PHYSIOLOGICAL CHARACTERISATION OF THE NEONATAL MENINGITIC BACTERIUM *CRONOBACTER SAKAZAKII*

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ABSTRACT

The *Cronobacter* genus is a member of the Enterobacteriaceae family that comprises 7 species. A multiple locus sequence typing (MLST) scheme was designed for *Cronobacter* and was used to identify over 400 sequence types (STs). Notably, *C. sakazakii* ST4 and its single/double loci variants, which together form clonal complex 4 (CC4), are predominantly associated with neonatal meningitis. The present PhD study aimed to investigate the diversity of *C. sakazakii* especially CC4 using MLST analysis, physiological characterisation of stress responses along with genomic analysis associated genes.

In the first part of the study, MLST was used to analyse three collections of environmental bacterial isolates. The isolates were from powdered infant formula, milk powder factory environments, and milk powder processing equipment; they had not been profiled prior to this study and had previously been identified as *E. sakazakii*. These environmental strains were obtained from geographically diverse countries. A total of 39 STs were identified across the studied isolates. The analysis revealed that despite their geographical and temporal spread, only 4 *Cronobacter* species were isolated from these three collections of strains, namely *C. sakazakii, C. malonaticus, C. turicensis,* and *C. muytjensii*. Interestingly, this study demonstrated that *C. sakazakii* CC4 isolates represent a predominantly stable lineage within the *Cronobacter* genus in these environments. This result led to further investigation to study the physiological factors in the CC4 clone associated with its persistence in the environment. This included desiccation stress, heat tolerance, acid resistance and serum resistance assays. The desiccation and serum resistance assays indicated that all of the tested *C. sakazakii* CC4 and non-CC4 strains showed equal resistance against desiccation and serum. The experiments also indicated that *C. sakazakii* CC4 strains were more heat tolerant than non-CC4 strains to 100°C heat. The most interesting observation was that *C. sakazakii* CC4 strains were significantly more acid-resistant than non-CC4 strains to pH 3.5, which is the pH of the neonatal stomach. The results were supported by the outer membrane protein (OMP) profiling where *C. sakazakii* strains revealed more OMPs than non-CC4 at pH 3.5.

Capsule production assays showed that most of the *C. sakazakii* CC4 and non-CC4 strains produced mucoid capsules when cultured on milk agar. The motility of the tested strains was associated with the presence of the *fliRQPON* flagellar genes. An exhaustive BLAST search of sequenced strains to identify genes associated with virulence or with environmental fitness found no significant difference between the virulence potential of *C. sakazakii* CC4 and *C. sakazakii* non-CC4 strains.

The present study provides important insights into neonatal meningitis *C. sakazakii* CC4. Further studies are warranted to characterise the OMPs that are predominant in *C. sakazakii* CC4 and elucidate their significance towards virulence of this important neonatal health-associated pathogen.

