003) found *E. sakazakii* pathogenic for suckling mice inoculated orally and interperitoneally and that *E. sakazakii* appears to produce an enterotoxin-like compound. Duffy *et al.* (1997) found *Enterobacter* spp. to be strongly associated with elevated levels of endotoxin (lipopolysaccharide), an indicator of NEC disease, in stool filtrates from neonates. A head CT scan is recommended early in the management of cases with *E. sakazakii* in the blood or cerebral spinal fluid. Almost all cases have abnormalities due to bacterial invasion and hydrocephalus may later develop in cases with no initial CT abnormalities (Lai, 2001). The bacterium causes cystic changes, abscesses, fluid collection, dilated ventricles and infarctions.

Antibiotic susceptibility

Farmer *et al.* (1980) found all strains were susceptible to gentamicin, kanamycin, chloramphenicol, and ampicillin; 87% or over were susceptible to nalidixic acid, streptomycin, tetracycline, and carbenicillin; 71 and 67% were susceptible to sulfadiazine and colistin respectively; only 13% were susceptible to cephalothin. All strains were resistant to penicillin; only 1 of over 100 strains tested showed multiple antibiotic resistance. Muytjens and van der Ros-van der Repe (1986) found the MICs for 90% of 195 *E. sakazakii* strains tested against 25 antibiotics were at least twofold lower than those for *E. cloacae*. There was however resistance to cephalothin and sulfamethoxazole. Willis and Robinson (1988) recommended the combined use of ampicillin and gentamicin for the treatment of *E. sakazakii* meningitis, however gentamicin may be unable to achieve adequate concentrations in the cerebral spinal fluid. Nazarowec-White and Farber (1999) found the type strain (ATCC 29544), 5/8 food and 8/9 clinical strains were only resistant to sulphisoxazole and cephalothin. The other clinical strain showed susceptibility to all agents while the other three food isolates were also resistant to chloramphenicol. Two of the chloramphenicol resistant food isolates were also resistant to tetracycline and one was also resistant to ampicillin. Kuzina, Peloquin, Vacek and Miller (2001) found *E. sakazakii* isolated from the guts of Mexican fruit flies resistant to ampicillin, cephalothin, erythromycin, novobiocin, and penicillin. Lai (2001) found all *E. sakazakii* isolates were resistant to ampicillin, cefazolin and extended spectrum penicillins, but were susceptible to the aminoglycosides and trimethoprim-sulfamethoxazole. Whereas sensitivity to 3rd generation cephalosporins and the quinolones was variable. Subsequently Lai (2001) proposed the use of carbapenems or 3rd generation cephalosporins with an aminoglycoside or trimethoprim with sulfamethoxazole. This treatment regime has improved the outcome of *E. sakazakii* meningitis though the resistance of *Enterobacter* spp. to these antibiotics is increasing (Lai, 2001). Dennison and Morris (2002) have reported an *E. sakazakii* infection that was resistant to multiple antibiotics, including ampicillin, gentamicin and cefotaxamine. In their study of the antibiotic resistance of bacteria found in dairy farm soil, Burgos and Varela (2002) found genomic DNA from *E. sakazakii* contained multiple antibiotic resistance (mar) operons.

Risk management

There are five principal control measures relevant to *E. sakazakii* in infant formula milk powder.

(1) Control initial levels in raw materials on receipt

The source of *E. sakazakii* in IFM powder is uncertain. However it is an environmental organism and hence can be present in many raw ingredients as well as milk (Table 2). This control measure can be improved in the future with the development of validated detection methods as well as greater awareness in the food industry.

(2) Reduce levels during heat treatment of raw milk and related ingredient

It has often been stated that *E. sakazakii* has a high thermal resistance in comparison to other members of the *Enterobacteriaceae* and that this may explain their high prevalence in powdered and prepared formula milk. However, this statement needs careful clarification. It is principally based on three studies reported by Kindle, Busse, Kampa, Meyer-Koenig, and Daschner (1996) and Nazarowec-White and Farber (1997b and c).

Kindle *et al.* (1996) reported that the *E. sakazakii* viable count was reduced from 10^5 to 20 cfu/ml after exposure to microwaves (85 s, 82°C in Milumil infant formula). Whereas no *E. coli*, *Ps. aeruginosa* or poliovirus survived this treatment. However closer inspection of the data shows that *E. sakazakii* was not recoverable in other infant formulas with treatments from which other organisms did survive. A comparison of *D*-values in milks for several *Enterobacteriaceae*, by Nazarowec-White and Farber (1997b), indicated that *E. sakazakii* is one of the most thermotolerant organisms of this group. For example D_{72^0C} for *E. sakazakii* was 1.3 compared with 0.22, 0.16 and 0.07 for S. Typhimurium, *E. coli* and *C. jejuni* respectively. Nazarowec-White and Farber (1997b) calculated *D*-values ranging from 54.8 min at 52°C to 2.5 min at 60°C in reconstituted infant formula for pooled strains of *E. sakazakii* (5 clinical and 5 from infant formulae). However, the *z* value was 5.82°C which is within the range reported for most non-spore

forming bacteria (4–6°C). A 4-7D kill log reduction of micro-organisms is required for process control in many thermal or pasteurisation regimes, with a $D_{60}{}_{^\circ C}$ value of 2.5 min, heat treatment of 60°C for 17.5 min would be required to obtain a 7 log reduction. A minimum HTST pasteurisation of 15 s at 71.7°C would result in >11 D kill of *E. sakazakii* in dried infant formula therefore the organism should not survive the pasteurisation process. Nazarowec-White, McKellar, and Piyasena (1999) clarified the heat-resistance by constructing a predictive model of *E. sakazakii* inactivation following HTST treatment of milk and showing that *L. monocytogenes* was more heat resistant.

(3) Prevent an increase in levels by avoiding post-processing contamination

To simulate breast milk, cow's milk is modified to reduce protein and mineral content, increase the amount of whey protein, increase the carbohydrate content and increase the Ca/P ratio. The fat is modified and vitamins are added. In the 'dry procedure' for producing IFM, all ingredients are blended in the dry form. Skimmed milk is pasteurised and then evaporated before fat, whey, vitamins, emulsifiers, and stabilisers are added and blended. The mixture is pasteurised at 110°C for 60 s and then spray dried. This method is more prone to bacterial contamination and the ingredients are harder to mix uniformly and can separate on vibration of the cans. In the 'wet procedure' the liquid skimmed milk and premix of skimmed milk and fat are treated at 80 and 82°C respectively for 20 s; the total mixture is heated at 107–110°C for 60 s, concentrated using a falling-film evaporator, and heat treated again at 80°C before finally being spray dried. These 'wet' and 'dry' methods can be combined with the more soluble components added to milk before drying. Pathogens can gain access from the environment, the addition of ingredients at the powder stage and from post-processing contamination. The drying and filling are often the principle contamination site for dried products. The bacterial pathogens associated with dried milk powders include Salmonella (Mettler, 1994).

(4) Application of microbiological criteria

The FAO's code of hygienic practice for foods for infants and children (Codex Alimentarus CAC/RCP 21-1979) requires a minimum of 4–5 samples with <3 coliforms/g and a maximum of 1/5 control samples with >3 but ⩽20 coliforms/g. This criteria has not been exceeded by the numbers of *E. sakazakii* present in IFM including those associated with outbreaks. Muytjens *et al.* (1988) found levels ranging from 0.36 to 66.0 cfu/100 g with no *Enterobacteriacae* exceeding 1 cfu/g. Similarly Simmons *et al.* (1989) found 8 *E. sakazakii* cells/100 g in an open can of IFM associated with an outbreak. Nazarowec-White and Farber (1997c) found levels of *E. sakazakii* in positive IFM samples to be 0.36 cfu/100 g, but did not enumerate Enterobacteriacae or coliforms. Nestlé upgraded their facilities and applied more stringent release criteria for dietetic specialities of <0.3 coliforms/g, 0 *E. sakazakii* isolates/10 g following an outbreak of NEC due to a batch of Alfaré milk which met the FAO standards (van Acker *et al.*, 2001).

In March 2002, a fatal case of neonatal meningitis due to *E. sakazakii* was linked to Nestlé's Beba 1 infant formula in Belgium and involved a previously healthy 5–day old boy who became ill on release from hospital. The Federal Agency for Food Safety in Belgium, asked Nestlé Belgium to recall the batch in question. Although the level of contamination (1 sample 20 coliforms/g, 4 samples <1/g) was well below the acceptable international standard it was above Belgium statutory limits (<1 coliform/g in all samples; van Acker *et al.*, 2001). An outbreak in 2001 of meningitis in Tennessee (USA) resulted in the recall of Portagen Powder (Mead Johnson Nutritionals), a nutritional product for adults, toddlers and infants with rare digestive diseases (Himelright *et al.*, 2002). The microbiological quality of each batch of Portagen was assessed using validated methods, and its product specifications were consistent with those proposed by the US Food and Drug Administration and other US regulatory organizations. Hence it is probable that IFM powders associated with the various *E. sakazakii* outbreaks have met the current statutory criteria. Therefore these criteria must be reviewed and possibly more stringent statutory ones enforced. It could be argued that a criteria of <1 enteric pathogen cell/25 g should be adopted by IFM manufacturers. This is level of microbial contamination control is achievable and is already applied for the presence of *Salmonella*. In order to implement effective microbiological criteria it also will be necessary to validate the recommended and newly developed detection methods (Fig. 2).

(5) Provide appropriate information and preparation instructions

Bottled liquid infant formula is prepared as a sterile product. However IFM powders are not a sterile product, and therefore once rehydrated there is the risk of infectious organisms growing over prolonged periods of storage and a susceptible infant being given an infectious dose.

The temperature growth range of *E. sakazakii* was reported by Farmer *et al.* (1980) for 57 strains. There was no growth at 4 or 50°C but most strains grew at 47°C. Later, Kindle *et al.* (1996) found *E. sakazakii* and *Klebsiella pneumoniae* had higher growth rates in reconstituted infant formula than other organisms tested (*Pseudomonas aeruginosa*, *E. coli*, *Staphylococcus aureus*, *Mycobacterium terrae* and *Candida albicans*). Nazarowec-White and Farber (1997c) confirmed the

wide temperature range using five clinical isolates, five food isolates and the species type strain. The maximum growth temperature reported was between 41 and 45°C and the minimum growth temperature was 5.5–8°C (cf. Fig. 2). The generation and lag times for *E. sakazakii* were found to be similar to those reported for other *Enterobacter* but shorter than those of other organisms found in milk products. The generation time of *E. sakazakii* in infant formula was 40 min at room temperature.

Skladal *et al.* (1993) found UHT milk cartons inoculated with 10–15 cfu *E. sakazakii* 500 ml^{-1} and incubated at 30°C produced a very fast fermentation yielding D-lactate and subsequent acidification. The infective dose for *E. sakazakii* in infant formula is not known and the relatively short generation time and ability to grow at temperatures often found in domestic refrigerators makes the presence of *E. sakazakii* in infant formula particularly hazardous.

Risk communication

Due to epidemiological studies and the fact that powdered infant formulas are not commercially sterile products, the US FDA has proposed interim recommendations on the preparation of powdered infant formula in neonatal intensive care units. These recommendations include the preparation of the feed by trained personnel, immediate refrigeration (temperature not stated), to discard the feed if not used within 24 h, continuous enteral feeding (hang time) should not exceed 4 h and that alternatives to the powdered from should be chosen when possible. However there is clearly a need for a review of the accepted criteria for *E. sakazakii* in infant food products and for a rapid test that is feasible for use in the food manufacturing industry.

Conclusion

A newborn child is so susceptible to infections that the products such as infant formula milk require high levels of microbiological quality control during production, distribution and usage. Therefore IFM preparation and use requires the diligence of both the manufacturer and 'user' in domestic and hospital environment. Where the source has been traced to contaminated milk formula the levels of *Enterobacteriacae* were below the acceptable limit. Since the level of milk powder contamination with *E. sakazakii* is generally low (<0.36 cells/100 g formula milk) and the standard period (<24 h) of storage in a refrigerator would not permit the growth to an infectious levels (1000 cells based on *E. coli* O157 and *L. monocytogenes* 4b) it is probable that outbreaks are due to gross temperature abuse or poor hygienic practice. Statutory microbiological criteria needs to be revised and possibly more stringent ones enforced.

Despite the publicised outbreaks and product recalls, infant deaths due to *E. sakazakii* infection of the brain is fortunately rarely reported. Nevertheless there is a significant amount of further information that is required before a full risk assessment can be constructed. The prevalence and concentration of the organism in powdered milk products needs to be re-determined using validated detection methods. Heat-processing should be investigated in more detail, for example the role of the capsule as a factor enabling the organism to resist the lethal affects of desiccation and long term storage. Routes of infection and virulence factors are virtually unknown for this organism.

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The growth profile, thermotolerance and biofilm formation of *Enterobacter sakazakii* grown in infant formula milk

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ABSTRACT

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Aims: To study the growth, thermotolerance and biofilm formation of the emergent pathogen *Enterobacter sakazakii* in infant formula milk (IFM).

Methods and Results: The temperature range, death kinetics and biofilm formation of *E. sakazakii* were determined using impedance microbiology and conventional methods. In IFM the organism grew as low as 6°C and optimally at 37–43°C. In faecal coliform tests, 23% of strains ($n = 70$) produced gas from lauryl sulphate broth (LSB) at 44°C after 48 h incubation. Three strains failed to grow in LSB at any of the temperatures. The D-value of cells suspended in IFM was determined between 54 and 62°C. The resultant z-value was 5·7°C. The organism was able to adhere and grow on latex, polycarbonate, silicon and to a lesser extent stainless steel.

Conclusions: *Enterobacter sakazakii* was able to grow at refrigeration temperatures and on infant-feeding equipment. The thermotolerance of the organism was similar to other Enterobacteriaceae and should be killed during standard pasteurization treatment.

Significance and Impact of the Study: *Enterobacter sakazakii* has been associated with infant meningitis through consumption of contaminated IFM. *Enterobacter sakazakii* is able to grow in IFM during storage at refrigeration temperatures and attach to infant-feeding equipment, which may become reservoirs of infection.

Keywords: D-value, emergent pathogen, impedance, neonate.

INTRODUCTION

Enterobacter sakazakii is a contaminant of powdered infant formula milk (IFM; Simmons *et al.* 1989) that has been associated with necrotizing enterocolitis (van Acker *et al.* 2001), bacteraemia (Muytjens *et al.* 1983) and a rare form of infant meningitis (Bar-Oz *et al.* 2001; Himelright *et al.* 2002). Mortality rates vary from 10 to 80% (van Acker *et al.* 2001; Lai 2001) and meningitis survivors suffer severe neurological sequelae such as hydrocephalus, quadriplegia and retarded neural development. Recently the International Commission for Microbiological Specifications for Foods ranked the organism as 'severe hazard for restricted populations, life threatening or substantial chronic sequelae or long duration' (ICMSF 2002). The FAO/WHO is revising the Recommended International Code of Hygienic Practice for Foods for Infants and Children and this includes reassessing the testing of specific pathogens associated with IFM (Codex Alimentarius Commission 2003).

Multiple cases of *E. sakazakii* necrotizing enterocolitis and meningitis have occurred in neonatal intensive care units (NICU) because of contamination and persistence of *E. sakazakii* in IFM and feed preparation equipment (Simmons *et al.* 1989; van Acker *et al.* 2001). In one study, *E. sakazakii* has been isolated from 20 of 141 infant formula products (Muytjens *et al.* 1988). The organism has also been isolated from a wide range of other foods including cheese, meat, vegetables, grains, herbs and spices (Iversen and Forsythe 2003).

Nazarowec-White and Farber (1997) reported that *E. sakazakii* was more thermotolerant than most other

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Enterobacteriaceae which may contribute to its survival of heat treatments, and subsequent presence in desiccated products. The lowest temperature of growth was 5·5°C and therefore potentially the organism can grow during refrigerated storage (Nazarowec-White and Farber 1997). However, the rate of growth of the organism at ambient and subambient temperatures is required to predict possible ingested doses after the infant feed has been rehydrated and stored. In a NICU, prolonged feeding time at ambient temperature and repeated use of enteral feed bags have been identified as risk factors (Oie and Kamiya 2001). *Enterobacter sakazakii* produces viscous capsular material (Scheepe-Leberkühne and Wagner 1986) and therefore the organism could form a biofilm on feeding equipment and contact surfaces (i.e. stainless steel).

Hence the temperature growth range, thermotolerance and biofilm formation of *E. sakazakii* need to be better understood to limit the occurrence of neonate infection.

MATERIALS AND METHODS

Strains and growth media

Enterobacter sakazakii NCTC 11467 (type strain) was obtained from the NCTC (London). Seven strains were kindly donated by Franco Pagotto (Health Canada), including the clinical isolates LA, LB and SK90. Five strains were from Nestlé Research Centre (Lausanne, Switzerland). The remaining strains were food, clinical and environmental isolates from the culture collection at Nottingham Trent University. *Enterobacter sakazakii* strain 823 was the only capsulated strain.

Lauryl sulphate broth (LSB), brilliant green bile broth (BGBB), tryptone soya broth (TSB) and brain–heart infusion (BHI) were from Oxoid Ltd (Basingstoke, UK). Whitley impedance broth (WIB) was from Don Whitley Scientific Ltd (Shipley, UK). Cow & Gate Premium 1 ready-made 200 ml IFM cartons were purchased from local commercial retailers.

Microbiological methods

Upper growth temperature was determined by inoculating 10 ml volumes of TSB, LSB and BGBB (containing Durham tubes for gas collection) with 10^4 stationary phase *E. sakazakii* cells. The broths were incubated between 37 and 47°C, and optical density (O.D. 650 nm) was recorded after 24 and 48 h incubation.

Specific growth rates were measured in non-selective (BHI, TSB, WIB) microbiological media and IFM using the Rapid Automated Bacterial Impedance Technique (RABIT™; Don Whitley Scientific Ltd). The impedance calibration curves (direct and indirect methods) were established using plate counts on TSA at 37°C for 24 h as according to the manufacturer's instructions. To determine specific growth rates, 0·1 ml decimal dilutions of overnight *E. sakazakii* cultures were inoculated into 2 ml media using the direct impedance technique (Silley and Forsythe 1996) and incubated at temperatures ranging from 6 to 49°C.

The thermotolerance of *E. sakazakii* was determined by suspending 1 ml overnight culture in 20 ml of temperature equilibrated TSB and IFM in water baths between 54 and 62°C. At timed intervals 0·1 ml aliquots were transferred to 2 ml TSB at room temperature and the number of survivors determined using the direct impedance technique (Hilton *et al.* 2001). The time required at each temperature for the viable count to decrease by 1 log cycle (termed the decimal reduction time, *D*-value) was determined by plotting the $\log_{10}$ number of survivors against time using linear regression analysis. The *z*-value, the increase in temperature required for the *D*-value to decrease 10-fold, was determined by plotting the best line of fit of $\log_{10}D$-value against temperature. Each *D*-value determination was in triplicate using three separate batches of cells.

Biofilm formation was determined using sterilized 10 × 10 × 0·5–1·0 mm pieces of latex, silicon and polycarbonate cut from infant-feeding bottles and stainless steel. The pieces were placed in 10 ml IFM, inoculated with 10^4 *E. sakazakii* cells in the stationary phase and incubated for 24 h at 37°C. The pieces were washed by shaking for 1 min in 10 ml sterile distilled water followed by drying for 3 h in a class 1 airflow cabinet. The pieces were then placed directly in the impedance tubes for enumeration of the microbial load as previously described by Druggan *et al.* (1993). The viable counts were analysed using the Box-Whisker plot option of SlideWrite Plus for Windows (Advanced Graphics Software Inc., Encinitas, CA, USA).

Electron microscopy

The stainless steel, latex, silicon and polycarbonate squares were incubated, washed and dried as described above for biofilm formation before mounting on 12·5 × 10 mm aluminium stubs (TAAB, Aldermaston, UK) using 12 mm self-adhesive carbon discs (TAAB). The samples were sputter-coated with gold and scanned on a JEOL-840A scanning electron microscope (JEOL Ltd, Tokyo, Japan). Representative photographs were taken at various magnifications.

RESULTS

Temperature range of growth and thermotolerance

All strains ($n = 70$) grew to *ca* 10^9 CFU ml^{-1} overnight in TSB at 37 and 44°C. No growth was observed for any strain after 24 h at 47°C. However, minimal growth was observed

for 34% of strains after 48 h. The ability of *E. sakazakii* to grow and ferment lactose in the coliform broths LSB and BGBB was determined at 37 and 44°C after 48 h. At 37°C, 80 and 76% of strains produced gas from lactose in LSB and BGBB, respectively. Whereas the corresponding values were only 23 and 11% at 44°C. Three *E. sakazakii* strains did not grow in LSB or BGBB at any of the temperatures, although viability was confirmed by growth in TSB at 37 and 44°C after 48 h.

Six clinical and food strains were chosen for further detailed growth rate studies. All strains grew between 6 and 45°C, the optimum being 37–43°C depending on the medium. The mean specific growth rates for all strains in all media are shown in Fig. 1. The mean doubling time at 37°C was 22 min and ranged from 14 to 29 min. In IFM the range was less broad (19–21 min). The doubling time in IFM at 6 and 21°C were 13·7 and 1·7 h, respectively. The type strain grew slower in all media than the other strains. The capsulated strain was able to grow at 47°C, but only in IFM. It was noted that the exopolysaccharide production by the capsulated strain made the IFM very viscous.

D-values for the type and capsulated strains, suspended in TSB and IFM, were determined from 54 to 62°C (Table 1). At 54°C the *D*-values ranged from 10·2(±3·56) to 16·4(±0·67) min. The *D*-values were between 0·2 (±0·13) and 0·4 (±0·08) min when the treatment temperature was raised to 62°C. Due to the large standard deviation, no statistical difference was shown in the *D*- and *z*-values between the *E. sakazakii* type strain and capsulated strain. Neither was any statistical difference shown between the *D*-values obtained when the cells were heat treated in TSB compared with IFM. The average *z*-value for both strains was 5·7°C.

Biofilm formation

When grown in IFM *E. sakazakii* adhered to silicon, latex and polycarbonate to a greater extent than to stainless steel (Fig. 2). For example the viable count (median values) of

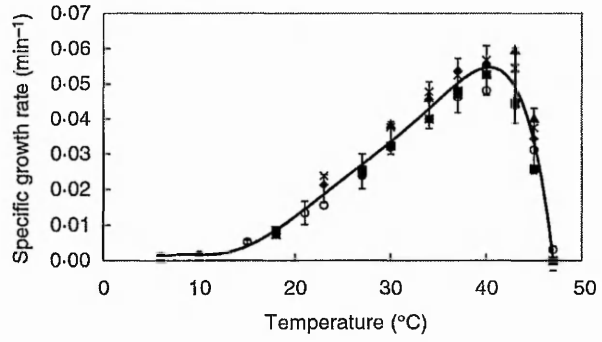

Fig. 1 Mean specific growth rate of six *Enterobacter sakazakii* strains in four media: (◆) WIB, (×) BHI, (○) IFM, (▲) TSB. Trend line is the mean specific growth rate in all media ($R^2 = 0{\cdot}997$). Error bars represent standard deviation of the mean specific growth rate in all media

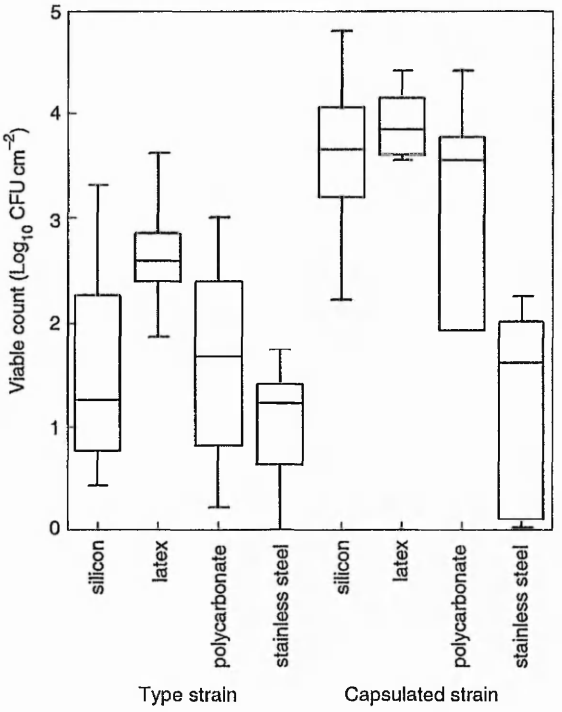

Fig. 2 Biofilm formation by *Enterobacter sakazakii* in infant formula milk on materials commonly used for infant-feeding equipment and work surfaces. The box shows the range between the 25th and 75th percentiles with a horizontal line at the median value. The whiskers extend to the 5th and 95th percentile of the data

Medium	Strain	*D*-value (min) 54°C	56°C	58°C	60°C	62°C	*z*-value (°C)
TSB	Type	14·9 ± 0·65	2·7 ± 0·08	1·3 ± 0·28	0·9 ± 0·17	0·4 ± 0·08	5·6 ± 0·13
	Capsulated	10·2 ± 3·56	1·2 ± 0·01	1·7 ± 0·38	0·2 ± 0·06	0·2 ± 0·13	5·6 ± 0·50
IFM	Type	16·4 ± 0·67	5·1 ± 0·27	2·6 ± 0·48	1·1 ± 0·11	0·3 ± 0·12	5·8 ± 0·40
	Capsulated	11·7 ± 5·80	3·9 ± 0·06	3·8 ± 1·95	1·8 ± 0·82	0·2 ± 0·11	5·7 ± 0·12

Table 1 Decimal reduction times (*D*-values) and *z*-values (±standard deviation) for *E. sakazakii* type strain and capsulated strain in TSB and IFM

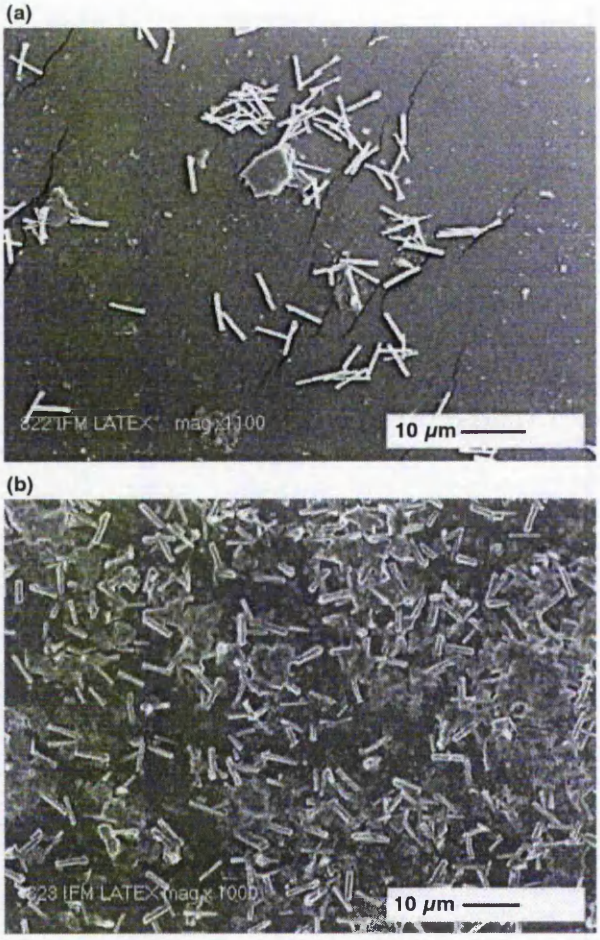

Fig. 3 Scanning electron microscopy of *Enterobacter sakazakii* adhering to latex during growth in infant formula milk: (a) type strain and (b) capsulated strain

the capsulated strain on latex was 8×10^3 CFU cm^{-2} compared with 50 CFU cm^{-2} on stainless steel. The capsulated strain formed denser biofilms on silicon, latex and polycarbonates than the type strain; median 6×10^3 CFU cm^{-2} compared with 20–400 CFU cm^{-2}. Scanning electron microscopy was used to visualize the adherence of both strains to latex (Fig. 3).

DISCUSSION

Enterobacter sakazakii grew between 6 and 45°C in all media, with one strain being able to grow at 47°C but only in IFM (Fig. 1). Farmer *et al.* (1980) reported that 70–90% of *E. sakazakii* strains produced gas from lactose in EC medium at 35°C compared with 58% of strains at 44·5°C. Similarly in our study, 76% of strains produced gas from lactose at 37°C and only 23% of strains produced gas at 44°C. Since carbohydrate metabolism in a large number of *E. sakazakii* strains may be sensitive to temperatures of 44°C and above, the organism will not necessarily be detected using coliform broths.

The thermotolerance of the organism, as determined by *D*- and *z*-value determination (Table 1), was similar to other Enterobacteriaceae such as *Salmonella* in rehydrated milk powder (Read *et al.* 1968). The *D*-values for *E. sakazakii* were between those previously published. For example the *D*-value at 58°C was 2·4 min compared with 4·2 min (Nazarowec-White and Farber 1997) and 0·4 min (Breeuwer *et al.* 2003). The *z*-value for the organism (5·7°C) was nearly identical to the 5·8°C of Nazarowec-White and Farber (1997) and greater than the 3·4°C of Breeuwer *et al.* (2003). The experiments of Breeuwer *et al.* (2003) were in phosphate buffer with a cold shock after heat treatment and this may account for some of the differences in the *D*- and *z*-values between groups. Using the decimal reduction time for the organism in IFM at 60°C of 1·1 min and the *z*-value of 5·7°C (Table 1), it is predictable that the decimal reduction time at 71·2°C is 0·7 s. Therefore the standard high temperature short treatment pasteurization process of 15 s at 71·7°C will result in *ca* a 21-log reduction in the viable count of the organism. Hence any contamination of IFM powder will probably be due to failure in hygiene standards after pasteurization.

The organism grew in IFM at refrigeration temperatures with a doubling time of *ca* 13 h. Although the infectious dose of the organism is unknown, it is unlikely that sufficient multiplication will occur under refrigeration conditions to cause an infection. However, when grown in IFM the organism formed a biofilm on surfaces such as latex, silicon and to a lesser extent stainless steel. These materials are commonly used for infant-feeding equipment and in preparation areas. Attachment and adherence may be enhanced because of the production of exopolysaccharide capsular material previously described by Scheepe-Leberkühne and Wagner (1986). Neonatal infections have been associated with *E. sakazakii* colonization of food preparation equipment such as brushes, blenders and spoons (Simmons *et al.* 1989; Bar-Oz *et al.* 2001). Therefore all bottles and utensils should be cleaned thoroughly as soon as possible after use to deter *E. sakazakii* biofilm formation, which could become a source of infection.

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A selective differential medium for *Enterobacter sakazakii*, a preliminary study

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Abstract

Enterobacter sakazakii can cause fatal invasive infection of neonates associated with the presence of this organism in powdered infant milk formula. A new chromogenic medium (Druggan–Forsythe–Iversen agar, DFI) is described for the selective detection of this emergent pathogen. The medium is based on the α-glucosidase reaction which is detected using 5-bromo-4-chloro-3-indolyl-α,D-glucopyranoside (XαGlc). *Ent. sakazakii* hydrolyses this substrate to an indigo pigment, producing blue-green colonies on this medium. DFI was compared with the current method of detection on violet red bile glucose agar (VRBGA) followed by pigment production on tryptone soy agar (TSA) after 48–72 h at 25 °C and subsequent biochemical profile determination using Biomerieux API20E. Ninety-five clinical and food strains of *Ent. sakazakii* were detected on the DFI chromogenic medium 2 days sooner than the alternative method. The characteristics of 148 strains representing 17 genera of non-*Ent. sakazakii* Enterobacteriaceae were compared using the two methods. Only 16/18 *Escherichia vulneris* strains, 2/3 strains of *Pantoea* spp. and 1/8 *Citrobacter koseri* strains gave false positive results on DFI agar.

Eight α-glucosidase positive strains were identified as *Pantoea* using their API20E biochemical profile, but had higher percentage identification as *Ent. sakazakii* using ID32E. Therefore the DFI medium enables the detection of *Ent. sakazakii* within mixed cultures of Enterobacteriaceae, whereas the organism could be missed when using VRBGA since the latter is a general Enterobacteriaceae selective medium. In addition, the common use of API20E to check yellow pigmented colonies on TSA may lead to false negative results and consequently the acceptance of a batch of infant formula milk (IFM) that contains *Ent. sakazakii*.

Keywords: *Enterobacter sakazakii* medium; Chromogenic; Dried milk; Infant formula

1. Introduction

The presence of *Enterobacter sakazakii* in powdered infant formula milk (IFM) has been associated with outbreaks of a rare form of infant meningitis, necrotising enterocolitis, bacteraemia and neonate deaths (Simmons et al., 1989; Lai, 2001; van Acker et al., 2001; Himelright et al., 2002). Early studies in the 1980s isolated the organism at low levels (0.36 cells/100 g) from 20 out of 141 infant formula products (Muytjens et al., 1988). In addition to IFM, *Ent. sakazakii* has been isolated from a wide range of foods including cheese, meat, vegetables, grains,

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herbs and spices (Iversen and Forsythe, 2003). To date detection methods have primarily involved pre-enrichment in buffered peptone water (BPW), enrichment in EE broth which inhibits the growth of non-Enterobacteriaceae such as lactic acid bacteria, plating on violet red bile glucose agar (VRBGA), picking five Enterobacteriaceae colonies on to tryptone soy agar (TSA) plates which are incubated at 25 °C for 48–72 h to observe yellow pigment production; a typical trait of *Ent. sakazakii* (FDA; Nazarowec-White and Farber, 1997). However, other Enterobacteriaceae might outgrow *Ent. sakazakii* during the pre-enrichment and enrichment stages, leading to relatively few *Ent. sakazakii* colonies on VRBGA and subsequently a reduced chance of picking the organism onto TSA. Thus false negative results would be obtained and *Ent. sakazakii* contaminated IFM distributed. Therefore a more reliable, specific medium is required for screening infant foods.

Isolation methods based on raised temperature (~ 44 °C) have been used for 'faecal coliforms' (Alonso et al., 1999). This approach would be inappropriate for *Ent. sakazakii*, however, since previous workers reported that not all strains grew at this temperature (Gavini et al., 1983; Nazarowec-White and Farber, 1997). During the past decade there has been a significant increase in the use of chromogenic substrates in isolation media (Manafi, 2000). In particular, the indolyl-substrates have been used to differentiate a number of important organisms such as *Escherichia coli* from their competitors (van Poucke and Nelis, 2000). A major advantage of these substrates is that strong colours are produced that do not diffuse out from colonies and therefore even small positive colonies are visible in the presence of more numerous competitors.

It has been reported that 100% of *Ent. sakazakii* ($n=129$) were positive for α-glucosidase and that 100% of other *Enterobacter* species ($n=97$) were negative for this enzyme (Muytjens et al., 1984). Based on this observation the indolyl substrate 5-bromo-4-chloro-3-indolyl-α,D-glucopyranoside (XαGlc) was added to a basal medium to differentiate *Ent. sakazakii* colonies from other members of the Enterobacteriaceae. The enzyme α-glucosidase hydrolyses XαGlc liberating the aglycone, 5-bromo-4-chloro-indolol, which dimerizes in the presence of oxygen to form the pigment bromo-chloro-indigo. In addition, a hydrogen sulphide indicator (sodium thiosulphate and ammonium iron citrate) was incorporated to differentiate weak α-glucosidase, H_2S positive organisms (such as *Proteus vulgaris*) from *Ent. sakazakii*. Sodium deoxycholate was the selective agent for Enterobacteriaceae.

2. Materials and methods

2.1. Druggan–Forsythe–Iversen (DFI) medium

Sodium deoxycholate (1 g l^{-1}, CAS 302-95-4, Sigma-Aldrich, UK), 5-bromo-4-chloro-3-indolyl-α,D-glucopyranoside (0.1 g l^{-1}, CAS 108789-36-2, Glycosynth, Warrington, UK), sodium thiosulphate (1 g l^{-1}, CAS 7772-98-7, BDH, UK) and ammonium iron(III) citrate (1 g l^{-1}, CAS 1185-57-5, BDH, UK) were dissolved in distilled water before addition of the basal medium Tryptone Soya Agar (TSA, 40 g l^{-1}) (Oxoid, UK). The complete medium (pH 7.3) was heated in a boiling water bath to dissolve the components before autoclaving at 121 °C for 15 min. Presence of inducible α-glucosidase in selected non-*Ent. sakazakii* strains was determined by testing them on DFI with the addition of maltose (1 g l^{-1}).

2.2. Test organisms

The sensitivity of the medium (as defined below) was tested using 95 strains of *Ent. sakazakii* comprising; four strains from national culture collections, including the type strain ATCC 29544, and seven from Health Canada (donated kindly by Franco Pagotto) and five from Nestlé Research Centre, Switzerland. The remaining strains were food (IFM, baby foods, dried food, milk powders, herbs and spices) and environmental isolates from the culture collection at Nottingham Trent University and Oxoid. The specificity of the medium (as defined below) was tested using 148 strains of other Enterobacteriaceae which represented 17 genera as listed in Table 1. The strains were streaked onto DFI medium and incubated at 37 °C for 24 h. Only colonies that were entirely blue-green after 24 h incubation at 37 °C were considered presumptive positives.

Following the standard FDA method, all 243 strains were streaked onto VRBGA (37 °C, 24 h) for colony morphology, and TSA (25 °C, 48–72 h)

Table 1
Strains used in this study

Organism (number of strains)	Source
Buttiauxella agrestis (1)	Lactose
Chryseomonas luteola (1)	Infant formula milk
Citrobacter braakii (3)	Herbs and spices, meat, vegetables
Citrobacter freundii (3)	Environmental, herbs and spices, vegetables
Citrobacter koseri (9)	ATCC 25408, ATCC 25409, ATCC 25410, ATCC 27026, herbs and spices, vegetables
Citrobacter youngae (1)	Herbs and spices
Edwardsiella tarda (1)	NCTC 10396
Enterobacter aerogenes (6)	NCTC 10006, Baby food, herbs and spices
Enterobacter amnigenus (2)	Environmental, herbs and spices
Enterobacter cloacae (31)	ATCC 13047, ATCC 23355, NCTC 10005, clinical, dried food, environmental, herbs and spices, milk powder
Enterobacter sakazakii (95)	ATCC 12868, ATCC 29544, ATCC 51329, NCTC 11467, infant formula milk, baby food, clinical, dried food, herbs and spices, milk powder
Escherichia coli (6)	Fresh food, herbs and spices, meat
Escherichia hermanii (4)	Dried food, milk powder
Escherichia vulneris (18)	Infant formula milk, baby food, dried food, herbs and spices, meat
Hafnia alvei (2)	Environmental, raw milk
Klebsiella ornitholytica (1)	Herbs and spices
Klebsiella oxytoca (5)	Herbs and spices, vegetables
Klebsiella ozaenae (1)	Herbs and spices
Klebsiella planticola (1)	Herbs and spices
Klebsiella pneumoniae (12)	Dried food, herbs and spices, milk powder, vegetables
Klebsiella terrigena (8)	Herbs and spices, milk powder
Kluyvera spp. (1)	Vegetables
Leclercia adecarboxylata (4)	Herbs and spices, milk powder
Morganella morganii (1)	NCTC 235
Pantoea spp. (3)	Herbs and spices
Pantoea agglomerans (3)	Meat, milk
Proteus vulgaris (6)	NCTC 4636, environmental, clinical
Providencia rettgeri (2)	Environmental
Providencia stuartii (1)	Environmental
Rahnella aquatilis (2)	Infant formula milk
Salmonella Arizonae (1)	Herbs and spices
Salmonella enterica serovar Enteritidis (1)	NCTC 3046
Serratia ficaria (4)	Baby food, herbs and spices
Serratia fonticola (1)	Herbs and spices
Serratia marcescens (3)	NCTC 2487, clinical

Total number = 243.

for pigment production. The biochemical profiles of all strains were determined using API20E and ID32E (bioMérieux UK) in parallel. Where there was a discrepancy between the identification using the two biochemical test panels, the highest % identification was chosen and is reported in Table 1.