DECLARATION

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LIST OF ABBREVIATIONS

ABC ATP binding cassette

ATP Adenosine triphosphate

ANN Artificial neural networks

BLAST Basic local alignment search tool

BPW Buffered peptone water

BCA Bicinchoninic acid

CC Clonal complex

CDC Centres for disease control and prevention

CFU Colony forming units

CGH Comparative genomic hybridization

COSHH Control of substances hazardous to health

CSF Cerebrospinal fluid

DDH DNA-DNA hybridization

DFI Druggan-Forsythe-Iversen

DLV Double locus variant

DNA Deoxyribonucleic acid

EDTA Ethylenediamine tetra-acetic acid

EE Enterobacteriaceae enrichment

FAO Food and Agriculture Organization of the UN

FDA Food and Drug Administration

FUF Follow up formula

HBMEC Human brain microvascular epithelial cells

ISO International Organization for Standardization

ITS Internal transcribed spacer

kDa Kilodalton

MEGA Molecular Evolutionary Genetics Analysis

MLEE Multilocus enzyme electrophoresis

MLSA Multilocus sequence analysis

MLST Multilocus sequence typing

MLVA Multi-locus variable-number tandem-repeat analysis

MPN Most probable number

NCBI National Centre for Biotechnology Information

NCTC National Collection of Type Cultures

NEC Necrotising enterocolitis

NGS Next generation sequencing

NICU Neonatal intensive care unit

NTU Nottingham Trent University

OD Optical density

O-LPS Oligo-lipopolysaccharide

OM Outer membrane

OMP Outer membrane proteins

ORF Open reading frame

PBS Phosphate buffered saline

PCR Polymerase chain reaction

PFGE Pulsed field gel electrophoresis

PIF Powdered infant formula

RAPD Random amplification of polymorphic DNA

RFLP Restriction fragment length polymorphism

SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel -electrophoresis

SLV Single locus variant

ST Sequence type

TLV Triple locus variant

TSA Trypticase soy agar

TSB Trypticase soy broth

UV Ultraviolet

VRBGA Violet red bile glucose agar

WHO World Health Organization

TEMED N,N,N'N'-tetramethyl-ethylenedamine

APS ammonium persulphate

CHAPTER 1: General introduction

1.1 Taxonomy

Bacteria in the *Cronobacter* genus, which was formerly called *Enterobacter sakazakii*, represent a fundamental cause of fatal neonatal infections such as necrotizing enterocolitis, meningitis, and brain abscesses. These infections typically develop as the result of ingestion of contaminated reconstituted powdered infant formula (PIF). However, *Cronobacter* spp. infections are rare, and not all cases can be connected to the consumption of reconstituted infant formula (Forsythe *et al*. 2014). Bacteria in the *Cronobacter* genus are Gram-negative, rod-shaped, facultative anaerobic bacteria that belong to the Enterobacteriaceae family and to the Gammaproteobacteria class. The currant taxonomy of the *Cronobacter* genus comprises 7 species: *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. universalis*, *C. muytjensii*, *C. dublinensis*, and *C. condimenti* (Iversen *et al.* 2008; Joseph *et al.* 2011). *Cronobacter* bacteria are residents of plants, which may help the bacteria acquire environmental fitness characteristics that help them survive stressful conditions during food manufacturing processes (Iversen and Forsythe, 2003; Osaili and Forsythe, 2009).

A well-defined bacterial classification system is a basic requirement for *monitoring* the bacterium, since detection techniques must be based on a full and correct understanding of the different species of the target bacterium as well as on an understanding of closely-related organisms (Forsythe *et al.* 2014). Next-generation sequencing (NGS) was recently used to analyse *Cronobacter* genes, thereby advancing our knowledge of the taxonomy of this genus and helping to establish the *Cronobacter* PubMLST database (http://pubmlst.org/cronobacter/). This database, which comprises > 1000 isolates and over 230 complete genomes, helped define the sequences, contributed to genomic analyses, and enhanced our understanding of how best to control this important bacterial pathogen that has garnered international attention. Importantly, NGS and the database have facilitated the identification of particular clonal lineages that are associated with infections in both neonates (e.g. meningitis) and adults (Forsythe *et al.* 2014).

The *Cronobacter* genus is closely related to the newly-defined genus *Kosakonia* and to the well-characterised *Citrobacter* and *Pantoea* genera. Before the taxonomic revision in 2007, some isolates of *Enterobacter hormaechei* and *Enterobacter ludwigii* were misidentified as *Cronobacter*, leading

to confusion in the literature (Iversen *et al.* 2008*;* Joseph *et al.* 2012a). In addition, another Enterobacteriaceae member, *Citrobacter koseri*, is reported to cause invasive meningitis and brain abscess formation in neonates. The presentation of *Citrobacter koseri* infections is the same as that of infections caused by *Cronobacter* spp. but is unlike neonatal meningitis caused by the pathovar *E. coli* K1. Like many *Cronobacter* isolates, some of the *Pantoea* genus isolates are plant-borne pathogens, and they mostly form yellow pigmented colonies (Kucerova *et al.* 2011).

E. sakazakii was initially identified as *Enterobacter cloacae*, an organism that also produces yellow colonies. Based on the phenotype analyses and DNA-DNA hybridization (DDH) studies Farmer *et al.* (1980) were the first to identify and describe *E. sakazakii* as an independent named species.

Iversen and co-workers (2004a) used the 16S rDNA and hsp60 sequences to analyse the diversity and phylogenetic relationships of *Cronobacter* strains, which were known as *E. sakazakii* at that time. Their analysis clustered the strains into four phylogenetic groups and indicated that these strains comprised a separate genus within the Enterobacteriaceae family (Iversen *et al.* 2004a). Iversen *et al.* first described the taxonomy of the *Cronobacter* genus in 2007. By 2008, the *Cronobacter* genus included 6 species: *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. dublinensis*, *C. muytjensii*, and *C. genomospecies* 1 (Iversen *et al.* 2008).

Iversen *et al.* (2008) initially differentiated these 6 *Cronobacter* species depending on 16 *E. sakazakii* biotypes: *C. sakazakii* has biotypes 1–4, 7, 8, 11, and 13; *C. dublinensis* has biotypes 6, 10, and 12; *C. turicensis* has biotypes 16, 16a, and 16b; *C. malonaticus* has biotypes 5, 9, and 14, and *C. muytjensii* has only biotype 15. The biotyping system is considered to be unreliable for characterisation of *Cronobacter* species (Joseph *et al*. 2013) because the use of biotype index strains that were misidentified previously led to incorrect classification of *Cronobacter* species (Baldwin *et al.* 2009).

The use of 16S rDNA sequence analysis could not distinguish between two *Cronobacter* species, namely *C. sakazakii* and *C. malonaticus*, due to the presence of 7 copies of the rDNA in the *Cronobacter* genus. In addition, intrageneric variances can produce uncertain and unreliable base calls, leading to errors in the GenBank records. The multilocus sequence typing (MLST) scheme for

Cronobacter spp. was initially introduced by Baldwin *et al.* in 2009. This method exploits the sequences of 7 housekeeping genes that have a concatenated sequence length of 3036 bp to distinguish between different *Cronobacter* species within the genus (Baldwin *et al,* 2009). The 7 genes are the ATP synthase beta chain (*atpD*), DNA gyrase subunit B (*gyrB*), elongation factor G (*fusA*), glutamate synthase large subunit (*gltB*), glutaminyl tRNA synthetase (*glnS*), translation initiation factor IF-2 (*infB*), and phosphoenolpyruvate synthase a (*ppsA*). MLST analysis is more robust and discriminatory than 16S sequence analysis and revealed that *C. sakazakii* and *C. malonaticus* are distinct organisms (Baldwin *et al.* 2009; Yan *et al.* 2012).