2.3. Sensitivity and specificity determination of DFI medium

The sensitivity and specificity of the DFI agar was determined by comparison with the FDA recommendations for the isolation and identification of *Ent. sakazakii* from IFM; yellow pigmentation after incubation on TSA at 25 °C for 48–72 h and identification by API20E. Sensitivity was defined as the number of true positives divided by the sum of true positives plus false negatives, expressed as a percentage. Specificity was defined as the number of true negatives divided by the sum of false positives plus true negatives, expressed as a percentage (Greenhalgh, 1997).

3. Results

3.1. Identification of test organisms

The identity of some Enterobacteriaceae strains was dependent upon the biochemical test kit used. Of 12 strains identified as *Pantoea* spp. using API20E, eight gave higher % identity as *Ent. sakazakii* and one gave higher % identification as *Klebsiella pneumoniae* using ID32E and are consequently listed as such in Tables 1 and 2. Three strains identified as *Ent. sakazakii* by API20E were identified as *Enterobacter amnigenus*, *Enterobacter cloacae* or *Enterobacter gergoviae* by ID32E. Detailed biochemical profiles and identities are given in Table 2.

3.2. Specificity and sensitivity of DFI medium

All 95 *Ent. sakazakii* strains produced colonies that were an entirely blue-green colour on DFI after 24 h incubation (Fig. 1). In general, other Enterobacteriaceae produced white colonies. The exceptions were some *Serratia* spp. that were pink-red and *Escherichia hermanii* that were yellow. Also, *Salmonella enterica* strains, *P. vulgaris*, *Morganella mor-*

Table 2
Differences in strain identification using API20E and ID32E biochemical profiles

Strain	Profile	API20E analysis % identification	Organism	Profile	ID32E analysis % identification	Organism
1	3305573	95.1	*Ent. cloacae*	34076743211	54.9	*Ent. amnigenus*
2	3305573	95.1	*Ent. cloacae*	34074743211	52.2	*Ent. amnigenus*
3	3305573	95.1	*Ent. cloacae*	34074743231	60.8	*Ent. amnigenus*
4	3305173	51.1	*Ent. sakazakii*	34074743011	61.7	*Ent. cloacae*
5	3305173	51.1	*Ent. sakazakii*	14074743211	88.5	*Ent. amnigenus*
6	3305173	51.1	*Ent. sakazakii*	71074747011		*Ent. cloacae* or *gergoviae*
7	1004153	61.5	*Esch. vulneris*	00674561061	unacceptable	*B. agrestis*
8	3004153	95.9	*Esch. vulneris*	34774563051	unacceptable	*B. agrestis*
9	1006523	20.3	*Pantoea* sp. 1	30074773050	98.1	*Ent. sakazakii*
10	1205773	42.2	*Pantoea* sp. 2	45076777330	98.2	*K. pneumoniae*
11	0005173	66.7	*Pantoea* sp. 3	34674773050	99.9	*Ent. sakazakii*
12	0005173	66.7	*Pantoea* sp. 3	30674773050	99.9	*Ent. sakazakii*
13	1005153	74.7	*Pantoea* sp. 3	34674563051	94.9	*Ent. sakazakii*
14	1005153	74.7	*Pantoea* sp. 3	34674563051	94.9	*Ent. sakazakii*
15	1006312	99.5	*Pantoea* sp. 3	00274513011	99.8	*Pantoea* sp. 1
16	0004153	53.2	*Pantoea* sp. 4	30676563051	unacceptable	*Ent. sakazakii*
17	1004173	64.0	*Pantoea* sp. 4	34276377050	99.9	*Ent. sakazakii*
18	1004173	64.0	*Pantoea* sp. 4	34674773051	99.9	*Ent. sakazakii*
19	1004173	64.0	*Pantoea* sp. 4	30674773050	99.9	*Ent. sakazakii*
20	1006153	40.3	*Pantoea* sp. 4	34774563051	unacceptable	*Ent. sakazakii*

ganii, *Providencia* and *Citrobacter* spp. and one *Serratia marcescens* strain produced grey to black colonies due to hydrogen sulphide production. Some non-*Ent. sakazakii* organisms produced white colonies on DFI, but developed small (<0.5 mm diameter) blue-green centres on prolonged incubation (>30 h). Four isolates developed blue centres within the 24-h incubation period, these were *Leclercia adecarboxylata, Citrobacter koseri* and two strains of *P. vulgaris*.

The DFI medium had 100% sensitivity (Table 3) for *Ent. sakazakii* due to the constitutive expression of α-glucosidase. Using the given range of Enterobacteriaceae, the specificity of the medium was 87.2%. The false positive organisms were 16/18 *Escherichia vulneris* strains, 2/3 *Pantoea* spp. strains and 1/8 *C. koseri* strains. The addition of maltose to DFI as an inducer resulted in entirely blue-green colonies for the culture collection strains of *C. koseri* as well as *Ent. aerogenes* and *K. pneumoniae*, whereas without maltose colonies were white.

All 243 strains used in this study gave typical Enterobacteriaceae colonies on VRBGA (1–2 mm red with or without red halo) and therefore could have been selected using the standard FDA method for further analysis of pigment production on TSA. Whereas only *Ent. sakazakii*, most *Esch. vulneris*, a few *Pantoea* and one *C. koseri* strain gave entirely blue-green colonies on DFI agar (Fig. 1).

Of the 148 strains of non-*Ent. sakazakii* used in this study 31 were yellow pigmented after 48–72 h incubation at 25 °C on TSA—giving 31 false positive results using the standard method, as opposed to the 19 false positives on DFI.

4. Discussion

It is essential that detection methods for *Ent. sakazakii* are established that are robust and reliable to ensure the safety of infant formula milk. The current method for the detection of *Ent. sakazakii* is based on yellow pigment production and originated from pioneering work of Muytjens et al. (1988). The method uses sample enrichment in EE broth, followed by plating on VRBGA to isolate Enterobacteriaceae. Five colonies are selected and streaked on TSA for pigment production. This method however does not select for *Ent. sakazakii* and the combined use of EE broth and VRBGA could allow other Enterobacteriaceae to outgrow *Ent. sakazakii* and give false negative results. It is not possible to select for *Ent. sakazakii*

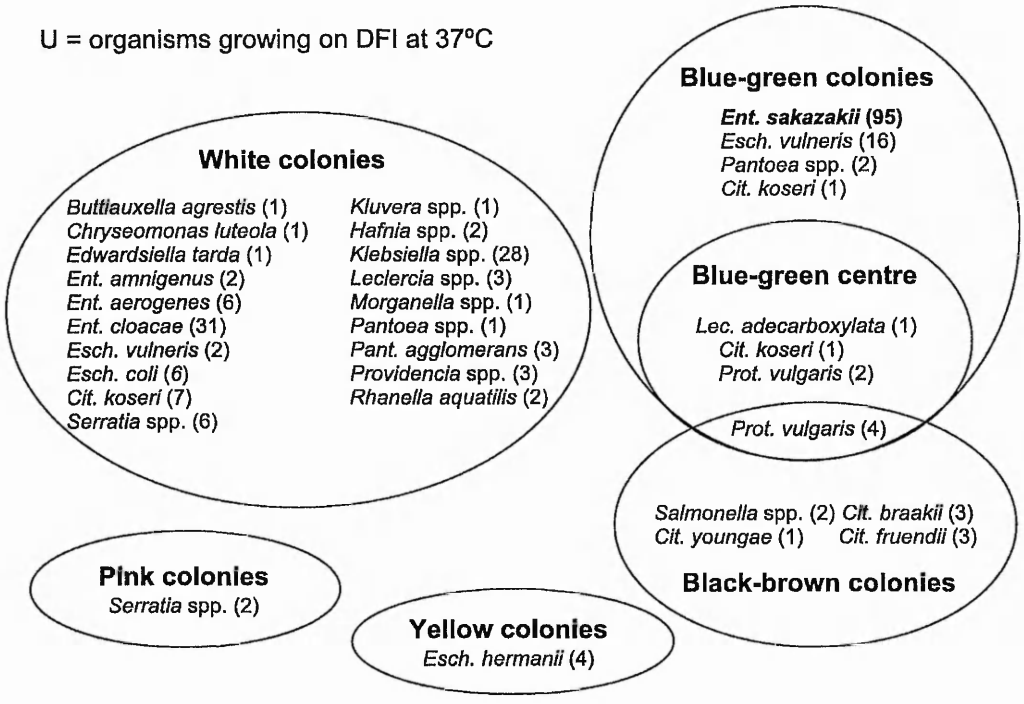

Fig. 1. Venn diagram of colony morphology on DFI agar for organisms which grow in EE broth (bile-tolerant). The number of strains is indicated in parentheses.

colonies from VRBGA plates on the basis of colony morphology. In this study, all 243 strains from 17 genera of Enterobacteriaceae produced similar small red colonies.

Table 3
Specificity of DFI agar and FDA method for the identification of *Ent. sakazakii*

DFI agar (37 °C, 24 h)	FDA method (TSA 25 °C, 48–72 h; API20E)	
	Positive	Negative
Positive	95[a]	19[b]
Negative	0[c]	129[d]

[a] Number of presumptive *Ent. sakazakii* strains by both methods which were confirmed.

[b] Number of non-*Ent. sakazakii* strains that were presumptive *Ent. sakazakii* on DFI agar and not by FDA method.

[c] Number of non-*Ent. sakazakii* strains the were presumptive *Ent. sakazakkii* by FDA method and not on DFI agar.

[d] Number of non-*Ent. sakazakii* strains that were not presumptive *Ent. sakazakii* by either FDA method or DFI agar.

DFI agar differs from the previous media in that the chromogenic substrate indolyl substrate 5-bromo-4-chloro-3-indolyl-α,D-glucopyranoside is incorporated into the medium to detect α-glucosidase activity. All 95 strains of *Ent. sakazakii* produced entirely blue-green colonies on DFI, confirming the constitutive production of α-glucosidase by this organism (Muytjens et al., 1988; Table 1, Fig. 1). Other members of the Enterobacteriaceae are also α-glucosidase positive. However, many of these organisms (such as *P. vulgaris*) are differentiated from *Ent. sakazakii* due to their production of hydrogen sulphide and consequent blackening of the colony (Fig. 1).

Organisms giving false positive results (entirely blue-green colonies) on DFI were *Esch. vulneris* (16/18 strains), *Pantoea* spp. (2/3 strains) and *C. koseri* (1/8 strains). Although *P. vulgaris* is α-glucosidase positive (60%), it was expected that only a small percentage would produce false positive colonies on DFI as 95% of strains are H_2S positive (Farmer, 1999). In contrast, 40% of *C. koseri* strains give α-

glucosidase positive reaction and all are H_2S negative, and hence would produce entirely blue-green colonies. However, most *C. koseri* strains (7/8 strains) were easily distinguished from *Ent. sakazakii* as the XαGlc substrate did not appear to be preferentially utilised. *C. koseri*, *Ent. aerogenes* and *K. pneumoniae* produced entirely blue-green colonies only on DFI supplemented with maltose. This suggests that in these organisms α-glucoside permease and hydrolase are not constitutively expressed but are inducible and therefore they would not present as false positives on DFI medium.

It should be noted that *C. koseri* has also been associated with neonate meningitis through infant formula milk (Kleiman et al., 1981) and was isolated from three out of 141 samples by Muytjens et al. (1988). The organism produces brain abscesses similar to those caused by *Ent. sakazakii* (Kline, 1988). The false positive result with *Esch. vulneris* was expected since this species was reported to be 25% positive for α-glucosidase and 50% positive for yellow pigment production (Farmer, 1999). It is currently unclear how significant this organism would be in screening IFM as Muytjens et al. (1988) only isolated one *Esch. vulneris* strain from 141 batches of milk powder. However, more recent databases would classify the *Ent. agglomerans* they isolated from 35 out of 141 samples as either *Pantoea* sp. or *Esch. vulneris* (Farmer, 1999) or possibly *Ent. sakazakii* (see Table 2).

The difference in identification for certain strains (Table 2) according to the biochemical profile reflects the remaining taxonomic uncertainty of *Enterobacter* and *Pantoea* organisms (Janda and Abbott, 1998). Ten out of 12 *Pantoea* strains identified using the API20E profile had higher % identification as *Ent. sakazakii* using the ID32E panel. It would therefore appear that the more limited number of tests used in the API20E kit may be inadequate to identify all *Ent. sakazakii* isolates and may lead to a false negative result. In turn, this could lead to errors in the release of batches of infant formula milk and milk powders that would otherwise be withheld from the market. However, the standard FDA protocol uses API20E.

This study has shown the potential for a chromogenic medium to be used for the detection of *Ent. sakazakii* that requires shorter incubation period than the standard protocol, can differentiate the organism in the presence of other Enterobacteriaceae, and has a higher probability of detecting *Ent. sakazakii* amongst competing *Enterobacteriaceae* from enrichment media.

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Isolation of *Enterobacter sakazakii* and other Enterobacteriaceae from powdered infant formula milk and related products

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Abstract

The presence of *Enterobacter sakazakii* and other Enterobacteriaceae was surveyed in 82 powdered infant formula milk (IFM) and 404 other food products. The presence of *Ent. sakazakii* was detected using the conventional method (growth on violet red bile glucose agar plus yellow pigment production on TSA) and a new chromogenic medium (Druggan–Forsythe–Iversen agar, DFI) which enables results to be obtained 2 days earlier than the conventional method. *Ent. sakazakii* was isolated from 2/82 powdered IFM, 5/49 dried infant foods, 3/72 milk powder, 2/62 cheese products and various dry food ingredients, especially herbs and spices (40/122). *Ent. sakazakii* was isolated from 67 samples using the DFI medium, however only 19 of the samples were positive following the conventional method. The largest difference in isolation between the two methods was with dry food ingredients.

Although Enterobacteriaceae were enumerated from one powdered IFM sample (*Klebsiella ozaenae*, 200 cfu/g), 7/82 had detectable Enterobacteriaceae after enrichment in EE broth. Using the ISO 6579 2002 method and immuno-magnetic separation technique no *Salmonella* serovars were isolated from powdered IFM, dried infant foods or milk samples. Therefore hygienic production of powdered IFM and milk production as monitored by control of *Salmonella* and enumeration of Enterobacteriaceae did not control *Ent. sakazakii*.

Keywords: *Enterobacter sakazakii*; Enterobacteriaceae; Infant formula milk

1. Introduction

Enterobacter sakazakii has been associated with outbreaks of a rare form of infant meningitis, necrotizing enterocolitis (NEC), bacteraemia and neonate deaths (Arseni et al., 1987; Simmons et al., 1989; Noriega et al., 1990; Bar-Oz et al., 2001; Lai, 2001). The organism has been isolated from a wide range of foods including cheese, meat, vegetables, grains, herbs and spices (Iversen and Forsythe, 2003). However, its presence in powdered infant formula milk (IFM) for use with newborn babies is of particular concern (Clark et al., 1990; van Acker et al., 2001; Himelright et al., 2002).

The International Commission for Microbiological Specifications for Foods (ICMSF, 2002) has ranked *Ent. sakazakii* as 'Severe hazard for restricted populations, life threatening or substantial chronic sequelae or long duration'. Due to the raised awareness of the organism, FAO/WHO requested a review be undertaken and two risk profiles of the organism have been published (Codex Alimentarius Commission, 2003a, b; Iversen and Forsythe, 2003). A full risk assessment of the organism will require greater knowledge of its presence in food, especially those consumed by neonates and infants. However the only detailed survey of *Ent. sakazakii* and other Enterobacteriaceae in IFM was published 15 years ago (Muytjens et al., 1988). One hundred and forty-one powdered IFM samples from 36 countries were examined. *Ent. sakazakii* was isolated at low levels (0.36 cells/100 g) from 20 out of 141 infant formula products. A smaller survey by Nazarowec-White and Farber (1997) involved 5 manufacturers (each supplying 24 samples) and reported the prevalence of the organism to be between 0% and 12% of the samples per manufacturer.

The method used by Muytjens et al. (1988) and Nazarowec-White and Farber (1997) was (a)

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pre-enrichment in Buffered Peptone-Water (BPW), (b) enrichment in Enterobacteriaceae enrichment (EE) broth, (c) plate on to Violet Red Bile Glucose Agar (VRBGA), (d) picking five Enterobacteriaceae colonies on to TSA plates and incubate at 25°C for 48–72 h to observe yellow pigment production (a typical trait of *Ent. sakazakii*) and finally (e) confirmation by biochemical profile according to API20E (bioMérieux UK Ltd.). This method has been adopted by the FDA for isolation of *Ent. sakazakii* from IFM and milk powders with the modification of pre-enrichment in distilled water rather than BPW (URL: http://www.vm.cfsan.fda.gov/~comm/mmesakaz.html). Unfortunately, *Ent. sakazakii* could be outgrown by other Enterobacteriaceae during the pre-enrichment and enrichment stages, leading to relatively few *Ent. sakazakii* colonies on VRBGA and subsequently a reduced chance of picking the organism onto TSA. Consequently the chromogenic Druggan–Forsythe–Iversen (DFI) medium was developed which shows better sensitivity (87.2%) and specificity (100%) than the Muytjens et al. (1988) procedure (Iversen et al., 2004). The medium uses the chromogen indolyl substrate 5-bromo-4-chloro-3-indolyl-α,D-glucopyranoside (XαGlc) to differentiate *Ent. sakazakii* colonies (100% α-glucosidase positive) from glucosidase negative Enterobacteriaceae. The method obtains results two days sooner than the conventional method (Iversen et al., 2004).

Since the Muytjens et al. (1988) survey may not reflect the microbiological content of current powdered infant formula products, the prevalence of *Ent. sakazakii* in powdered IFM and dried infant foods was assessed using samples collected in the period March to October 2003. Twenty-five gram samples were analysed as standard for specified enteric pathogens (ICMSF, 2002) and as previously used by Heuvelink et al. (2001) for *Ent. sakazakii* in milk powders. Other sources of the organism also investigated included dried infant foods, lactose powder, cheese products and dry food ingredients. In order to assess the general hygienic status of the samples, the presence of other Enterobacteriaceae, including where appropriate *Salmonella*, was also determined.

2. Methods

2.1. Food sample preparation

The survey was composed of 82 powdered IFM, 49 dried infant foods, 72 milk powders, 8 lactose powders, 62 cheese products (40 hard cheese, 17 soft cheese, 3 cheese sauces, 2 cheese spreads), 25 fresh foods (7 meats, 18 salads), 122 herbs and spices and 66 non-herb or spice dry food ingredients (e.g. nuts, fruits and grains). A total of 387 brands from 89 suppliers were surveyed. All samples except for 12 herbs and spices, 1 lactose, 35 IFM and 18 milk powders were purchased from retailers across the UK. The remaining IFM and milk powders were purchased from other European countries, Asia, Africa and USA. The IFM, dried infant foods and milk powders were manufactured in South Africa, South Korea, Holland, Spain, Switzerland, USA, Belgium, Ireland, Slovenia and UK.

Twenty-five grams of each sample was homogenized for 1 min at medium speed in a Seward Stomacher® 400 Laboratory Blender (Seward, Thetford, UK) in 225 ml buffered peptone-water (CM 509 Oxoid Ltd., Basingstoke, UK). An aliquot (1 ml) of the homogenate was removed for aerobic plate count and Enterobacteriaceae enumeration (described below). The remaining material was incubated overnight at 37°C for *Ent. sakazakii* and *Salmonella* detection (described below).

2.2. Aerobic plate count (APC) and Enterobacteriaceae enumeration

Decimal dilutions (0.1 ml) of the food homogenate in BPW were used to inoculate PCA (CM325, Oxoid Ltd.) and Violet Red Bile Glucose agar (VRBGA, Oxoid Ltd.) using the surface spread method. The plates were incubated at 37°C for 24 h. On VRBGA Enterobacteriaceae form small red colonies (~1 mm diameter), with or without a red precipitate.

2.3. Salmonella isolation

The presence or absence of *Salmonella* in 25 g samples in IFM, dried infant foods and milk powder samples was assessed according to the ISO 6579 (2002) standard and immuno-magnetic separation (IMS) methods. For the ISO 6579 method samples were pre-enriched in BPW, as described above, enriched in Rappaport–Vassiliadis Soya Peptone (RVS) broth (CM866 Oxoid Ltd.) and Müller–Kauffmann tetrathionate-novobiocin (MKTTn) broth (CM343 Oxoid Ltd. supplemented with 19 ml iodine–iodide solution, 9.5 ml 0.1% brilliant green solution and 0.04 g novobiocin per litre) for 24 h at 37°C and 42°C, respectively. The enriched samples were streaked on XLD medium (CM469 Oxoid Ltd.) and Brilliant Green Agar (BGA, CM329 Oxoid Ltd.) and incubated at 37°C for 24 h. An automated IMS system (Bead Retriever™, Dynal Biotech Ltd., Preston, UK) was also used to recover *Salmonella* according to the manufacturer's instructions. The IMS procedure was conducted on 24 h BPW broth enrichment samples and the anti-Salmonella Dynabeads™ were plated on XLD and BGA (Mansfield and Forsythe, 1996). Presumptive *Salmonella* isolates were confirmed using API20E biochemical profile (bioMérieux UK Ltd., Basingstoke, UK). *Salmonella* Enteritidis NCTC 3046 was used as the positive control organism.

2.4. Conventional *Ent. sakazakii* isolation procedure

The conventional *Ent. sakazakii* isolation method was as according to Muytjens et al. (1988). Ten millilitres of the BPW pre-enrichment was added to 90 ml EE broth (CM317 Oxoid Ltd.) and further incubated for 24 h. EE broth contains bile salts and brilliant green to suppress the growth of non-Enterobacteriaceae. The overnight EE broth was streaked on VRBGA (CM485 Oxoid Ltd.) and colony morphology observed after incubation at 37°C for 24 h. Five red colonies were picked off and streaked on TSA (CM131 Oxoid Ltd.). Colonies that produced yellow pigment after incubation at 25°C for 48–72 h were termed presumptive *Ent. sakazakii* and their identity was confirmed using API20E (bioMérieux UK Ltd.). Non-yellow colony types were also picked and identified using API20E (bioMérieux) to determine which other Enterobacteriaceae were present in the sample after enrichment. *Ent. sakazakii* strain NCTC 11467 was used as the positive control organism.

2.5. Isolation of *Ent. sakazakii* using Druggan–Forsythe–Iversen (DFI) medium

The incubated EE broth samples were streaked on DFI (an *Ent. sakazakii* specific chromogenic medium, composition described below) and incubated at 37°C. Presumptive *Ent. sakazakii* colonies appear entirely blue-green after 24 h incubation on DFI agar. The identification of presumptive *Ent. sakazakii* isolates was determined using API20E biochemical profiles. *Ent. sakazakii* strain NCTC 11467 was used as the positive control organism. To determine which other Enterobacteriaceae were present in the sample after enrichment, non-blue-green colonies were also picked from DFI and identified using API20E (bioMérieux).

2.6. DFI medium

Sodium deoxycholate (1 g/l, CAS 302-95-4, Sigma-Aldrich Co. Ltd., UK), 5-bromo-4-chloro-3-indolyl-α,D-glucopyranoside (0.1 g/l, CAS 108789-36-2, Glycosynth Ltd., Warrington, UK), sodium thiosulphate (1 g/l, CAS 7772-98-7, BDH Ltd., UK) and ammonium iron(III) citrate (1 g l, CAS 1185-57-5, BDH Ltd., UK) were dissolved in distilled water before addition of the basal medium Tryptone Soya Agar (TSA, 40 g/l) (Oxoid Ltd., UK). The complete medium (pH 7.3) was heated in a boiling water bath to dissolve the components before autoclaving at 121°C for 15 min. The sensitivity and specificity of DFI medium has already been described by Iversen et al. (2004).

3. Results

3.1. General flora and Enterobacteriaceae enumeration

The general microbial flora (APC) and Enterobacteriaceae counts for the 486 food samples are given in Table 1. In general the microbial loads for powdered IFM, milk powder and dried infant foods were lower than that for the other products (Table 1). The APC for the majority (149/203) of IFM, milk powder and dried infant food samples was 10^2 cfu/g or less, and no Enterobacteriaceae were enumerated from milk powders, lactose or dried infant foods. One powdered IFM sample had Enterobacteriaceae (*Klebsiella ozaenae*) at 200 cfu/g. No bacteria were isolated from any of the lactose samples tested. In contrast the majority of APC and Enterobacteriaceae counts of unprocessed foods (meat, salads, herbs and spices) were $> 10^3$ cfu/g (Table 1).

3.2. Enterobacteriaceae isolated from enriched IFM, dried infant foods and milk powders

Table 2 tabulates the Enterobacteriaceae isolated after EE broth enrichment of 203 powdered IFM, dried infant foods and milk powder samples. The most frequently isolated organisms were *Ent. cloacae* (15 samples), *Pantoea* spp. (11 samples) and *Ent. sakazakii*

Table 1
Variation in aerobic plate count and Enterobacteriaceae counts

Sample	Aerobic plate count (cfu/g)					Enterobacteriaceae (cfu/g)				
	$<10^2$	10^2	$>10^2$–10^3	$>10^3$–10^4	$>10^4$	$<10^2$	10^2	$>10^2$–10^3	$>10^3$–10^4	$>10^4$
Powdered IFM (82)[a]	56[b]	22	14	6	2	99	0	1	0	0
Dried infant food (49)	74	14	6	6	0	100	0	0	0	0
Milk powders (72)	40	18	22	20	0	100	0	0	0	0
Lactose powders (8)	100	0	0	0	0	100	0	0	0	0
Cheese products (62)	68	2	18	11	1	92	5	0	3	0
Fresh foods (25)	0	0	8	8	84	0	0	8	4	88
Herbs and spices (122)	22	4	7	26	41	65	4	12	6	13
Dry food ingredients other than herbs and spices (66)	46	9	21	15	9	82	3	8	4	3

[a] Number of samples analysed are given in parenthesis.
[b] Percentage of samples.

Table 2
Enterobacteriaceae isolated from powdered infant formula milk, milk powder and dried baby foods after enrichment in EE broth

Organism	Powdered infant formula milk (82)[a]	Dried infant food (49)	Milk powder (72)	Total
Enterobacter cloacae	1	1	13	15
Ent. sakazakii	2	5	3	10
Ent. amnigenus	0	0	4	4
Pantoea spp.	2	5	4	11
Escherichia hermanii	0	0	3	3
Esch. vulneris	0	2	1	2
Esch. coli	0	1	0	1
Leclercia adecarboxylata	0	0	2	3
Klebsiella ozaenae	1	0	1	2
K. pneumoniae	0	0	1	1
Raultella terrigena	0	0	2	2
Serratia ficaria	1	0	2	3
Rahnella aquatilis	1	0	0	1
Citrobacter freundii	1	0	0	1

[a] Number of samples analysed is given in parenthesis.

Table 3
Presumptive and true positive samples for *Enterobacter sakazakii*

Sample	No. of samples	Presumptive positive		*Ent. sakazakii* positive	
		DFI[a]	VRBGA[b]	DFI[c]	VRBGA[c]
Powdered IFM	82	2	6	2	2
Dried infant food	49	5	9	5	3
Milk powders	72	4	10	3	0
Lactose powders	8	0[d]	0	0	0
Cheese products	62	2	2	2	2
Fresh foods	25	3	1	0	0
Herbs and spices	122	69	24	40	11
Dry food ingredients other than herbs and spices	66	18	18	15[d]	1
Total	486	104	70	67	19

[a] Entire blue-green colonies on DFI agar after 24 h incubation.
[b] Red colonies on VRGBA and yellow pigmentation on TSA.
[c] API 20E profile of presumptive isolate matched *Ent. sakazakii*.
[d] 0 indicates no presumptive isolates in 25 g sample.

(10 samples). *Salmonella* was not detected in any of samples.

3.3. *Ent. sakazakii* detection

Table 3 summarizes the presumptive and confirmed identification of *Ent. sakazakii* for 486 food samples. Sixty-seven samples were found to be positive for *Ent. sakazakii* using the DFI medium compared with only 19 using the conventional method. A greater proportion of presumptive isolates were also confirmed as *Ent. sakazakii* using the DFI medium than the conventional method (Table 3). There were 72.9% false positive presumptive *Ent. sakazakii* with the conventional method, compared with 38.5% with DFI agar. No samples that were positive by the conventional method were negative using DFI medium. In addition the DFI medium detected the organism 2 days before the conventional procedure, since a 2 day incubation period on TSA is not required prior to confirming identification using API20E. Eleven samples contained more than one *Ent. sakazakii* strain, as shown by different API20E profiles.

Both detection methods isolated *Ent. sakazakii* from the same two powdered IFM samples. No Enterobacteriaceae were isolated from either of these samples prior to enrichment in EE broth. The organism was isolated from five dried infant foods which contained rice, fruit or herbs. Only the DFI medium isolated *Ent. sakazakii* from three of the milk powders. Both methods isolated *Ent. sakazakii* from the same two cheese products. Forty herb and spice samples were positive for *Ent. sakazakii* using the DFI medium, whereas only 11 were positive by the conventional method. *Ent. sakazakii* was isolated from 15/66 dry ingredients including soy protein, almonds, coconut powder, pistachio, sunflower seeds, sesame seeds, lentils, ground maize, ground rice, sponge mix, soup, beanfeast, and vegetable suet. Compared with the conventional method, *Ent. sakazakii* was isolated from three milk powders, two additional dried infant foods, 29 herbs and spices and 14 additional dry food ingredients using the DFI medium compared to the conventional method.

4. Discussion

The APC of 75/82 powdered IFM samples was less than 10^3 cfu/g (Table 1). Sixteen were between 10^3 and 10^4 cfu/g and two were $>10^4$ cfu/g which is the maximum limit set by CODEX for dried and instant products (Codex Alimentarius Commission, 1979). The milk powder, lactose and dried infant food samples all had APC lower than the CODEX maximum (10^4 cfu/g), although 14/72 milk powders had APC between 10^3 and 10^4 cfu/g which is close to the maximum set criteria.

As expected, due to good manufacturing practice no Enterobacteriaceae were detected following direct

plating of rehydrated milk powder, lactose or infant foods on to VRGBA (Table 1). Sixteen out of 122 herbs and spices contained Enterobacteriaceae $>10^4$ cfu/g which exceeded the maximum acceptable level (10^4 cfu/g) during the shelf-life of the product (IFST, 1999). In contrast, most (54/66) non-herb and spice dry food ingredients did not contain Enterobacteriaceae, although five samples had $>10^3$ cfu/g, two containing numbers greater than 10^4 cfu/g. Microbiological criteria for good manufacturing practice of these ingredients would be <10 with a maximum of 10^3 Enterobacteriaceae/g (IFST, 1999). As would be expected for uncooked foods such as raw meat and salads, the Enterobacteriaceae count was predominantly $>10^4$ cfu/g.

Only one powdered IFM sample contained detectable Enterobacteriaceae without enrichment in EE broth (Table 1). This sample had a corresponding APC of 1.6×10^3 cfu/g which is higher than most of the other samples and *Ent. sakazakii* was not isolated from it. In contrast 59 strains of Enterobacteriaceae were recovered from powdered IFM, dried infant foods and milk powders after enrichment in EE broth (Table 2). Forty-nine samples contained only one Enterobacteriacae, four contained two species. These Enterobacteriaceae could have been below the initial plate count detection limit (100 cfu/g) or were sublethally injured and unable to form colonies on VRBGA without resuscitation. The Enterobacteriaceae isolated included *Enterobacter* species, *Pantoea* species, *Esch. coli* and *Klebsiella* species. The most frequently isolated organisms being *Ent. cloacae* and *Pantoea* spp. (Table 2). Muytjens et al. (1988) reported that 52.5% of powdered IFM contained Enterobacteriaceae and the two species most frequently isolated were *Ent. agglomerans* (35/141) and *Ent. cloacae* (30/141). However the identity of *Ent. agglomerans* in the Muytjens paper is uncertain since the *Enterobacter–Pantoea–Citrobacter* group has been revised and the former *Ent. agglomerans* description now encompasses both *Pantoea* spp. and *Esch. vulneris* (Janda and Abbott, 1998). The FAO/WHO (Codex Alimentarius Commission, 2003a) are evaluating the organisms of concern in powdered IFM and therefore it is important to recognize that various members of the Enterobacteriaceae may be present in high-risk foods despite the absence of recovery by direct enumeration.

Ent. sakazakii was isolated from 2/82 powdered IFM samples (Table 3) by both detection methods. One of the two *Ent. sakazakii* positive samples had a negative APC while the other had greater than $>10^4$ cfu/g, Enterobacteriaceae were not detected in either of the samples by direct plating. *Ent. sakazakii* was isolated from five out of 49 dried infant foods. Two of the positive samples contained herbs, one was fruit-based, one was rice-based and one contained both rice and fruit. Although these products have not been associated with outbreaks of *Ent. sakazakii* infection, they are nevertheless a potential source of the pathogen (Table 3). Although no Enterobacteriaceae were enumerated from 72 milk powder samples, *Ent. sakazakii* was isolated from 3 samples after enrichment (Tables 1 and 3). The manufacture of lactose powder (an ingredient of IFM) is at such a high level of hygiene that no bacteria were isolated directly or after enrichment procedures.

Ent. sakazakii was isolated from 40/122 herbs and spices and 15/66 other dried food ingredients. Although many of these ingredients would be heat-treated prior to ingestion, the high prevalence of the organism and the potential for cross-contamination means that these could be a source of *Ent. sakazakii* in a food manufacturing process. It is notable that *Ent. sakazakii* was frequently isolated from plant related material and this may reflect the ecology of the organism in common with *Pantoea* and other *Enterobacter* species (Janda and Abbott, 1998).

The conventional method only detected 19 samples containing *Ent. sakazakii* whereas the DFI method detected 67. For the conventional method five colonies are selected from the Enterobacteriaceae medium VRGBA and streaked on TSA (25°C, 48–72 h) for pigment production. This method however does not select for *Ent. sakazakii* and the combined use of EE broth and VRBGA could allow other Enterobacteriaceae to outgrow *Ent. sakazakii* leading to false negative results. It is not possible to select for *Ent. sakazakii* colonies from VRBGA plates on the basis of colony morphology. Therefore the greater the number of competitors the less chance that one of the 5 colonies picked will be *Ent. sakazakii*.

The powdered IFM, dried infant foods and milk powder samples were also tested for *Salmonella* using both the ISO 6579, 2002 method and IMS technique. No *Salmonella* serovars were isolated. Therefore during IFM, dried infant foods and milk powder production control of *Salmonella* did not result in the control of *Ent. sakazakii*. Organisms that were found in powdered IFM included *Ent. cloacae*, *Klebsiella* spp., and *Citrobacter freundii* (Table 2). These organisms have been associated with neonatal infections including necrotizing enterocolitis (NEC), which is the most common important gastrointestinal illness in the newborn. NEC has an incidence of 2–5% in premature infants and 13% in those weighing less that 1.5 kg at birth and is 10 times as common in babies fed formula milk compared with those fed breast milk (Lucas and Cole, 1990). In a study of 125 neonates with NEC, *Enterobacter* spp. were the most common organisms being isolated from 29% of patients (Chan et al., 1994). Three studies have shown that colonization with *Klebsiella* spp. increased the risk of NEC (Westra-Meijer et al., 1983). In addition NEC outbreaks due to multi-resistant *Klebsiella* strains have been reported (Blahova et al., 1998; Ben-Hamouda et al., 2003). There are no reports of infant feeds being

included in epidemiological investigations of *Klebsiella* related neonatal disease. However, powdered IFM is a potential source of neonatal colonization by *Klebsiella* and other genera of Enterobacteriaceae and may therefore be a risk factor to vulnerable infants. Infant food has been implicated as the source in a nosocomial outbreak due to *Citrobacter freundii* contamination (Thurm and Gericke, 1994).

It is essential that detection methods for *Ent. sakazakii* are established that are robust and reliable to ensure the safety of IFM. These results show that *Ent. sakazakii* is present in a wide range of food products, and may only be recoverable using the chromogenic DFI medium and not the conventional method (Table 3). The DFI medium requires a 2 day shorter incubation period than the standard protocol, can differentiate the organism in the presence of other Enterobacteriaceae, and has a higher probability of detecting *Ent. sakazakii* amongst competing Enterobacteriaceae from enrichment media.

This survey shows that *Ent. sakazakii* is found in high-risk products such as powdered IFM, dried infant food and milk powder as well as various plant related ingredients. The organism was isolated frequently from dried material, indicating an ability to survive desiccation and persist for long periods of time. Other Enterobacteriaceae were also present in these high-risk products. They were only detectable after enrichment in EE broth and not by direct enumeration on VRBGA which is the standard method of Enterobacteriaceae enumeration. It is therefore timely that the FAO/WHO is reviewing the Recommended International Code of Hygienic Practice for Foods for Infants and Children (Codex Alimentarius Commission, 2003a).

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Identification and Phylogeny of *Enterobacter sakazakii* Relative to *Enterobacter* and *Citrobacter* Species

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The phylogenetic relationships of *Enterobacter sakazakii* strains were investigated using 16S ribosomal DNA (rDNA) and *hsp60* sequencing. Each analysis distributed *E. sakazakii* strains among four clusters, indicating substantial taxonomic heterogeneity. The *E. sakazakii* type strain 16S rDNA sequence was 97.8% similar to that of *Citrobacter koseri* but 97.0% similar to that of *Enterobacter cloacae*.

In recent years, *Enterobacter sakazakii* has been associated with necrotizing enterocolitis, bacteremia, and infant meningitis through the ingestion of contaminated powdered infant formula milk (5). Since *E. sakazakii* has been reported to be 50% related to *Enterobacter cloacae* and *Citrobacter koseri* by DNA-DNA hybridization (3) and the latter organism produces brain abscesses similar to those caused by *E. sakazakii* (9), the 16S ribosomal DNA (rDNA) and *hsp60* housekeeping gene sequences from these organisms were compared.

A total of 126 strains identified as *E. sakazakii* by biochemical test kits (API20E and ID32E; bioMérieux) were sequenced. These included the type strain, NCTC 11467, and strains NCIMB 5920, NCIMB 8272, ATCC 51329, and ATCC 29004. Seven *E. sakazakii* strains were from European hospitals; seven strains were supplied by Jeff Farber, Health Canada; six strains were supplied by Nestlé-CRN, Vers-chez-les-Blanc, Lausanne, Switzerland; six strains were from J. Hoogkamp-Korstanje, St Radboud, Nijmegen, The Netherlands; and a further five strains were from the Centers for Disease Control and Prevention, Atlanta, Ga. All other strains were food and environmental isolates from the culture collection at Nottingham Trent University, Nottingham, United Kingdom (6). Nine *C. koseri* strains were also sequenced, including the type strain, ATCC 25408, and strains ATCC 25409, ATCC 25410, and ATCC 27026. Four additional *C. koseri* strains were from S. Townsend, Children's Hospital, Los Angeles, Calif., and one strain was obtained from a European hospital.

Comparative 16S rDNA sequence analysis using the MicroSeq 500 16S rDNA bacterial sequencing kit (Applied Biosystems) was performed according to standard procedures (2) at Accugenix (Newark, Del.). Sequencing of the *hsp60* gene was performed by AGOWA (Berlin, Germany) using the primer sequences GGT AGA AGA AGG CGT GGT TGC (forward) and ATG CAT TCG GTG GTG ATC ATC AG (reverse) (4) to generate a 341-nucleotide PCR product. Additional 16S and *hsp60* DNA sequences from type strains of *Enterobacter* species and *Citrobacter freundii* were obtained from GenBank. Sequences were aligned, corrected for multiple base changes (Jukes-Cantor method), and compared by using Bionumerics version 3.0 (Applied Maths, Kortrijk, Belgium). Relationships between strains were depicted in dendrogram form using the neighbor-joining method. Gaps and unknown bases were not considered in the analysis. The statistical significance of the branch points was evaluated by use of bootstrap analysis using 1,000 replicates. The trees were rooted to *Pantoea agglomerans*, and the scale bars (see Fig. 1) represent percentage sequence divergence as measured by the length of horizontal lines connecting any two strains. Bootstrap values derived from 1,000 replicates indicating the statistical significance of the branch points are also shown.