In 2012, Joseph *et al.* performed multilocus sequence analysis (MLSA) of these 7 loci in strain collections that were representative of the 7 *Cronobacter* species. This approach overcame the problems that arose when strains were clustered according to the phenotyping method (Baldwin *et al.* 2009). As a result of this study, 2 new species of *Cronobacter* were identified, namely *C. condimenti* and *C. universalis* (Joseph *et al.* 2012a).

These recent studies of the development of *Cronobacter* taxonomy were prompted by the use of the MLST method, which is based on sequencing analysis. However, techniques such as the use of PCR probes need to be re-evaluated. Notably, in 2013 Brady and colleagues proposed three *Enterobacter* spp. namely: *E. pulveris*, *E. helveticus* and *E. turicensis* using only 4 MLSA loci: *atpD*, *gyrB*, *infB*, and *rpoB* (Brady *et al*. 2013). This led to these organisms being defined as *C. pulveris*, *C. helveticus* and *C. zurichensis,* respectively. The number of loci that were used in this particular re-examination was lower than the standard accepted number of 5, which is questionable. In addition, this re-evaluation of *Cronobacter* taxonomy led to problems in controlling *Cronobacter* spp. internationally in powdered infant formula, since several detection systems used the three *Enterobacter* species identified by Brady *et al.* (2013) as negative controls (Masood *et al.* 2013a; Jackson *et al.* 2014). Later, Stephan and co-workers corrected this misidentification in the taxonomy of these three species, stating that based on whole genome SNP analysis, there should be two new genera that included *Franconibacter helveticus*, *Siccibacter turicensis* and *Franconibacter pulveris* (Stephan *et al.* 2014). The phylogenetic tree in **Figure 1.1** shows that the use of 7-loci MLSA confirmed the revised *Cronobacter* taxonomy, as the 3 new species are not clustered with the 7 *Cronobacter* species.

Notably, after the taxonomic revision, and until now, some strains were identified incorrectly due to the use of phenotypic and biochemical identification methods; therefore, these methods are not reliable. One example is a study of an outbreak in a neonatal intensive care unit (NICU) in France that resulted in a fatal neonatal sepsis infection attributed to *Cronobacter* spp. that were re-identified as *E. cloacae* (Caubilla-Barron *et al.* 2007). Townsend *et al.* (2008) re-identified *Cronobacter* spp. isolated from a NICU outbreak in the U.S. as *E. hormaechei*. Also, *C. sakazakii* strains that caused infections linked to infant formula in Mexico were re-classified as *E. hormaechei* and *Enterobacter* spp. (Flores *et al.* 2011; Jackson *et al.* 2015).

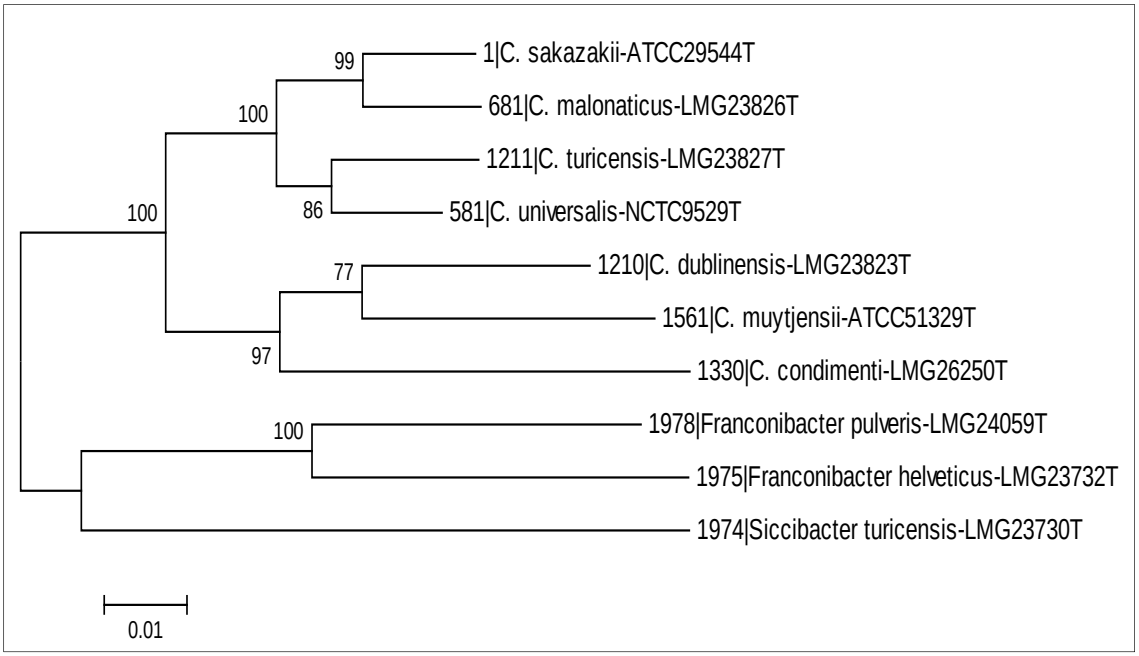


Figure 1. 1 A maximum likelihood tree of 7 MLST loci of *Cronobacter* spp.

The tree was constructed using 7 MLST loci (concatenated length 3036) of the 7 *Cronobacter* species and closely-related Enterobacteriaceae (*Franconibacter* and *Siccibacter*). The NTU ID number of the isolates is displayed on top of each branch, the type strains number are displayed in the end of each branch and the tree is drawn to scale using MEGA v5.2 with 1000 bootstrap.

1.2 Phenotypes and detection methods

It is essential to have accurate detection and identification techniques for *Cronobacter* isolates from different sources. Notably, the association of *Cronobacter* species with rare cases of neonatal illness has elicited the attention and concern of both food manufacturers and regulatory authorities. This is due mainly to the association of ingestion of contaminated reconstituted powdered infant formula

(PIF) with some neonatal infections. The U.S. Food and Drug Administration (FDA) has established a standard technique for isolating the *Cronobacter* spp. from PIF, which involves bacterial enrichment followed by biochemical characterization. Previous methods for detecting isolated *Cronobacter* strains were similar to the methods used to detect *Salmonella* species in milk powder samples (FDA, 2002). The methods usually included the following steps: 1) pre-enrichment (225 ml of buffered peptone water (BPW) or distilled water + 25 g PIF); 2) enrichment by culture overnight at 37°C in Enterobacteriaceae enrichment (EE) broth; 3) streaking or spreading the sample onto violet red bile glucose (VRBG) agar plates for overnight culture at 37°C. After the last step, the plates were observed to detect the classic colony morphology of *Enterobacteriaceae. Cronobacter* forms purple colonies on this medium due to precipitation of bile acids, which produce a purple halo on the plate. In a fourth step, 5 probable positive colonies are picked and grown on TSA plates at 25°C to see whether the colonies are yellow. The fifth and last step is to confirm the identification of the colonies using API20E strips and to determine the most probable number (MPN).

When used for *Cronobacter* species, this method has some limitations. Initially, the protocol is not selective for *Cronobacter* spp., as all Enterobacteriaceae are enriched in the EE broth and grow on VRBGA. This is likely to cause overgrowth of *Cronobacter*. In addition, the protocol can result in false negative identifications, as only 80% of *Cronobacter* spp. strains are yellow on TSA plates at 25^{O}C (Iversen and Forsythe, 2007). Finally, the phenotype databases that are based on commercial biochemical characterisation kits do not sufficiently cover the genus, giving contradictory results. In addition, the technique is both time-consuming and labour-intensive, requiring almost 5 days to perform. In 2012, the FDA approved a revised procedure that overcame the limitations of the previous method, which was used only in the U.S. (Chen *et al*. 2012). The revised method contains the following steps: 1) pre-enrichment of PIF for 24 hours in sterile BPW followed by centrifugation; 2) transfer of the pellets into phosphate buffered saline (PBS); 3) culture of aliquots, followed by screening to detect *Cronobacter* species. The screening is performed using chromogenic media such as R&F agar and Druggan-Forsythe-Iversen (DFI) agar, real-time PCR assays, and biochemical tests (Rapid ID32E or VITEK 2.0). This method can confirm the identification of *Cronobacter* spp. in just two days (Chen *et al*. 2010; Chen *et al*. 2011a).

The International Organization for Standardization (ISO) procedure is used everywhere except the U.S. to detect *Cronobacter* spp. in PIF. In this method, a pre-enrichment step is carried out in BPW, followed by inoculation of 100 µl of the bacteria into 10 ml of a modified lauryl sulphate (mLST) broth (which is a selective enrichment medium) and incubation at 44°C for 24 hours. Then a sample of the culture is streaked onto chromogenic *Enterobacter sakazakii* isolation agar (ESIA) for growth at 44°C, followed by sub-culture of typical colonies on TSA plates at 25°C to check for pigmentation. The yellow colonies are chosen for biochemical characterisation (Anonymous, 2006).

1.3 Molecular typing and identification techniques

In recent years, important progress has been made in the identification of *Cronobacter* species. In particular, several molecular typing techniques have been developed to identify and characterise *Cronobacter* strains. Similar to the analysis used to identify most Enterobacteriaceae members, most of the molecular methods used for the identification of *Cronobacter* isolates are based on analysis of 16S rDNA sequences. Many researchers have created probes for genes either to detect isolates or to identify the organism (Keyser *et al*. 2003; Lehner *et al*. 2004; Iversen *et al*. 2007). One of these techniques is the TaqMan real-time PCR assay (Malorny and Wagner, 2005).