According to 16S rDNA sequence comparisons, the type strain of *E. sakazakii* was closer to *C. koseri* (97.8% similar) than to any other *Enterobacter* species, although *E. cloacae* was 97.0% similar and *C. freundii* was 96.0% related by this method. Our findings are consistent with the polyphyletic nature of *Enterobacter* spp. revealed by both 16S rDNA and *gyrB* sequence comparisons (1). In the absence of defined phylogenetic criteria for delineating genera, the results observed with *Citrobacter* and *Enterobacter* species suggest that further studies are warranted to clarify their relationship. The 16S rDNA-based analysis divided the *E. sakazakii* strains into four clusters (Fig. 1). The majority (110 strains) were in cluster 1, with 0.1 to 1.2% difference from the type strain. These included 17 clinical strains and 3 nonpigmented strains. Nine strains (including one clinical strain) exhibited 1.6 to 1.9% sequence divergence from the type strain and formed a second cluster closely related to *E. sakazakii* (Fig. 1). The third cluster contained five strains (including one clinical strain), which despite being identified as *E. sakazakii* by the API20E and ID32E kits were more closely related (97.5 to 97.8% similar) to taxa including *Enterobacter pyrinus*, *Enterobacter hormaechei*, and *C. koseri* (Fig. 1). This cluster included the Preceptrol strain ATCC 51329, which was 3.0% divergent from the *E. sakazakii* type strain. The fourth cluster contained two strains, one of which was a clinical isolate. These strains were also identified as *E. sakazakii* by API20E and ID32E, but their 16S rDNA sequences were just

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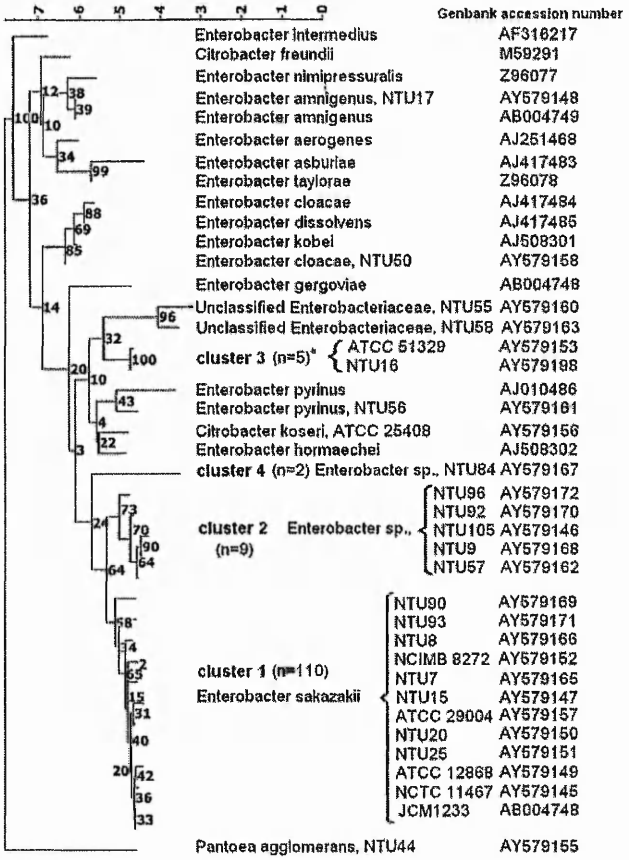

FIG. 1. Neighbor-joining tree of *E. sakazakii* and related organisms based on partial 16S rDNA sequences. Strains presented are representative of the total strains sequenced. Numbers in parentheses indicate the number of strains in a given cluster.

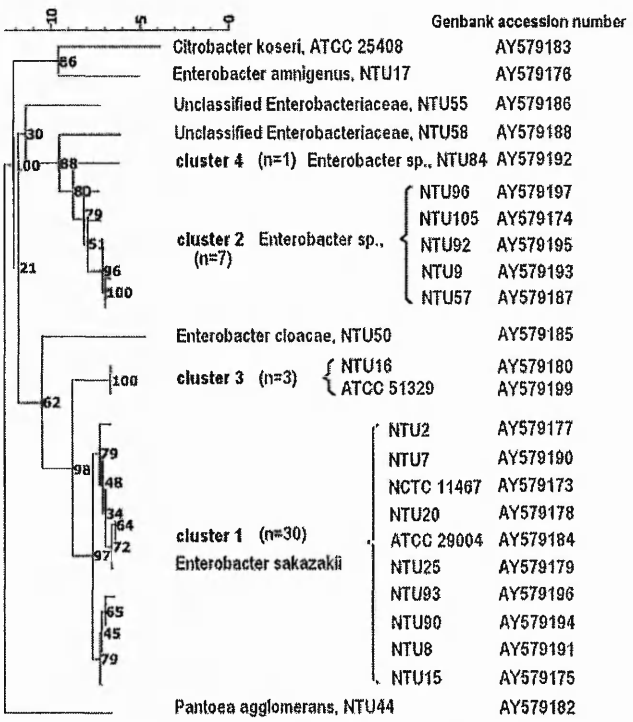

FIG. 2. Neighbor-joining tree of *E. sakazakii* and related organisms based on partial *hsp60* sequences. Strains presented are representative of the total strains sequenced. Numbers in parentheses indicate the number of strains in a given cluster.

96.5% similar to the type strain of that species. Our 16S rDNA sequence analysis also confirmed that several strains identified as *E. sakazakii* by commercial biochemical kits belonged to distinct species, including *Enterobacter amnigenus* and *E. cloacae* (7), as well as potentially novel taxa (Fig. 1).

hsp60 sequences were obtained for 47 representative strains, including 41 strains of *E. sakazakii* that were representative of the different strain origins and of the four different 16S rDNA clusters (Fig. 2). Although the bootstrap values for the 16S rDNA-defined *E. sakazakii*-like clusters were somewhat low (Fig. 1), strains assigned to each of the 16S rDNA-based clusters also formed distinct clades in the *hsp60* analysis (Fig. 2), although the tree topologies differed, reflecting the different similarity values obtained. Cluster 1 strains remained closely related (97.6 to 99.4% similar) to the *E. sakazakii* type strain, but cluster 2 (89.2 to 90.4% similar) and cluster 4 (88.8% similar) strains were more distinct. By contrast, cluster 3 isolates shared 95.2% *hsp60* sequence homology with the *E. sakazakii* type strain, and their position in the *hsp60*-derived dendrogram supports our classification of these strains as *E. sakazakii*-like strains. In contrast, the distinct positions of strains NTU55 and NTU58 suggest that these strains should not be considered to be *E. sakazakii* despite their identification as this species by some biochemical test kits.

This investigation has demonstrated that *E. sakazakii* is genetically diverse and potentially more taxonomically complex than hitherto realized. Clusters 2, 3, and 4 described here may represent novel species closely related to *E. sakazakii*; further study is necessary to confirm their taxonomic positions. This need is due to the nonlinear relationship often observed between 16S rDNA sequence similarities and the "gold standard" for species delineation, i.e., quantitative DNA-DNA hybridization data (8). Nonetheless, the implications of our study for accurate identification of *E. sakazakii* are self evident and relevant to clinicians, epidemiologists, and infant formula manufacturers.

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Identification of *Enterobacter sakazakii* from closely related species: The use of Artificial Neural Networks in the analysis of biochemical and 16S rDNA data

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Abstract

Background: *Enterobacter sakazakii* is an emergent pathogen associated with ingestion of infant formula and accurate identification is important in both industrial and clinical settings. Bacterial species can be difficult to accurately characterise from complex biochemical datasets and computer algorithms can potentially simplify the process.

Results: Artificial Neural Networks were applied to biochemical and 16S rDNA data derived from 282 strains of *Enterobacteriaceae*, including 189 *E. sakazakii* isolates, in order to identify key characteristics which could improve the identification of *E. sakazakii*. The models developed resulted in a predictive performance for blind (validation) data of 99.3 % correct discrimination between *E. sakazakii* and closely related species for both phenotypic and genotypic data. Three main regions of the partial rDNA sequence were found to be key in discriminating the species. Comparison between *E. sakazakii* and other strains also constitutively positive for expression of the enzyme α-glucosidase resulted in a predictive performance of 98.7 % for 16S rDNA sequence data and 100% for phenotypic data.

Conclusion: The computationally based methods developed here show a remarkable ability in reducing data dimensionality and complexity, in order to eliminate noise from the system in order to facilitate the speed and reliability of a potential strain identification system. Furthermore, the approaches described are also able to provide valuable information regarding the population structure and distribution of individual species thus providing the foundations for novel assays and diagnostic tests for rapid identification of pathogens.

Background

Enterobacter sakazakii is an emergent pathogen associated with ingestion of infant formula milk that can lead to neonatal meningitis, necrotising enterocolitis and sepsis [1-5]. The International Commission for Microbiological Specifications for Foods [6] has ranked *E. sakazakii* as 'Severe hazard for restricted populations, life threatening or substantial chronic sequelae or long duration'. Therefore as there is no accepted gold standard methodology, the correct definition and identification of *E. sakazakii* is important for powdered infant formula manufacturers, as well as regulators, clinicians and epidemiologists.

In 1980, Farmer and co-workers [7] defined the species and described fifteen biogroups according to biochemical profiles. A defining characteristic has been activity of the α-glucosidase enzyme. Consequently selective, differential media incorporating chromogenic or fluorogenic α-glucosides such as the indolyl substrate 5-bromo-4-chloro-3-indolyl-α, D-glucopyranoside have been developed [8,9]. It has been reported that 100% of *E. sakazakii* (n = 129) were positive for α-glucosidase in comparison to 0% of other *Enterobacter* species (n = 97) [10]; however a small number of other *Enterobacteriaceae* test positive for this enzyme.

Recently 16S rDNA sequencing has revealed that commercial biochemical test kits identified more than one species as '*E. sakazakii*' [11], and that there are at least four genetically and biochemically distinct subgroups of *E. sakazakii*. In this study we applied Artificial Neural Networks (ANNs) [12-14] to biochemical and 16S rDNA data in order to identify key phenotypic characteristics and nucleotide sequences which could improve the identification of *E. sakazakii* in respect to, a) other *Enterobacteriaceae*, and b) non-*E. sakazakii* α-glucosidase positive *Enterobacteriaceae*.

ANNs are adaptive, non linear forms of Artificial Intelligence (AI) inspired by the way the human brain learns and processes information in order to solve specific problems, such as pattern recognition and classification problems. The multi-layer perceptron (MLP) ANN is a form of feed-forward ANN architecture which contains several layers, with each node in one layer being connected to every node in the next by a series of weighted links. When used with the back-propagation algorithm, this type of ANN learns in a fashion analogous to the way learning in the human brain is carried out, that is, by example. In humans, learning involves minor adjustments being made to the synaptic connections between neurons, in ANNs, learning is achieved by updating the weights that exist between the processing elements that constitute the network topology.

ANNs were applied to biochemical and 16S rDNA data derived from 282 strains of *Enterobacteriaceae*, including 189 *E. sakazakii* isolates, in order to identify key characteristics which could improve the identification of *E. sakazakii*. Results show that ANNs have the potential to identify key features from the data, both for biochemical tests and sequence data. These key features may then be used to form the basis of novel rapid identification systems, which have the ability to classify samples by strain and eliminate the risk of false positive and negative results.

Results

Food, clinical and environmental isolates of *E. sakazakii* were shown by 16S rDNA analysis to form four clusters. A summary of the main cluster groups is shown in Figure 1. The clusters that were positive for constitutive X-α-glucoside metabolism were the four *Enterobacter sakazakii* groups, *Buttiauxella noakiae*, and two clusters of *Enterobacteriaceae* (groups 5 and 6 in Figure 1) that could not be matched, either by genomic or biochemical profile, to any currently named species.

Model development and classification analysis

A MLP ANN was used together with the back-propagation algorithm. Inputs to the network represented biochemical test results or sequence ID; two hidden nodes were used in the hidden layer for mathematical feature detection and a single output node was used to represent species class, with a class assignment of 1 representing *E. sakazakii* strains, and 2 representing all other strains. Models were developed utilising a random sample cross validation approach where 100 random training/test/validation submodels were run and evaluated. This repeated random sampling guarantees that all samples are treated as blind data a number of times, to ensure model generality and to enable confidence intervals to be calculated for each sample. For each of the models a full analysis was conducted including sensitivity analysis to determine the importance ratio of each input. This process removed all of the inputs singularly from the model. The error of predictions was then measured for each of the inputs removed. The sensitivity ratio was then calculated based on the performance with and without the given input. The hypothesis here is that if a given input is important its removal will have strong negative effect on predictive performance. Therefore a sensitivity ratio greater than one indicates an input whose removal is detrimental for the model. Additionally, the analysis of predictive performance was performed to evaluate model accuracy, sensitivity and specificity, and assessment of the raw ANN predictions was conducted for the positioning of individuals within the population.

Phenotypic data

Using the phenotypic data, the models developed resulted in a predictive performance for blind (validation) data of

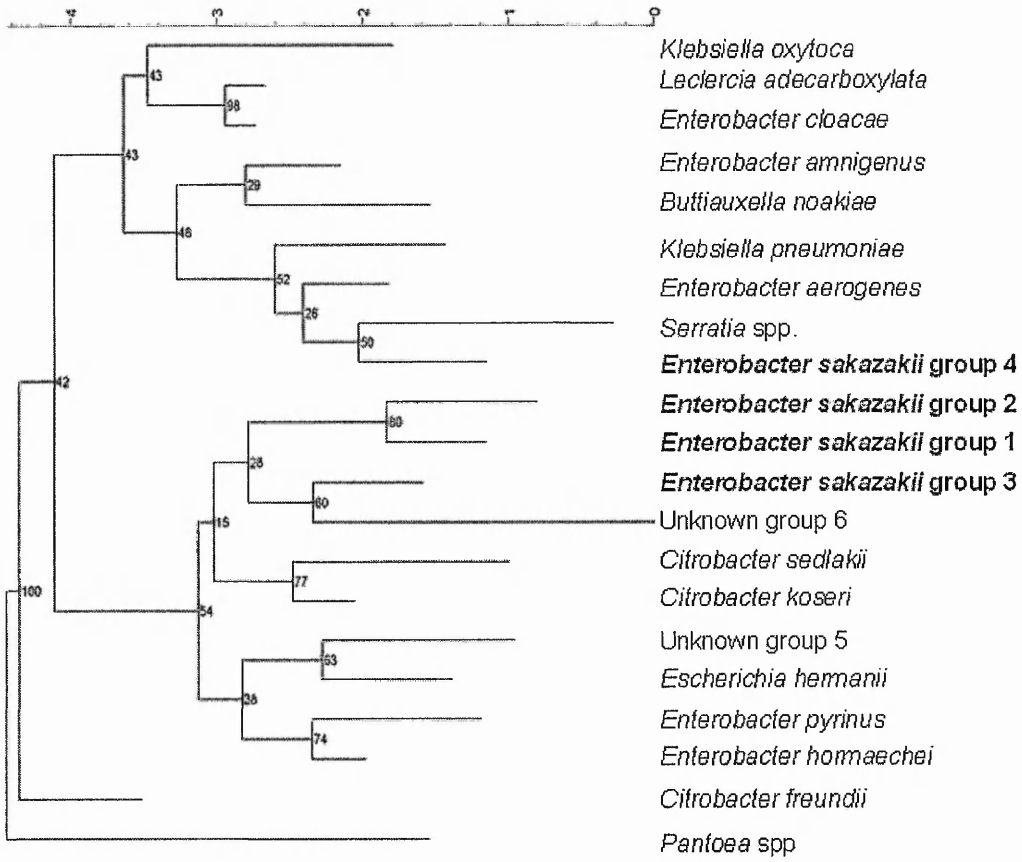

Figure 1
Summary partial 16S rDNA sequence Neighbour Joining tree of *E. sakazakii* and related organisms. Bootstraps were derived from 1000 replicates and the Jukes-Cantor correction was applied.

99.3 % (sensitivity of 100 % and specificity of 97.6 %) correct discrimination between *E. sakazakii* and closely related species. The population distribution was also examined by plotting the individual predictions from the ANN models (Figure 2). A model prediction of one indicates a sample is *E. sakazakii* whilst a two is indicative of another species, so as this value increases from one to two, the more characteristic a sample is of non-*E. sakazakii* origin. This distribution shows the variation that is present not only between the same strains, but also across species, which is why correct identification of pathogens can often be extremely difficult, with strains having the potential for frequent mutation and change.

16S rDNA sequence data

The analysis was also repeated using 16S rDNA sequence data to identify any areas of the sequence that could potentially be used to differentiate between the different species. The models developed produced predictive performances for blind (validation) data to an accuracy of once again, 99.3 % (with sensitivity and specificity values of 99.5 and 98.9 % respectively) of samples correctly identified as *E. sakazakii* or other species. There were three main regions of the sequence which were key in discriminating between the species. These regions all occur amongst regions that vary structurally among domains (see secondary structure Figure 3). Table 1 shows the 20 nucleotides with greatest relative importance and it is evident that they all appear to be derived from these focal positions in the sequence.

Classification of α-glucosidase positive strains

Furthermore, the study was expanded in order to elucidate whether the ANNs could be used to differentiate between

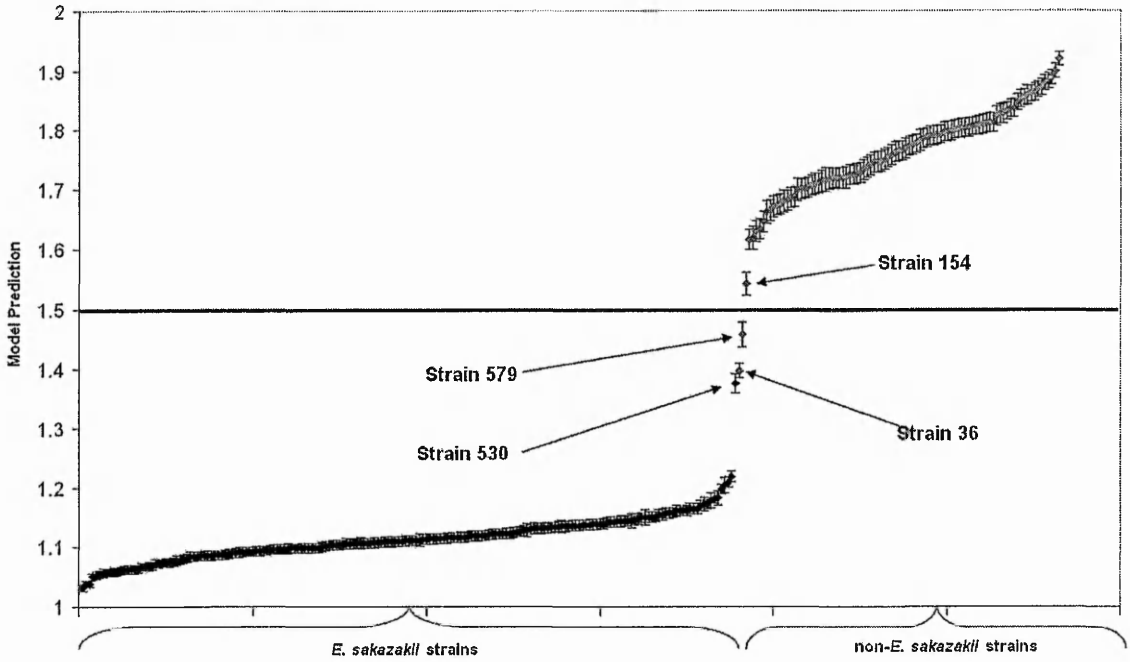

Figure 2
Population distribution of samples from the biochemical test data. Strains coloured blue represent *E. sakazakii* samples, whilst those in red represent non-*E. sakazakii*. The line at a predicted value of 1.5 represents the threshold for class prediction. Error bars indicate 95 % confidence intervals, and labelled samples highlight those which were either misclassified or close to being so.