The 16S rDNA and hsp60 sequences were used by Iversen and co-workers (2004a) to analyse the diversity and phylogenetic relationships of *Cronobacter* species (which were known as *E. sakazakii* at that time). This analysis clustered the strains into four phylogenetic groups and strongly indicated that these strains formed a separate genus within the Enterobacteriaceae family (Iversen *et al.* 2004a). Another study used artificial neural networks (ANN) to identify particular regions in *E. sakazakii* in the partial 16S rDNA sequence. Specifically, a 528-bp sequence can be used for to identify the strains (Iversen *et al.* 2006). In contrast, phylogenetic analysis uses the complete 16S rDNA sequences, which includes over 1300 bp. Variations in the 16S rDNA sequence have been used to determine both species (3%) and genus (5%) boundaries in prokaryotes. However, 16S rDNA sequence analysis has some limitations with regard to closely-related bacteria since the sequences do not vary as much. In addition, there are multiple copies of the 16S rDNA operon in most of the organisms, so variations in their sequences may lead to inconsistent results (Acinas *et al*. 2004). In the case of *Cronobacter* species, this is the case for distinguishing between *C. sakazakii* and *C. malonaticus*. The 16S rDNA

sequences for these two species indicate 99.7% resemblance, while the sequences are >70% related using the DDH (DNA-DNA hybridization) method. During the reclassification of *E. sakazakii* species to the *Cronobacter* genus, *C. malonaticus* was categorised initially as a subspecies of *C. sakazakii* (Iversen *et al*. 2007). Later, it was characterised as separate species (Iversen *et al*. 2008).

Many studies have used oligonucleotide arrays and TaqMan Real-time PCR probes to target the internal transcribed spacer (ITS) regions in order to detect *Cronobacter* strains in infant formula (Liu *et al*. 2006a; Liu *et al*. 2006b). The ITS region consists of nucleotide sequences between the 16S and 23S rDNA in the bacterial genome. Another real-time PCR assay was developed to detect *Cronobacter* spp. in PIF. This method determines the sequence of the partial macromolecular synthesis operon, particularly the *rpsU* and primase (*dnaG*) genes (Seo and Brackett, 2005). Notably, in these methods, the probes were designed using a small number of *E. sakazakii* isolates. As a result, the probes have not been validated, even after the taxonomic revision of *Cronobacter*. A number of molecular techniques have been used to reclassify *E. sakazakii* as a separate genus (i.e. as *Cronobacter*), and these methods helped clarify variations within the strains. In addition to the basic biochemical tests that are needed to validate the phenotypic features of the species, methods like ribotyping, full-length 16S rDNA sequencing, f-AFLP, and DDH using *EcoRI* and *TaqI* have also been used to characterise the *Cronobacter* genus (Iversen *et al*. 2007; Iversen *et al*. 2008).

The PFGE technique is based on a PulseNet-validated protocol for *Salmonella* (Swaminathan *et al*. 2001) that uses the most common restriction endonuclease, *XbaI*. For years, this method was the most reliable technique for outbreak investigation, source tracking, and surveillance of the organism. (Nazarowec-White and Farber, 1997; Caubilla-Barron *et al*. 2007; Craven *et al*. 2010; CDC, 2011). Although PFGE has shown a high level of discrimination of *Cronobacter* spp. isolates in several studies, it is labour-intensive and time-consuming. As recommended for *Salmonella,* a recent study also recommended the uses of a second enzyme, *SpeI* in *Cronobacter* PFGE to confirm the *Xbal* results (Brengi *et al*. 2012). A study conducted by Caubilla-Barron *et al*. (2007) used PFGE to analyse 30 *Cronobacter* strains isolated from a fatal NICU outbreak in France. The analysis indicated that strains isolated from some infants included two pulsotypes (Caubilla-Barron *et al*. 2007). PFGE profiling has also been used to analyse *Cronobacter* spp. isolated from milk powder and infant

formula manufacturing plants to study the distribution of the organisms in different manufacturing areas (Craven *et al*. 2010; Jacobs *et al*. 2011).

Phenotyping and genotyping methods were used by Nazarowec-White and Farber (1999) to analyse a number of *Cronobacter* spp. isolates (before *E. sakazakii* reclassification), who assessed whether these methods were suitable for characterizing the isolates. Specifically, Nazarowec-White and Farber used PFGE, random amplification of polymorphic DNA (RAPD), and ribotyping methods and found that the RAPD and PFGE techniques were the most effective for distinguishing between isolates (Nazarowec-White and Farber, 1999).

A study by Mullane *et al*. (2008a) established a multi-locus variable-number tandem-repeat analysis (MLVA) scheme for *Cronobacter* and evaluated 112 isolates. This technique was based on the genome sequence of *C. sakazakii* BAA-894, and suggested that *E. sakazakii* must be classified as a separate genus, i.e as *Cronobacter* (Iversen *et al*. 2007). The study showed significant variations in the isolates in agreement with the re-classification. However, the method was not re-analysed after this taxonomic revision.

Gram-negative bacteria have O antigens, which are considered very important characteristics of the cell wall. Bacterial variations in the production of O antigens lead to *various* bacterial serotypes. A PCR-based technique to detect genes involved in O-antigen synthesis, termed serogrouping, is considered the most advanced non-MLST profiling method (Sun *et al*. 2011, 2012; Jarvis *et al*. 2011, 2013). Generally, Enterobacteriaceae have an O-antigen synthesis region located at the *rfb* locus. This locus encodes dTDP-D-glucose 4,6-dehydratase between the *gnd* and *galF* genes, which encode UDP-glucose pyrophosphorylase and gluconate-6-phosphate dehydrogenase, respectively.