E. sakazakii and a number of other *Enterobacteriaceae* which test positive for constitutive metabolism of X-α-glucoside. The same approach was used as above, with both phenotypic tests and 16S rDNA sequencing used as inputs in the ANN models. Once again analysis using the ANN based approach proved to be extremely successful. Using the 16S rDNA sequence data as inputs, the predictive performance of the ANN models was 98.7 % (92.9 % sensitivity, and 100 % specificity). This improved further still when the biochemical test data results were used as inputs into the model. Here, 100 % of the strains were correctly predicted into their respective classes, further highlighting the capabilities of ANN modelling in bacterial identification, which could potentially reduce the risk of false positive identification. The most relevant biochemical tests are summarised in Table 2, showing percent positive strains for *E. sakazakii* as well as other α-glucosidase positive and negative *Enterobacteriaceae*.

Discussion

Models have been developed to identify (i) key biochemical tests and (ii) important areas of the DNA sequence which can be used in the accurate discrimination of *E. sakazakii* from other closely related species. Furthermore, the study was expanded to differentiate between *E. sakazakii* strains and other α-glucosidase positive *Enterobacteriaceae*. To date methods for the isolation and identification of *E. sakazakii* have used the α-glucosidase reaction and production of yellow pigment as presumptive differentiating characteristics. However these methods can result in presumptive false positives due to groups of as yet undefined non-*E. sakazakii Enterobacteriaceae* which are also positive for both of these characteristics. Use of yellow pigment as a defining characteristic can also result in false negatives due to the occurrence of non-pigmented *E. sakazakii* and the occasional transient nature of this trait. While there is no single test that can be used to differentiate *E. sakazakii* from other species we identified biochemical profiles that may help to improve the likelihood of correct species identification.

Deriving a population distribution (Figure 2) from the analysis of the influence of biochemical tests in sample classification showed samples to appear in distinct clus-

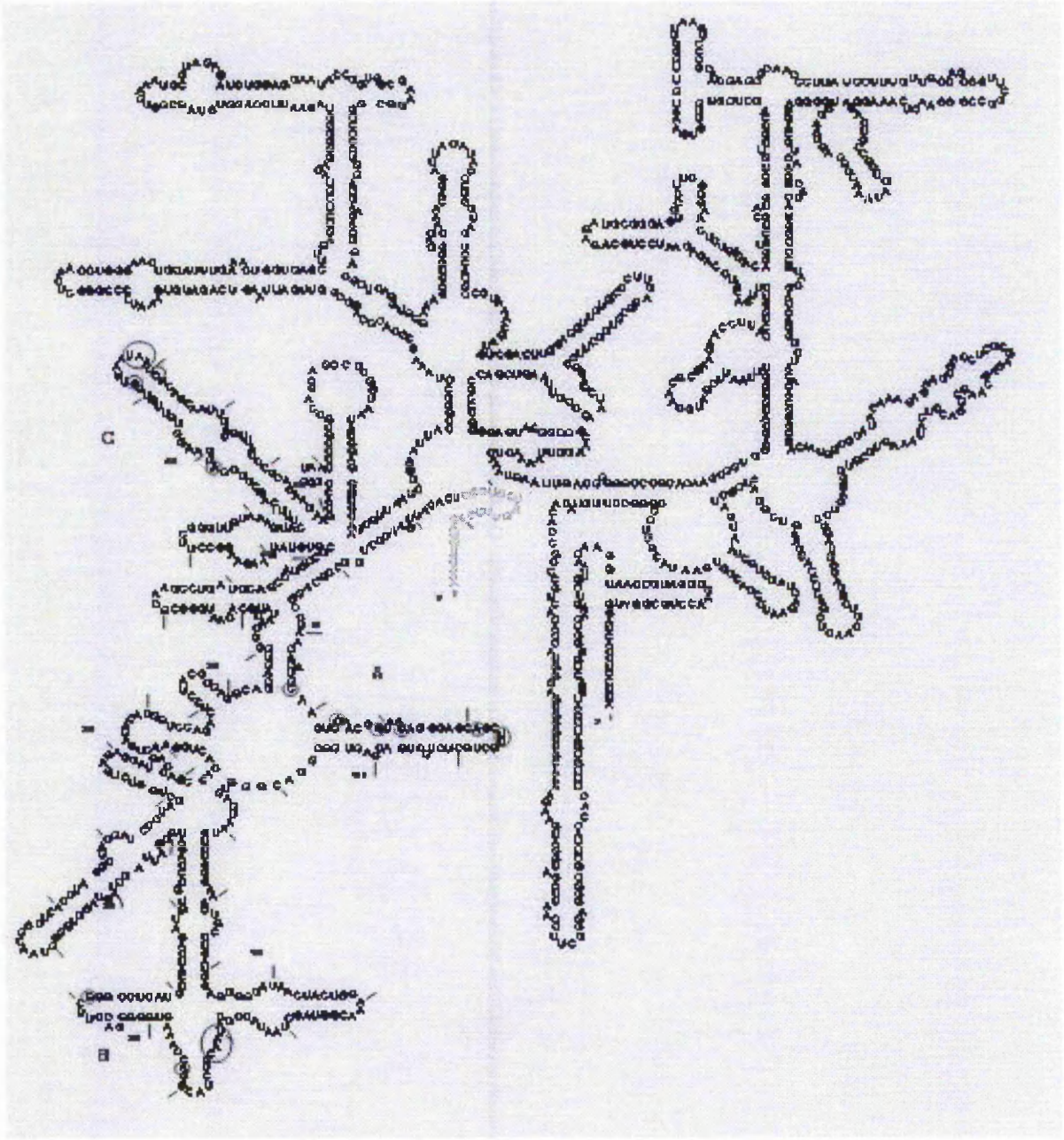

Figure 3
Secondary structure: small subunit ribosomal RNA of *E. sakazakii* NCTC 11467. Nucleotides in green and blue represent primer sequence targets. Pink circles denote regions of importance as determined by Artificial Neural Networks. Nucleotide numbering follows the Reference Numbering System used for *E. coli* J01695 [15]. Every 10th nucleotide is marked with a red tick mark and every 50th nucleotide is numbered. (Structure courtesy of Doug Smith, Accugenix, DE, USA).

Table 1: Top 20 nucleotides involved in classification from partial 16S rDNA data. Regions shown in this table are highlighted in Figure 3.

REGION		
A	B	C
58	180	448
64	181	462
69	182	467
72	183	468
81	192	469
84	211	471
85		

ters. This supports the interpretation of the partial 16S rDNA clustering (Figure 1). The ANN model incorrectly identified two of the non-*E. sakazakii* strains as being *E. sakazakii*. The identities of these strains are highlighted in Figure 2, and these samples may provide a basis for further studies because they are being incorrectly classified as a result of them displaying characteristics of both of the two groups, but are being determined to be more related to the *E. sakazakii* group. Alternatively, since the 16S identification is based on differences between a number of nucleotide bases, the combinations of these is different for different species. The ANN models search for common elements of these bases, which are consistently represented in each class, and classifies based on these commonalities. Considering this, together with the incorrectly identified non- *E. sakazakii* strains, leads to the view that there may not be one base, or a series of bases, that are unique to *E. sakazakii*, and in some strains, such as those incorrectly identified by the model, common elements exist between *E. sakazakii*, and other strains.

Results from the analysis of the 16S rDNA data indicate that the key inputs identified were present in three distinct areas of the sequence and these areas were subsequently all regions that varied structurally among domains (Figure 3).

Conclusion

ANNs display their potential use in reducing model dimensionality and complexity, in order to facilitate the speed and reliability of a potential strain identification system. These methods are also able to provide valuable information regarding the population structure and distribution of individual species. These technologies may provide the foundations for novel assays and diagnostic tests for rapid identification of pathogens, and subsequently reducing the risk of incorrect diagnosis due to the occurrence of false positive and negative test results.

Methods

Genotypic and phenotypic data was collected for 282 strains of *Enterobacteriaceae*, including 189 *E. sakazakii* isolates and 39 other α-glucosidase positive strains. Strains were from diverse food, clinical and environmental sources worldwide. Clinical isolates were from cases occurring over the last 25 years. At least one original strain from each of the biogroups described when the *E. sakazakii* species was designated were included [7].

Phenotypic data

Biochemical characteristics were derived from commercial test kits (API 20E and ID32E, bioMérieux UK Ltd.; Biolog GN2, Biolog, CA; and Microbact 24E, Oxoid UK Ltd.) and conventional manual tests as per standard protocols. Tests were performed in triplicate on separate days. Motility was determined at 37 °C after 24 h and 48 h using motility medium (tryptose 10 g l^{-1}, NaCl 5 g l^{-1}, agar 5 g l^{-1}, pH 7.2

Table 2: Biochemical tests for the differentiation of *E. sakazakii*.

Biochemical Tests	*E. sakazakii* (n = 189)	other α-glucosidase positive strains (n = 39)	other Enterobacteriaceae (n = 54)
α-glucosidase	100 *	100	0
Arginine dehydrogenase	97	13	67
Citrate	99	15	80
D-saccharic acid	0	23	33
Dulcitol	8	80	28
glucose-1-phosphate	0	59	83
glucose-6-phosphate	0	46	82
Lipase	96	44	4
Methyl Red	5	95	57
Ornithine decarboxylase	91	0	74
Pyruvate	3	92	50
Raffinose	100	15	63
Sucrose	100	21	47
Voges Proskauer	96	0	44
Yellow pigment	98	90	28

* denotes percent strains from the data set which were positive for the test

± 0.2. Acid production from carbohydrates was tested in phenol red broth base (10 g l^{-1} peptone, 1 g l^{-1} yeast extract, 5 g l^{-1} NaCl, 0.018 g l^{-1} phenol red) with addition of filter-sterilized carbohydrate solution (final concentration 0.5%). Gas production was determined by collection in Durham tubes. The methyl red test was performed at 48 h on cultures grown in MR-VP broth (VWR, 1.05712.0500). The Voges-Proskauer test was performed at 24 h by addition of 40% potassium hydroxide in water and 5% 1-naphthol in ethanol to cultures grown in MR-VP broth. Indole production was measured at 24 h by addition of Kovacs reagent (5 g p-dimethylaminobenzaldehyde, 25 ml HCl, 75 ml pentanol-1-ol) or James Reagent (70542 bioMérieux) to cultures grown in Peptone Water (CM0009 Oxoid Ltd). Nitrate reduction was measured by addition of 1% sulphanilamide in 1 M HCl and 0.02% N-1 naphthylene diamine HCl in water. Zinc dust was added to negative tubes to confirm the presence of unreduced nitrate. Constitutive metabolism of X-α-glucoside was determined by formation of blue-green colonies on media containing 5-bromo-4-chloro-3-indolyl-α, D-glucopyranoside (Chromogenic Enterobacter sakazakii medium (DFI formulation) CM1055, Oxoid Ltd.; and ESIA, AES Laboratoire, France).

Comparative 16S rDNA sequencing

This was performed by Accugenix (Newark, DE, USA) using the MicroSeq™ 500 16S rDNA Bacterial Sequencing Kit (Applied Biosystems). DNA was prepared for PCR by quick-heat lysis by removing one colony into a tube of PrepMan Ultra™ (Applied Biosystems) and placed at 99°C for 10 min. Two microlitres of genomic DNA was amplified in 50 μl of a master mixture consisting of 0.4 μM TGGAGAGTTTGATCCTGGCTCAG and TACCGCGGCTGCTGGCAC primers, 200 mM deoxynucleoside triphosphates, PCR buffer, 0.3 U of AmpliTaq DNA polymerase, and 10% glycerol. PCR conditions were 95°C for 10 min; 30 cycles each of 95°C for 30 s, 60°C for 30 s, and 72°C for 45 s; and a final step at 72°C for 10 min. Purification of the PCR product to remove excess primers and nucleotides was performed using Montage SEQ_{96} filter plates (Millipore). Cycle sequencing was performed with the sequencing module, and after removal of excess dyes using Montage SEQ_{96} filter plates (Millipore), the labelled extension products were separated on an ABI 3100 16 capillary genetic analyzer (Applied Biosystems). Partial sequencing was performed for all isolates, the length of the partial rDNA was 528 nucleotides, and in addition the full sequence for the *E. sakazakii* type strain (NCTC 11467) was obtained.

The data was analysed using Bionumerics (Applied Maths, Belgium) to construct Neighbour Joining trees, bootstraps were derived from 1000 replicates and the Jukes-Cantor correction applied.

The full 16S sequence was used for the representation of the secondary structure of the small subunit ribosomal RNA of E. sakazakii NCTC 11467. Nucleotide numbering follows the Reference Numbering System used for E. coli J01695 [15].

List of abbreviations

AI Artificial Intelligence

ANN Artificial Neural Network

MLP Multi-Layer Perceptron

Authors' contributions

CI performed the biochemical characterizations, collated the test data and wrote the biochemical methods section and text relevant to *E. sakazakii*. LL co-developed and performed the computational analyses for the study, and drafted the manuscript. MW provided the 16S sequencing and wrote the 16S sequencing methods section. SF co-ordinated and managed the project. GB co-developed the analysis methods and co-ordinated the project. All authors read and approved the final manuscript.

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The biochemical differentiation of *Enterobacter sakazakii* genotypes

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Abstract

Background: *Enterobacter sakazakii* is an emergent pathogen that has been associated with neonatal infections through contaminated powdered infant milk formula. The species was defined by Farmer *et al.* (1980) who described 15 biogroups according to the biochemical characterization of 57 strains. This present study compares genotypes (DNA cluster groups based on partial 16S rDNA sequence analysis) with the biochemical traits for 189 *E. sakazakii* strains.

Results: Analysis of partial 16S rDNA sequences gave 4 well defined phylogenetic groups. Cluster group 1 was composed of the majority of strains (170/189) and included Biogroups 1–5, 7–9, 11, 13 and 14. Cluster 3 corresponded with Biogroup 15 and cluster 4 with Biogroups 6, 10 and 12. Cluster group 2 comprised a new Biogroup 16. For the isolates in this study, the four DNA cluster groups can be distinguished using the inositol, dulcitol and indole tests.

Conclusion: This study demonstrates an agreement between genotyping (partial 16S rDNA) and biotyping and describes a new biogroup of *E. sakazakii*.

Background

Enterobacter sakazakii is an emergent pathogen that is associated with neonatal infection [1,2]. Most reported outbreaks have occurred in neonatal intensive care units and some have been linked to the ingestion of contaminated powdered infant milk formula [3,4].

E. sakazakii was defined as a new species by Farmer *et al.* 1980. Using DNA-DNA hybridization the previously 'yellow pigmented *Enterobacter cloacae*' was shown to be 41–54% related to *Enterobacter* and *Citrobacter* species. Farmer *et al.* [5] described 15 biogroups of *E. sakazakii* based on biochemical profiles with the wild type Biogroup 1 being the most common. Since 1980 prokaryotic systematics has changed with the increasing use of 16S rDNA sequence analysis [6,7]. Harada and Ishikawa [8] used DNA sequence analysis of the *gro*EL operon to determine the phylogenetic relationship among *Enterobacter, Pantoea, Klebsiella, Serratia* and *Erwinia* species. Hoffmann and Roggenkamp [9] used *hsp*60 (*gro*EL homologue) DNA sequence variation to investigate *E. cloacae* polyphyletic groups. Previously, it has been shown by both partial 16S rDNA and *hsp*60 gene sequencing that isolates identified as *E. sakazakii* using commercial identification kits form at least four distinct clusters [10]. Recently, an Artificial Neural Network has been published which identified key bio-

chemical and 16S rDNA sequences that distinguish *E. sakazakii* from closely related organisms [11].

In this study, we compare the biogroups of 189 strains with the four 16S rDNA cluster groups. We also identified key biochemical characteristics for the differentiation of these four genotypes. This significantly extends the previous work of Farmer et al. [5], which used five strains to genetically define the species and a total of 57 strains to define biogroups.

Results

Biogroups

The biochemical profiles obtained for each strain were compared to the biogroups originally described by Farmer *et al* [5]. Clinical strains were distributed among fourteen of the biogroups (Table 1). The defining tests were motility, Voges-Proskauer, methyl red, indole, ornithine decarboxylase, reduction of nitrate to nitrite, production of gas from D-glucose, malonate utilization and production of acid from myo-inositol and dulcitol. Where strains could not be assigned to an original biogroup, a new biogroup or subgroup was designated (Table 2). The majority of isolates (60/189) were in Biogroup 1 with the *E. sakazakii* ATCC 29544^T type strain. These strains were motile, produced gas from glucose, produced acid from inositol, reduced nitrate and were positive for Voges-Proskauer and ornithine decarboxylase, but negative for methyl red, indole, malonate utilization and acid production from dulcitol. Biogroup 2 (n = 42) contained isolates negative for acid production from inositol; four of these were also non-motile. Biogroup 3 (non-motile) contained six strains and Biogroup 4 (ornithine negative) contained nine strains three of which were non-motile. Biogroup 5 (n = 16) was positive for malonate utilization and six of these were non-motile. Biogroup 6 (n = 2) was positive for indole and Biogroup 7 (n = 4) was negative for gas production from glucose. Biogroup 8 (n = 7) was defined by the inability to reduce nitrate. Two of these 7 strains were positive in the malonate test, three were negative for the inositol test and two were inositol negative but malonate positive. Biogroup 9 contained 13 strains that were inositol negative and malonate positive. Biogroup 10 contained one strain that was inositol negative and indole positive, while Biogroup 11 contained one strain that was inositol negative and dulcitol positive. Biogroup 12 was also represented by only one strain, which was indole and malonate positive. The seven isolates in Biogroup 13 were negative for the Voges-Proskauer reaction, three were non-motile, one was negative for methyl red and one was negative for ornithine. Biogroup 14 (n = 5) was negative for ornithine decarboxylase and inositol, with four of these strains being positive for malonate. Biogroup 15 was positive for all the tests performed except methyl red. A new group (Biogroup 16) had to be defined to accommodate 9 strains which were inositol and dulcitol positive, but indole negative. They were malonate positive, with the exception of one strain. Two strains were non-motile and one of these was also ornithine decarboxylase negative. Acid production from α-methyl-D-glucoside was included in the original study [1] and all biogroups were reported positive for this trait with the exception of Biogroup 15. As Biogroup 15 could be distinguished from the other biogroups without the α-methyl-D-glucoside test, this was not repeated for all strains in this study.

Phylogenetic analysis

Partial 16S rDNA sequences (528 bp) containing less than 1% undetermined positions were obtained for all strains in this study. The sequences were analysed using the maximum parsimony method, which is an evolutionary model that searches for the simplest tree that can be constructed using the fewest inferred changes between characters. The topology was optimised using simulated annealing, a heuristic that occasionally accepts a worse tree during the course of the search allowing it to escape local optima. This method is more economical than the more usual heuristic searches (stepwise addition and hill-climbing), which can require many randon re-starts, especially with large data matrices [12]. In agreement with previous reports [10], the isolates used in this study were divided into four genomic groups by 16S rDNA sequence analysis (Fig 1). The majority (170/189) of the presumptive *E. sakazakii* isolates in this study clustered with the type strain (Fig. 1*E. sakazakii* cluster 1). Cluster 1 was composed of the majority of biogroups, Biogroups 1–5, 7–9, 11, 13 and 14. The 9 isolates forming the new proposed Biogroup 16 corresponded with *E. sakazakii* cluster 2 (Fig. 1 cluster 2), with between 1.23–1.89% 16S rDNA sequence difference from the *E. sakazakii* type strain. No strains representative of this biochemical profile were included in the original study by Farmer *et al.* [5].

The six isolates in Biogroup 15 corresponded to *E. sakazakii* cluster 3 (Fig. 1 cluster 3). The four strains described as *E. sakazakii* cluster 4 (Fig. 1 cluster 4) represent Biogroups 6, 10 and 12. For this dataset, the four partial 16S rDNA cluster groups can be distinguished biochemically using the indole, dulcitol, and inositol tests (Table 4). Cluster 1 strains are variable for inositol, negative for indole, and dulcitol; with the exception of Biogroup 11, which is dulcitol positive and inositol negative. Cluster 2 – Biogroup 16 strains are positive for inositol and dulcitol but negative for indole. Cluster 3 – Biogroup 15 is positive for inositol, dulcitol and indole. Cluster 4 strains were also positive for indole but can be distinguished from Cluster 3 as they are negative for dulcitol. One of the strains in Cluster 4 was inositol negative (Biogroup 10) and one was malonate positive (Biogroup 12). There were insufficient

Table 1: Origins of *E. sakazakii* isolates used in this study.