Many molecular typing methods, such as the O-serotyping method, exploit the diversity and variation of O-antigen genes, and PCR-based O-serotyping techniques have been developed to analyse *Cronobacter* spp. (Mullane *et al*. 2008b; Jarvis *et al*. 2011; Sun *et al*. 2011). Mullane *et al*. (2008b) initially used PCR primers that detected the *wehI* and *wehC* genes in order to identify the O2 and O1 serotypes of *Cronobacter* as *C. sakazakii* serotypes O2 and O1, respectively (Jarvis *et al*. 2011). Later, this method was expanded to include identification of 10 additional serotypes to cover all

Cronobacter species. This included 4 serotypes for *C. sakazakii* (O3, O4, O5, O6, and O7), 2 serotypes for *C. malonaticus* (O1 and O2), 2 serotypes for *C. turicensis* (O1 and O2), and one serotype for *C. muytjensii* (O1) (Jarvis *et al*. 2011; Sun *et al*. 2011, 2012a and 2012b). Although serotyping has the advantages of identifying the bacterial serogroup and indicating the variety of lipopolysaccharides in the bacterium, this method also has some drawbacks. First, each serotype that is defined needs a specific set of primers for identification, which complicates the procedure. Second, only 18 serogroups have been identified to date, making serotyping less discriminating than MLST analysis, which has defined over 300 sequence types (STs) (Forsythe *et al.* 2015; Ogrodzki and Forsythe, 2015). Third, there are no PCR products when this method is used to analyse some strains, which indicates the existence of additional serogroups that have not yet been identified. Research by Jarvis *et al*. (2011) found that the serotypes of almost 20% of the *Cronobacter* spp. isolates that they tested could not be determined using the available range of primers. This supports the hypothesis that there is great variation in the O-antigen region (Jarvis *et al*. 2011). Therefore, to detect all of the differences in the region, this method would have to be re-evaluated and revised to detect a greater variety of serogroups.

PCR probes have been developed that are based on specific *Cronobacter* housekeeping genes, like *rpoB* and *gyrB* (Dauga and Breeuwer, 2008; Stoop *et al*. 2009). The PCR-based method for analysing the *rpoB* gene was reviewed recently to include the species that were identified most recently, which includes all 7 *Cronobacter* species. This scheme was first established using one gene to identify the species, and the typing method is performed using separate primers and different amplification conditions for each species (Lehner *et al*. 2012). Another PCR probe-based method has been established that detects C*ronobacter* in infant formula by targeting the virulence-associated outer membrane protein gene *ompA* (Mohan Nair and Venkitanarayanan, 2006). A previous study by Proudy *et al*. (2008) evaluated the use of BOX-PCR and PFGE as detection methods for 27 *Cronobacter* strains. BOX-PCR is a PCR-RFLP sequencing method that detects the flagellin gene, *fliC*. The results showed that BOX-PCR and PFGE are very similar in their ability to distinguish different isolates, but the *fliC* gene is not the best target for distinguishing between isolates (Proudy *et al*. 2008). A multiplex PCR assay for *Cronobacter* spp. was developed to target di-guanylate cyclise

gene (*cdgc1*). This method aimed to identify organisms at the species level, but it only recognised 6 of the 7 *Cronobacter* species (Carter *et al*. 2012).

The multilocus sequence analysis method (MLSA) is a technique based on the analysis of sequences of several housekeeping genes. MLSA has been used on some Enterobacteriaceae family members and has proved to be a good analysis tool (Lacher *et al*. 2007; Ibarz Pavón and Maiden 2009). The establishment of the 7-loci MLST method has advanced the systematic identification and characterisation of *C. sakazakii* and *C. malonaticus* (Baldwin *et al*. 2009). This method is based on an analysis of the partial sequences of 7 housekeeping genes, namely *ppsA*, *atpD*, *gltB*, *gyrB*, *fusA*, *glnS*, and *infB.* These 7 partial gene sequences encompass a total of 3036 nucleotides for analysis when they are grouped together. This technique can distinguish between *C. sakazakii* and *C. malonaticus* phylogenetically, which is hard to do using other approaches, like 16S rDNA sequencing.

According to Baldwin *et al*. (2009), *C. sakazakii* and *C. malonaticus* were previously confused, as they are difficult to differentiate by other methods like 16S rDNA. This identification failure was due to incorrect speciation of certain biotype reference strains. Baldwin *et al*. (2009) suggested that this error was first made when phenotyping was used to characterise the species. An open access MLST database for *Cronobacter* has been established and is available at http://www.pubmlst.org/cronobacter.

Importantly, some of these typing and detection techniques were developed during the same time period as when the microbiological safety of PIF was being questioned. In addition, other methods have been used to analyse poorly characterized strains of organisms, specifically *E. sakazakii* strains, which led to incorrect descriptions of bacterial diversity. Therefore, as a result of the taxonomic update, all techniques cannot be used to detect *Cronobacter* species until they are re-examined, since any such procedure will only be acceptable when it is shown to detect and recognise all of the identified species. This is because of the current international regulations that require that a 10-g sample of PIF is free of *Cronobacter* species.