Biogroup	Strain origin					Total
	Clinical	Powdered infant formula and infant food	Other dried food products	Environmental	Unknown	
1	7	16	33	2	2	60
2	4	12	23		3	42
3	4		1		1	6
4	4	1	1		3	9
5	2	2	11		1	16
6	1		1			2
7	2		1		1	4
8	1	2	2		2	7
9	10	2	1			13
10					1	1
11	1					1
12	1					1
13	3		3		1	7
14	1	2	2			5
15	1		3		2	6
16		1	7	1		9
Total	42	38	89	3	17	189

isolates in Cluster 4 biogroups to determine whether these biogroups could be further genomically divided.

Discussion

Farmer *et al.* [5] described 15 biogroups of 57 strains of *E. sakazakii*. Five strains were used to genetically define the species by DNA-DNA hybridization and the remaining strain definitions were phenotypic. This study defines isolates in terms of both phenotype and genotype and extends the initial 1980 [5] study with the analysis of 189 strains. According to partial 16S rDNA sequence analysis the majority of *E. sakazakii* strains clustered with the type strain (Figure 1), and within this cluster there was no clear further genomic division corresponding to biogroup. A second cluster of closely related strains was identified (cluster 2) which were biochemically distinct from cluster 1 and formed a biogroup that had not been previously described by Farmer *et al* [5]. These strains were not assigned a species match using the MicroSeq 500 or supplemental bacterial databases, but the nearest match was *E. sakazakii* (1.23–1.89%). Biogroup 15 [5] corresponds to *E. sakazakii* cluster 3, while Biogroups 6, 10 and 12 correspond to *E. sakazakii* cluster 4 [10,11].

Analysis of the full 16S sequence of strains corresponding to cluster groups 2 (biogroup 16) [13] and 3 (biogroup 15) [14] show these are less than 3% divergent from cluster group 1. Although DNA-DNA hybridization is the acknowledged standard for species delineation this technique was beyond the scope of this study, which focuses on evaluation of the biogroups reported when *E. sakazakii* was originally described. As well as defining a new biogroup (with three subgroups), this study found 10 subgroups within the original biogroups. The malonate and motility tests account for the majority of the subdivisions of the original biogroups.

In this study, the majority of food isolates belonged to Biogroups 1 and 2. All biogroups except 10 and 16 contained at least one strain from a clinical source. The greatest number of clinical strains (10 isolates) was found to belong to Biogroup 9 (Table 1). However, this included 9 isolates out of the ten from the same hospital. Therefore this may not be an indication of increased pathogenicity of this biogroup, but is likely due to an over representation of one clonal type from a single source. Most of the food isolates (including infant foods) belonged to Biogroups 1 and 2.

Conclusion

Biogroup 1 was the major group with approximately one third of the *E. sakazakii* strains (60/189). Cluster 1 was the major DNA cluster (170/189 strains), and was composed of Biogroups 1–5, 7–9, 11, 13 and 14. Cluster 3 is equivalent to Biogroup 15, and a new Biogroup 16 was designated for the strains in Cluster 2. For this dataset it is possible to differentiate the four genomic clusters using indole, dulcitol and inositol tests.

Methods

Sources of bacterial strains

A total of 189 *E. sakazakii* strains were analyzed in this study. Strains were from diverse food, clinical and environmental sources worldwide. The origin distribution of

Table 2: Assignment of strains to the biogroups originally defined by Farmer et *al.* [1]

Farmer Biogroup	Additional group	Phenotype[a]										No. of strains	16s rDNA cluster
		VP	MR	Nit	Orn	Mot	Ino	Dul	Ind	Malo	Gas		
1		+	-	+	+	+	+	-	-	-	+	60	1
2		+	-	+	+	+	-	-	-	-	+	38	1
	2a	+	-	+	+	-	-	-	-	-	+	4	1
3		+	-	+	+	-	+	-	-	-	+	6	1
4		+	-	+	-	+	+	-	-	-	+	6	1
	4a	+	-	+	-	-	+	-	-	-	+	3	1
5		+	-	+	+	+	+	-	-	+	+	10	1
	5a	+	-	+	+	-	+	-	-	+	+	6	1
6		+	-	+	+	+	+	-	+	-	+	2	4
7		+	-	+	+	+	+	-	-	-	-	4	1
8		+	-	-	+	+	+	-	-	-	+	0	1
	8a	+	-	-	+	+	-	-	-	-	+	3	1
	8b	+	-	-	+	+	+	-	-	+	+	2	1
	8c	+	-	-	+	+	-	-	-	+	+	2	1
9		+	-	+	+	+	-	-	-	+	+	12	1
	9a	-	-	+	+	+	-	-	-	+	+	1	1
10		+	-	+	+	+	-	-	+	-	+	1	4
11		+	-	+	+	+	-	+	-	-	+	1	1
12		+	-	+	+	+	+	-	+	+	+	1	4
13		-	+	+	+	+	+	-	-	-	+	2	1
	13a	-	+	+	+	-	+	-	-	-	+	3	1
	13b	-	+	+	-	+	+	-	-	-	+	1	1
	13c	-	-	+	+	+	+	-	-	-	+	1	1
14		+	-	+	-	+	-	-	-	+	+	4	1
	14a	+	-	+	-	+	-	-	-	-	+	1	1
15		+	-	+	+	+	+	+	+	+	+	6	3
New Biogroup	16	+	-	+	+	+	+	+	-	+	+	6	2
	16a	+	-	+	+	-	+	+	-	+	+	1	2
	16b	+	-	+	+	+	+	+	-	-	+	1	2
	16c	+	-	+	-	-	+	+	-	+	+	1	2

[a]VP, Voges-Proskauer; MR, methyl red; Nit, nitrate reduction; Orn, ornithine utilization; Mot, motility at 37°C; Ino, acid production from inositol; Dul, acid production from dulcitol; Ind, indole production; Malo, malonate utilization; Gas, gas production from glucose. All isolates positively reacted with α-methyl-D-glu, with the exception of biogroup 15 isolates.

the isolates is given in Table 1. At least one strain from each of the biogroups described when the *E. sakazakii* species was designated were included and representative strains for each biogroup are given in Table 3. The type strain (NCTC 11467^T) was purchased from the NCTC, London, UK, as were strains NCTC 9844, NCTC 9238 and NCTC 9846. Strain NCIMB 8272 was purchased from the NCIMB, Aberdeen, Scotland. Eleven strains, CDC 5960-70, 1895-73, 0743-75, 3523-75, 9363-75, 9369-75, 0407-77, 0996-77, 1058-77, 1716-77, and 3128-77 were donated by the CDC, Atlanta, GA, USA. Strains were also kindly donated by the following: – Nestlé Research Center, Lausanne, Switzerland; Health Products and Food branch, Health Canada; Children's Hospital Los Angeles CA, USA; Northern Foods, UK; Oxoid Ltd., Basingstoke, UK; Hospital Cèské Budéjovice, Czech Republic; Institut für Tierärztliche Nahrungsmittelkunde Milchwissenschaften, Justus-Liebig-Universität Gießen, Germany; Nottingham City Hospital Trust, Nottingham, UK and the Department of Medical Microbiology, Radboud, Nijmegen, Netherlands. All other strains were food and environmental isolates from the culture collection at Nottingham Trent University, Nottingham, UK.

Phenotypic characterisation

In addition to phenotypes derived from commercial biochemical test kits (Microbact GN2, Oxoid; API 20E and ID32E, BioMérieux) according to manufacturers' recommendations, the following tests were performed using conventional manual methods. Motility was determined at 37°C after 24 and 48 h using motility medium (tryptose 10 g l^{-1}, NaCl 5 g l^{-1}, agar 5 g l^{-1}, pH 7.2 ± 0.2). Acid

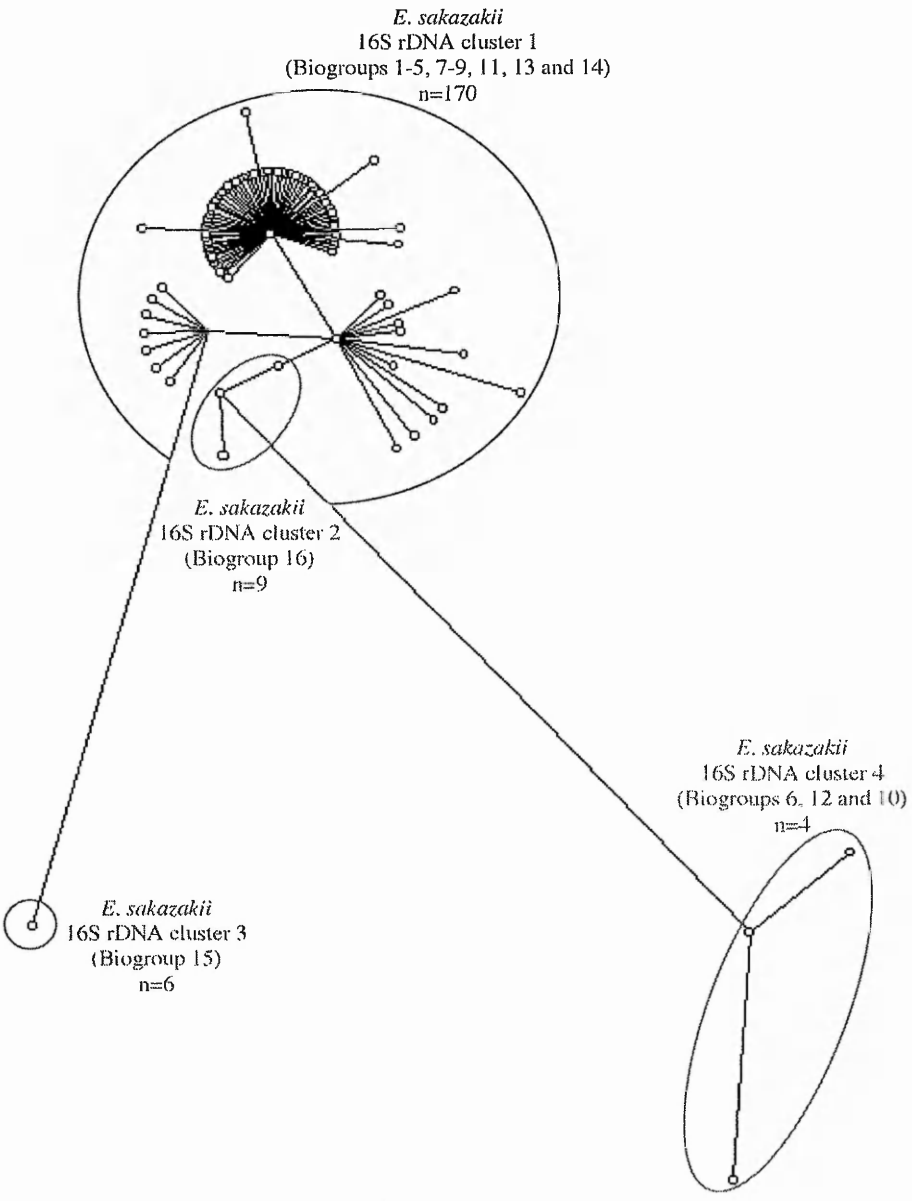

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Figure 1
Maximum parsimony tree (unrooted) showing the four genomic clusters of *E. sakazakii* based on partial 16S rDNA sequence analysis. Gaps were not considered an extra state, and the topology was optimised using simulated annealing.

Table 3: Representative strains from each of the biogroups used in this study.

Biogroup	NTU[a] strain number	Source	Origin
1	1	NCTC 11467[b]	Child's throat (Type strain) (ATCC 29544 = CDC 4562-70)
2	12	Cèské Budéjovice[c]	Faeces (adult 3753)
2a	512	Radboud[d]	Clinical (Prague 26248)
3	680	CDC 996-77[e]	Spinal fluid (New York, USA)
4	683	CDC 407-77	Sputum (Pennsylvania, USA)
4a	515	Radboud	Clinical (Prague 26706)
5	685	CDC 1716-77	Blood (Connecticut, USA)
5a	472	NTU	Infant formula (Korea)
6	564	CDC 5960-70	Blood (Florida, USA)
7	686	CDC 9369-75	Unknown
8a	22	FSM E321[f]	Infant formula (France)
8b	15	Cèské Budéjovice	Faeces (adult 2422)
8c	35	NTU	Infant cereal (Washington, USA)
9	681	CDC 1058-77	Breast abscess
9a	521	Radboud	Clinical (Prague 27288)
10	582	NCTC 9844	Unknown
11	684	CDC 3128-77	Sputum (Alabama, USA)
12	563	CDC 0743-75	Foot wound (Wisconsin, USA)
13	567	CDC 9363-75	Unknown
13a	535	Radboud	Milk powder (New Zealand)
13b	507	Radboud	Clinical (Prague 25569)
13c	101	NTU	Whole mixed spice
14	565	CDC 1895-73	Faeces (Maryland, USA)
14a	33	NTU	Baby rice (Herndon, USA)
15	566	CDC 3523-75	Bone marrow (Arizona, USA)
16	9	NTU	Infant food (cheese puffs)
16a	92	NTU	Herb (biota tops)
16b	57	NTU	Milk powder (UK)
16c	581	NCTC 9529	Water

[a]Nottingham Trent University, Nottingham, UK; [b]NCTC, London, UK; [c]Hospital Cèské Budéjovice, Czech Republic; [d]Department of Medical Microbiology, Radboud, Nijmegen, Netherlands; [e]CDC, Atlanta, GA, USA; [f]Nestlé Research Center, Lausanne, Switzerland.

production from carbohydrates was tested in phenol red broth base (10 g l^{-1} peptone, 1 g l^{-1} yeast extract, 5 g l^{-1} NaCl, 0.018 g l^{-1} phenol red) with the addition of filter-sterilized carbohydrate solution (final concentration 0.5%). Gas production from D-glucose was determined by collection in Durham tubes. Malonate utilization was determined using sodium malonate broth (sodium malonate 3 g l^{-1}, ammonium sulphate 2 g l^{-1}, bromothymol blue 0.025 g l^{-1}, NaCl 2 g l^{-1}, yeast extract 1 g l^{-1}, dipotassium hydrogen phosphate 0.6 g l^{-1}, potassium dihydrogen phosphate 0.4 g l^{-1}). The methyl red test was performed by addition of indicator (0.1 g methyl red per 300 ml 95% ethanol) to cultures grown for 48 h in 4 ml of MR-VP broth (VWR, 1.05712.0500). The Voges-Proskauer test was performed by the addition of 40% potassium hydroxide in water and 5% 1-naphthol in (95% ethanol) to cultures grown for 24 hours in MR-VP broth. Indole production was measured by the addition of

Table 4: Biochemical differentiation of *E. sakazakii* 16S rDNA clusters.

Farmer Biogroup	Phenotype [a]			No. of strains	Genomic cluster
	Ino	Dul	Ind		
1–5, 7–9, 13, 14	(+/-)	-	-	169	1
11	-	+	-	1	1
16	+	+	-	9	2
15	+	+	+	6	3
6, 10, 12	(+/-)	-	+	4	4

[a]Ino, acid production from inositol; Dul, acid production from dulcitol; Ind, indole production; (+/-), some stains positive, some strains negative, the test is used to define subgroups.

Kovacs reagent (5 g p-dimethylaminobenzaldehyde, 25 ml HCl, 75 ml pentanol-1-ol) or James Reagent (70542 BioMérieux) to cultures grown for 24 h in peptone water (CM0009 Oxoid). Nitrate reduction to nitrite was determined by the addition of 1% sulphanilamide in 1 M HCl and 0.02% N-1 naphthylene diamine HCl in water to nitrate test broths. Zinc dust was added to negative tubes of nitrate-negative strains to confirm the presence of unreduced nitrate.

Genotypic characterisation

Comparative 16S rDNA sequencing (528 bp) was performed as previously described [11] by Accugenix (Newark, DE, USA) using the MicroSeq™ 500 16S rDNA Bacterial Sequencing Kit (Applied Biosystems).

A maximum parsimony tree (Fig. 1) of 16S rDNA sequences was generated using Bionumerics (Applied Maths, Belgium). Gaps were not considered an extra state and the topology was optimized using simulated annealing.

Authors' contributions

CI carried out the biochemical tests, analyzed the data and wrote the initial manuscript. MW provided the 16S rDNA sequencing, assembly and editing.

JJF and SJF edited the manuscript; SJF managed the project. All authors read and approved the final manuscript.

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Comparison of Media for the Isolation of *Enterobacter sakazakii*[▽]

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***Enterobacter sakazakii* is associated with neonatal infections and is occasionally present at low levels (<1 CFU/g) in powdered infant formula milk (IFM). It has been previously reported that some *E. sakazakii* strains do not grow in standard media for *Enterobacteriaceae* and coliform bacteria; therefore, a reliable method is needed for recovery of the organism. Three *E. sakazakii* enrichment broths—*Enterobacteriaceae* enrichment broth (EE), *E. sakazakii* selective broth (ESSB), and modified lauryl sulfate broth (mLST)—were compared with a novel broth designed for maximum recovery of *E. sakazakii*, *E. sakazakii* enrichment broth (ESE). One hundred seventy-seven strains (100%) grew in ESE, whereas between 2 and 6% of strains did not grow in EE, mLST, or ESSB. *E. sakazakii* possesses α-glucosidase activity, and a number of selective, chromogenic agars for *E. sakazakii* isolation based on this enzyme have been developed. *E. sakazakii* isolation agar produced fewer false-positive colonies than did Druggan-Forsythe-Iversen agar. However, the latter supported the growth of more *E. sakazakii* strains. It was also determined that 2% of *E. sakazakii* strains did not produce yellow pigmentation on tryptone soya agar at 25°C, a characteristic frequently cited in the identification of *E. sakazakii*. The recovery of desiccated *E. sakazakii* (0.2 to 2000 CFU/25 g) from powdered IFM in the presence of a competing flora was determined with various enrichment broths and differential selective media. Current media designed for the isolation and presumptive identification of *E. sakazakii* do not support the growth of all currently known *E. sakazakii* phenotypes; therefore, improvements in the proposed methods are desirable.**

Enterobacter sakazakii is an occasional contaminant of powdered infant formula milk (IFM) and is a rare cause of neonatal infections (7, 9, 20, 22). Although not all cases have been attributed to the ingestion of IFM, the microbiological safety and preparation of IFM are of concern (4, 5). The Codex Alimentarius Commission is currently reviewing the code of hygienic practices for foods for infants and children, and the European Union has introduced microbiological criteria (2).

Several methods have been proposed for the enrichment and isolation of *E. sakazakii* (6, 11, 21). An integral part of all methods is the α-glucosidase test (16). However, a number of other *Enterobacteriaceae* are α-glucosidase positive (14, 15), and coisolation of these organisms lowers the efficiency of chromogenic media for the isolation of *E. sakazakii*. Although *E. sakazakii* can be recovered from 3 to 14% of IFM samples, reported levels have never exceeded 1 CFU g^{-1} (10, 17, 18). Therefore, specific and sensitive enrichment is required for isolation of the organism.

Farmer et al. (3) reported reduced plating efficiency of *E. sakazakii* strains on media commonly used in enteric bacteriology. It has also been noted that some *E. sakazakii* strains are unable to grow in lauryl sulfate broth (LST) or brilliant green bile broth (12). As the latter strains also failed to grow in *Enterobacteriaceae* enrichment broth (EE), it was deemed necessary to design a modified enrichment medium to aid comparison of the selective media. This study reports a comparison between currently proposed enrichment and isolation media for the detection of *E. sakazakii*.

(A preliminary report of this work was presented at the 105th General Meeting of the American Society for Microbiology [13]).