1.4 Physiology

A number of physiological characteristics have been identified as being of particular importance in *Cronobacter* spp. as a result of the incidence of *Cronobacter* strains in powdered infant formula (PIF). Thermotolerance is an important trait in *Cronobacter* spp. that has been studied extensively. Although the ability of different *Cronobacter* strains to resist heat varies, pasteurization temperatures of 72°C inactivate all bacterial isolates (Nazarowec-White and Farber, 1997; Nazarowec-White *et al*. 1999; Breeuwer *et al*. 2003; Iversen *et al*. 2004b). The guidelines for PIF preparation were reviewed after *Cronobacter* was found in PIF in order to minimise the risk of bacterial growth (FAO-WHO, 2004, 2006). A study by Adekunte *et al*. (2010) demonstrated that the number of *C. sakazakii* cells is reduced significantly when a combination of ultrasound and heat treatment is applied to reconstituted PIF, but this combination treatment required additional investigation. Iversen *et al*. (2004) demonstrated that *Cronobacter* can survive in reconstituted PIF in a temperature range of 5°C to 47°C. In the laboratory, *Cronobacter* isolates can grow in 16 to 18 hours at 37°C on various media, including tryptone soy agar (TSA) and MacConkey's agar.

The ability of the bacterium to resist dryness is another characteristic that is important for its survival. Gajdosova *et al.* (2011) found an association between thermotolerance in *C. sakazakii* and the presence of 22 open reading frames. Their research on the osmotolerance region showed that the *orfHIJK* operon was experimentally involved in thermotolerance, as there was a 2-fold increase in the *C. sakazakii* strain ATCC 29544 D_{58} values when the HIJK operon was cloned into *Escherichia coli* (Gajdosova *et al*. 2011).

A study by Caulbilla-Barron *et al*. (2007) showed that *Cronobacter* spp. can survive in a desiccated state for over two years. Notably, *Cronobacter* spp. can tolerate osmotic stress better than the other bacterial isolates found in PIF, like *Salmonella* spp., *Citrobacter* spp., and *E. coli* (Breeuwer *et al*. 2003). Many researchers have tried to elucidate the mechanisms underlying osmotic resistance in *Cronobacter.* For example, Riedel and Lehner (2007) used a proteomics approach and identified 53 proteins that were associated with the response of *Cronobacter* spp. to osmotic stress. The synthesis of trehalose is also thought to be a factor in the response of *Cronobacter* spp. to osmotic stress; notably, trehalose can act as a molecular chaperone by protecting proteins and cellular membranes from

denaturation, a function of trehalose that was first determined in *E. coli* isolates (Horlacher and Boos 1993; Breeuwer *et al*. 2003). A number of osmotolerance genes have been identified in *Cronobacter* and in other members of Enterobacteriaceae. Feeney and Sleator (2011) identified the system responsible for osmotolerance in the *C. sakazakii* BAA-894 genome, namely the low-affinity K^+ transport genes *trkG*, *trkH*, and *kup*. These genes are located near the *mscL* genes, which encode a mechanosensitive channel that might act as a co-regulator. Genes for other mechanosensitive channels have been identified in *Cronobacter* spp., including *mscL*, *mscS*, and *mscK*. These channels play important roles in the response to hypo-osmotic shock by allowing the efflux of osmolytes from microbial cells to decrease turgor stress in the cytoplasm. Feeney and Sleator (2011) also identified a system, the high-affinity K^+ uptake system Kdp in the *C. sakazakii* genome, which might play a role in transcriptional regulation. Seven copies of the osmolyte uptake system ProP were also found in the *C. sakazakii* BAA-894 genome, namely ESA-02131, ESA-04214, ESA-01706, ESA-pESA3p05450, ESA-00673, ESA-01226, and ESA-03328 (Feeney and Sleator, 2011). These proteins localize to the transmembrane and allow the uptake of osmoprotectants from the bacterial environment. Their roles in bacterial survival of desiccation have not yet been verified by laboratory studies. The genome also has a *proU* operon that encodes proVWX proteins that function as an ATP-requiring multicomponent osmoprotectant uptake system. The *C. sakazakii* genome also encodes the osmoprotectant (ATP-binding cassette) ABC transporter *OpuC* operon, which encodes the extracellular binding protein OpuCC; the membrane proteins OpuCB and OpuCD; and the ATP-binding protein OpuCA. Furthermore, the genome encodes proteins involved in the synthesis of proteins that are important for the uptake of osmoprotectants like trehalose, proline, and betaine (Feeney and Sleator, 2011). *Cronobacter's* ability to withstand desiccant conditions is due to an osmoprotectant accumulation (trehalose), since it protects cells from dying of low moisture contents (Breeuwer *et al*. 2003).

Yellow pigmentation is another important characteristic of some *Cronobacter* strains, since 80% of *Cronobacter* isolates are yellow when they are grown on TSA at 25°C (Iversen and Forsythe, 2007). This feature is assumed to be regulated by temperature, since a number of pigmented isolates show reduced yellow pigmentation when the temperature is changed to 37°C (Iversen and Forsythe, 2007). Osaili and Forsythe (2009) suggested that because *Cronobacter* spp. originate in plant material, the

yellow pigmentation might protect the bacteria from the oxygen radicals produced by sunlight (Osaili and Forsythe, 2009).