MATERIALS AND METHODS

Microbiological strains. Proposed media for the isolation of *E. sakazakii* were assessed with over 250 *Enterobacteriaceae* isolates from the culture collection of Nottingham Trent University, Nottingham, United Kingdom. The *E. sakazakii* isolates (n = 177) were from clinical, food, and environmental sources and have been described previously (10, 11). The competing α-glucosidase-positive *Enterobacteriaceae* were *Buttiauxella noakiae* and strains from two as-yet-unnamed species identified as distinct 16S cluster groups (14, 15). The remaining strains were *Enterobacter pyrinus*, *Enterobacter cloacae*, *Citrobacter koseri*, *Citrobacter freundii*, *Citrobacter braakii*, *Enterobacter asburiae*, *Enterobacter aerogenes*, *Enterobacter amnigenus*, *Escherichia hermanii*, *Escherichia coli*, *Hafnia alvei*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Klebsiella ozaenae*, *Raoultella terrigena*, *Kluyvera* sp., *Leclercia adecarboxylata*, *Pantoea* sp., *Proteus vulgaris*, *Providencia rettgeri*, *Salmonella enterica* serovar Enteritidis, *Serratia marcescens*, and *Serratia ficaria*.

Growth media. The following media were prepared according to the manufacturers' instructions: buffered peptone water (BPW) (CM0509; Oxoid, Basingstoke, United Kingdom), EE (CM0317; Oxoid), modified LST (mLST) (CM0451; Oxoid) with 0.5 M NaCl and 10 mg l^{-1} vancomycin) (6), violet red bile glucose agar (VRBGA) (CM0485; Oxoid), violet red bile lactose agar (VRBL) (CM0107; Oxoid), *E. sakazakii* chromogenic agar (Druggan-Forsythe-Iversen [DFI] formulation) (CM1055; Oxoid) (11), and tryptone soya agar (TSA) (CM0131; Oxoid). *E. sakazakii* isolation agar (ESIA) (AEB520010; AES Laboratoire) (8) and *E. sakazakii* selective broth (ESSB) (AEB611448; AES Laboratoire) were purchased as prepared media. Milk agar was prepared as follows: bacteriological agar (3.0 g) (LP0011; Oxoid) and ammonium sulfate were dissolved in 40 ml of distilled water. After autoclaving, 200 ml of warm (55°C) liquid IFM was added and the mixture was dispensed into petri dishes.

E. sakazakii enrichment broth (ESE) was composed of disodium hydrogen phosphate (6.5 g), potassium dihydrogen phosphate (2.0 g), yeast extract (1.5 g), neutralized peptone (4.0 g), base tryptone (12.0 g), sodium chloride (4.0 g), sucrose (100.0 g), and sodium deoxycholate (0.5 g) dissolved in distilled water (1,000 ml). The complete medium (pH 7.0 ± 0.1) was autoclaved at 121°C for 15 min.

Growth measurement. Enrichment broths were inoculated (1×10^4 CFU ml^{-1}) with overnight cultures diluted in sterile saline. Initially, growth was determined by measuring the change in optical density at 590 nm (OD_{590}) at

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TABLE 1. Methods for the recovery of desiccated *E. sakazakii* from powdered IFM

Method	Preenrichment	Enrichment	Primary isolation	Presumptive identification
FDA (21)	BPW (37°C)	EE broth (37°C)	VRBGA (37°C)	Yellow pigment on TSA at 25°C for 48-72 h
DFI (11)	BPW (37°C)	ESE broth (37°C)	DFI (37°C)	Blue-green colonies
mLST (6)	BPW (37°C)	mLST + vancomycin (45°C)	TSA (37°C with enhanced light exposure)	Yellow, α-glucosidase-positive colonies
AES	ESSB broth (37°C)		ESIA (44°C)	Blue-green colonies

37°C and 44°C with a TECAN SPECTRA Fluor instrument (TECAN United Kingdom Ltd., Reading, United Kingdom). Due to the inability to detect growth of some *E. sakazakii* strains in selective media with the OD measurements, 10 ml of EE, ESSB, and mLST were inoculated (1×10^7 CFU ml^{-1}) from overnight cultures into BPW (18 *E. sakazakii* strains and 21 strains of other *Enterobacteriaceae*). After a 24-h incubation, the viable counts were determined by decimal dilutions on TSA incubated at 37°C.

Recovery of desiccated *E. sakazakii* from powdered IFM containing competing organisms. Three strains of *E. sakazakii* (NCTC 11467^T, ATCC 12868, and SK90) were grown overnight on milk agar at 37°C. These strains are the species type strain, the ATCC Perceptrol quality control strain, and a clinical strain kindly supplied by Franco Pagotto (19), respectively. The cells were harvested from the plates and resuspended in sterile infant formula to give cell densities of ca. 10^{11} CFU/ml prior to freeze-drying. The freeze-dried samples were stored for 4 weeks prior to use for the bacterial concentrations to stabilize. The bacterial viable cell counts in the desiccated samples were estimated by a most-probable-number technique ($n = 8$) in BPW prior to inoculation of powdered IFM. Appropriate quantities of desiccated cells were used to inoculate triplicate 25-g quantities of commercial milk-based powdered infant formula (Cow & Gate Premium Stage 1) at 0.2 to 2,000 CFU $25g^{-1}$. The IFM contained endogenous *Bacillus* spp., and one aliquot contained endogenous *Raoultella terrigena*. All aliquots were additionally inoculated with yellow-pigmented *Enterobacteriaceae* isolates comprising an α-glucosidase-positive strain and an α-glucosidase-negative *Pantoea* strain at 0.4 CFU g^{-1}.

Four recovery methods were compared: FDA (http://www.cfsan.fda.gov/~comm/mmesakaz.html), DFI (11; also this study), mLST (6), and AES (http://www.aeslaboratoire.com/). For convenience, the preenrichment, enrichment, primary isolation, and presumptive identification steps for each of these methods

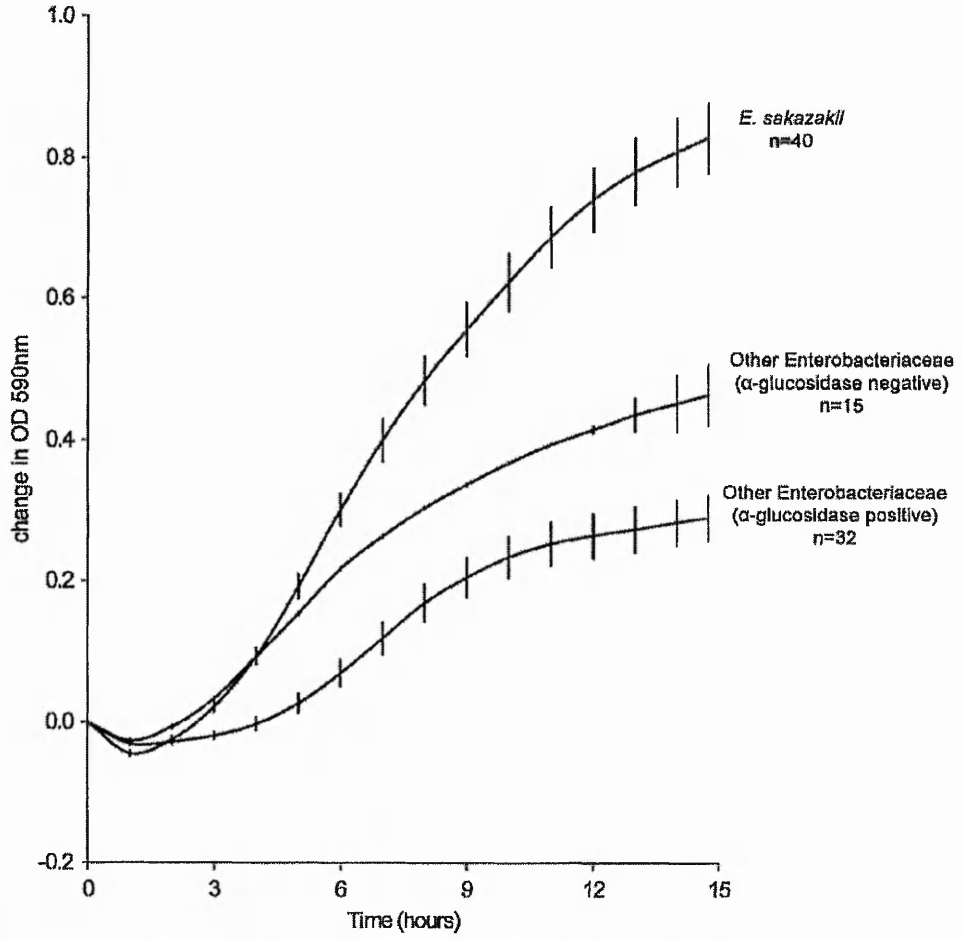

FIG. 1. Comparative growth of *E. sakazakii* and other organisms in ESE. Error bars represent the mean ± the standard error of the sample mean, calculated from the standard deviation of the sample mean divided by $\sqrt{n}$. A difference in OD_{590} of 0.5 is equivalent to ca. 0.5 $\log_{10}$ CFU ml^{-1}.

TABLE 2. Strains showing increases in OD after 24 h of incubation in enrichment media

Organism (no. of strains)	% of strains[a]							
	37°C				44°C			
	ESE	EE	mLST	ESSB	ESE	EE	mLST	ESSB
E. sakazakii (177)	100	97	96	96	99	95	94	87
α-Glucosidase-positive *Enterobacteriaceae* (40)	100	100	95	93	98	95	78	65
α-Glucosidase-negative *Enterobacteriaceae* (34)	100	100	100	97	100	100	91	88

[a] Values are percentages of strains showing increases in OD after 24 h of incubation at the indicated temperatures. See Materials and Methods for enrichment medium manufacturers' details.

are summarized in Table 1. Up to five presumptive isolates were selected and identified by biochemical profiles with an API20E instrument (bioMérieux United Kingdom Ltd.) according to the manufacturer's instructions.

RESULTS

Enrichment broth evaluation. Preliminary experiments had shown that a number of *E. sakazakii* strains were sensitive to brilliant green, lauryl sulfate, crystal violet, and/or novobiocin and that all *E. sakazakii* strains studied were able to ferment sucrose. In contrast, the majority of non-*E. sakazakii* α-glucosidase-positive *Enterobacteriaceae* did not utilize sucrose. Therefore, with sucrose in place of dextrose and/or lactose, *E. sakazakii* was able to outgrow other *Enterobacteriaceae* in ESE (Fig. 1). Sodium deoxycholate was included as a selective agent to suppress gram-positive organisms.

All 177 *E. sakazakii* strains grew well in ESE at 37°C (Table 2). In contrast, growth was not detected for 3 to 13% (n = 177) of *E. sakazakii* strains in EE, mLST, or ESSB at 37°C and 44°C. The viable cell counts after a 24-h incubation in selective broths are presented in Fig. 2. All *Enterobacteriaceae* strains grew in ESE. In the three selective enrichment broths—EE, mLST, and ESSB—the viability of four to six *E. sakazakii* strains decreased and some were unrecoverable (>6 log decline). mLST was the most selective broth, with only two non-*E. sakazakii* strains able to grow. *Bacillus cereus* (n = 1), *Bacillus subtilis* (n = 2), *Staphylococcus aureus* (n = 2), and *Lactobacillus* spp. (n = 2) were not recoverable from any broths (data not shown).

Selective agar assessment. Table 3 shows that 2% (n = 177) of *E. sakazakii* strains did not produce yellow pigmentation on TSA after 3 days of incubation at 25°C, a criterion which has been recommended for the presumptive identification of *E. sakazakii* (8, 21). All of the *E. sakazakii* strains grew at 37°C and produced characteristic colonies on the *Enterobacteriaceae* (VRBGA) and coliform (VRBL) agars; however, one strain (NTU 531) grew very poorly on these media. This isolate was also the only *E. sakazakii* strain in this study that did not grow and produced characteristic (blue-green) colonies on DFI after

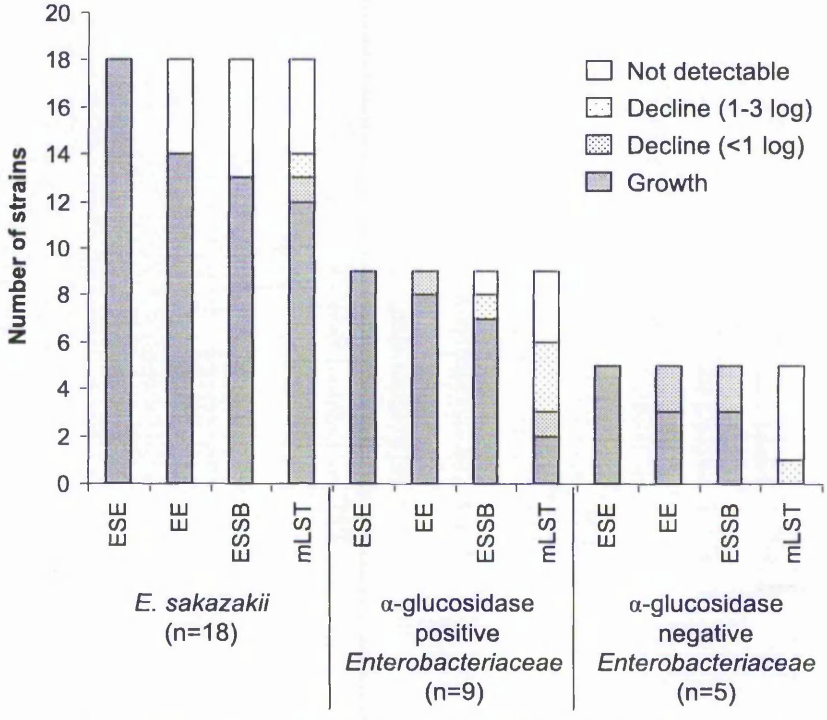

FIG. 2. Growth, persistence, and death of *E. sakazakii* and competitive organisms in enrichment broths.

TABLE 3. Growth of *E. sakazakii* and other *Enterobacteriaceae* on various selective and differential agars

Organism (no. of strains)	Yellow pigment production 25°C (TSA, 48–72 h)	% of strains[a]						
		Incubation temp for 24 h						
		37°C				44°C		
		TSA	DFI	VRBGA	VRBL	TSA	DFI	ESIA
E. sakazakii (177)	98	100	99	100	100	99	93	96
α-Glucosidase-positive *Enterobacteriaceae* (40)	93	100	100	100	92	95	45	63
α-Glucosidase-negative *Enterobacteriaceae* (34)	32	100	0	100	96	100	0	0

[a] Values are percentages of strains showing the following growth parameters: TSA, growth; DFI, blue-green colonies; VRBGA, red colonies; VRBL, red colonies. See Materials and Methods for agar manufacturers' details.

24 h of incubation at 37°C. NTU 531 appears to be more sensitive to sodium deoxycholate than the other isolates tested; decreasing the concentration of this compound in the media to 0.3g l^{-1} improved the growth of this strain. At 44°C, 1% (n = 177) of *E. sakazakii* strains did not grow on TSA, whereas on ESIA and DFI, three and five *E. sakazakii* strains (respectively) were unable to grow. In addition, rather than blue-green colonies, eight strains produced white or partially colored colonies on DFI and four strains produced mauve, rather than blue-green, colonies on ESIA at this temperature.

Recovery of desiccated *E. sakazakii* from powdered IFM. The numbers of samples positive for recovery of three desiccated *E. sakazakii* strains from powdered IFM are presented in Table 4. There were three replicates per strain at each inoculation level (2,000 to 0.2 CFU/25 g). The AES method recovered only the *E. sakazakii* type strain (NCTC 11467^T). Endogenous *R. terrigena* was the only organism recovered by the FDA method (21) for one of the samples inoculated with 2,000 CFU 25 g^{-1} *E. sakazakii*; therefore, *R. terrigena* had overgrown *E. sakazakii*. The mLST method recovered both *R. terrigena* and *E. sakazakii* in the corresponding sample, whereas the DFI method recovered only *E. sakazakii*. Fewer presumptive positive isolates were found to be false positive with the mLST method than with the other methods.

DISCUSSION

ESE broth was developed to facilitate comparison of the performance of *E. sakazakii* selective enrichment broths. Preliminary experiments had shown that all *E. sakazakii* strains were able to ferment sucrose, whereas the majority of non-*E. sakazakii* α-glucosidase-positive *Enterobacteriaceae* did not. Therefore, ESE broth was formulated to support good growth of *E. sakazakii* compared with competing organisms (Fig. 1). Although other *Enterobacteriaceae* also utilize sucrose, these are mainly α-glucosidase-negative organisms and so can be differentiated on current chromogenic media. As *E. sakazakii* has been shown to have greater desiccation tolerance than most other *Enterobacteriaceae* (1; J. Caubilla-Baron and S. J. Forsythe, submitted for publication), a high concentration of sucrose was incorporated into the broth to act as a humectant, lowering the available water. Sodium deoxycholate was incorporated to suppress the growth of gram-positive bacteria.

TABLE 4. Recovery of *E. sakazakii* from powdered IFM in the presence of competing flora by four isolation methods

E. sakazakii concn (CFU 25 g^{-1})	No. of samples positive for *E. sakazakii* recovery (n = 9) by the indicated method[a]			
	DFI (11)	FDA (21)	mLST (6)	AES[b]
2,000	9	7	9	3
200	8	6	8	1
20	6	3	3	1
2	3	1	0	0
0.2	0	0	0	0

[a] See Table 1 for details of the four methods.
[b] Only *E. sakazakii* NCTC 11467^T was recovered by this method.

All *E. sakazakii* isolates grew at 37°C in ESE, but 2 to 4% (n = 177) of the strains were undetected in EE, mLST, or ESSB. Assessment of the viability of these strains by standard plate counts showed that, for five strains, one or more of the selective media were bactericidal. There have been previous reports of *E. sakazakii* strains failing to grow in mLST (15), as well as in LST and brilliant green bile broth (12). Other α-glucosidase-positive organisms lost their viability in mLST to a greater extent than did *E. sakazakii*. Therefore, the selectivity of this medium is not necessarily a result of the increased growth of *E. sakazakii* but of the greater die-off of nontarget cells.

All strains produced characteristic red colonies on VRBGA and VRBLA. However, these media are selective only for *Enterobacteriaceae* and coliforms, respectively, and are not specific for *E. sakazakii*. They are therefore of use with respect to general hygiene monitoring but not for detection of specific pathogens, such as *Salmonella* and *E. sakazakii*. At the recommended incubation temperatures of 37 and 44°C, respectively, 99% of *E. sakazakii* strains grew on DFI agar but only 96% on ESIA. Incubation of DFI at 44°C (above the manufacturer's recommendation of 37°C) resulted in 7% of strains not showing the characteristic blue-green colony morphology. As 1% of *E. sakazakii* strains were unable to grow on nonselective medium (TSA) at 44°C, incubation at this temperature may not ensure the recovery of *E. sakazakii*.

Detection methods should be evaluated with desiccated *E. sakazakii* cells in the presence of competing flora to mimic environmental samples from manufacturing facilities. Comparison of four methods for the recovery of desiccated *E. sakazakii* from IFM indicated that the most sensitive method was preenrichment in BPW, followed by enrichment in ESE and plating on DFI agar (Table 4). However, at the lower inoculum levels, this method produced a large number of false-positive colonies on DFI. The presence of competing organisms reduced the sensitivity of the FDA method, and the AES method recovered only the *E. sakazakii* type strain. The mLST method was not as

sensitive as the DFI method at low inoculum levels but produced fewer presumptive false positives. As one of the strains used in this experiment was sensitive to lauryl sulfate, the ability of the mLST method to recover it at the higher inoculum levels suggests that this method works better in the presence of the sample matrix (IFM) than when used for pure cultures. This may be due to divalent cations in the IFM counteracting the effects of the lauryl sulfate. Therefore, the performance of the mLST method may be reduced if used for other sample matrices.

This study has used a large number ($n = 177$) of *E. sakazakii* strains to demonstrate that the levels of selective agents such as crystal violet, sodium lauryl sulfate, brilliant green, and sodium deoxycholate in media need to be reassessed to ensure the recovery of the organism, especially from mixed cultures. The use of sucrose (100 g/liter) in ESE promoted the growth of *E. sakazakii* relative to other α-glucosidase-positive *Enterobacteriaceae*. However, this broth is not selective enough to be considered a viable alternative enrichment method, and further development of effective media for the isolation of *E. sakazakii* is needed.

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