In order to cause infections in neonates and other immuno-compromised individuals, it might be reasonable to propose that *Cronobacter* strains should survive the stomach's high acidity level. Studies have shown *Cronobacter* is not only able to survive but also grow in such acidic environments. Acid resistance is another important physiological characteristic that has been studied in *Cronobacter* strains. In one report, at least 79.2% of the tested *Cronobacter* strains were able to grow at a pH of 3.9 (Dancer *et al.* 2009). Another study showed that *Cronobacter* was not only able to survive but even grow and multiply at a low pH 4.5 (Johler *et al*. 2009). A study by Kim and Beuchat (2005) reported the survival of *Cronobacter* spp. in some acidic fruits and vegetables for up to 48 hours at 25°C. Another study found that certain *Cronobacter* isolates exhibit moderate resistance to environments as acidic as pH 3.0. They also found that pre-exposure to non-lethal acidic pH increased the resistance of the strains to acid (Edelson-Mammel *et al*. 2006). A recent study by Alvarez-Ordones *et al*. (2014) proposed that the *ompR* gene is an essential factor in the adaptive response of *C. sakazakii* to highly acidic conditions. Al-Holy *et al*. (2010) showed that sub-lethal concentrations of copper in combination with lactic acid inhibited *Cronobacter* spp. growth in infant formula. In terms of food preservation, many studies use acids as antimicrobial factors since they inactivate *Cronobacter* spp. isolates, particularly in PIF. Back *et al*. (2009) demonstrated that adding organic acids, such as propionic and acetic acid, to liquids like baby food and fruit juice inhibited the growth and survival of *Cronobacter* isolates. Likewise, they showed the antimicrobial activity of red muscadine (*Vitis rotundifolia*) juice against *C. sakazakii* as a result of the combined activity of the malic, tartaric and tannic acids in the juice (Kim *et al*. 2010a).

The production of large quantities of capsular polysaccharides is another important characteristic that protects *Cronobacter* cells from desiccation. Caubilla-Barron and Forsythe (2007) investigated 27 Enterobacteriaceae strains and demonstrated that *Cronobacter* spp. strains that formed capsules survived and could be recovered from reconstituted PIF after more than 2.5 years of desiccation. In addition to desiccation survival, capsule formation is involved in *Cronobacter* biofilm formation.

Cronobacter possess the ability to make biofilms on surfaces, such as polycarbonate, stainless steel, polyvinyl, chloride, latex, and silicon (Iversen *et al*. 2004b; Lehner *et al*. 2005). Moreover, biofilm formation by *Cronobacter* was investigated in a study of the enteral feeding tubes of neonates in the NICU that was conducted by Hurrel *et al*. (2009a). Iversen and Forsythe (2003) suggested that properties such as desiccation resistance, capsule polysaccharide production, and yellow pigmentation are the result of the natural plant habitat of *Cronobacter* spp. The capsular polysaccharide helps the bacterium attach to plant surfaces, and its desiccation resistance allows it to survive in extreme environmental conditions. These characteristics might explain the survival of the bacterium in starch, which is a main component of products like PIF. Joseph *et al*. (2012) studied 14 *Cronobacter* spp. sequenced strains and found that colanic acid synthesis encoded by *wzABCKM* (ESA_01155-01175) genes were detected in all strains. The study suggested that these genes might contribute primarily to *Cronobacter's* biofilm formation (Joseph *et al*. 2012b).

1.5 Virulence and pathogenicity factors

Although *Cronobacter* spp. infections are rare, they are of great interest to clinicians and researchers because of the severity of the infections and the young age of the neonates that are most affected by them. Since the *C. sakazakii* BAA-894 and *C. turicensis* z3032 genomes were published, research efforts have focussed on genetic analysis of *Cronobacter* spp. in order to identify potential pathogenic mechanisms and genetic features that are associated with bacterial virulence (Kucerova *et al.* 2010; Stephan *et al.* 2011).

Notably, *Cronobacter* isolates and species vary in terms of their virulence factors and their properties in tissue culture (Caubilla-Barron *et al*. 2007; Townsend *et al*. 2007, 2008). Several studies have identified virulence factors, including the Type VI secretion system, blood-brain barrier penetration, iron acquisition, and enterobactin and aerobactin synthesis (Kucerova *et al*. 2011; Franco *et al*. 2011). Importantly, not all *Cronobacter* species are linked to neonatal and adult infections. In fact, *Cronobacter* infections in humans are limited to just three species: *C. sakazakii*, *C. turicensis*, and *C. malonaticus* (Kucerova *et al.* 2010). These *Cronobacter* species have the ability to attack human

intestinal cells, to replicate in macrophages, to invade the blood-brain barrier, and to survive in serum, although these abilities vary among the species (Townsend *et al*. 2007, 2008).

As noted above, the precise mechanisms underlying *Cronobacter* spp. pathogenicity have not been fully elucidated. Researchers have shown that *C. sakazakii* caused meningitis infections when the bacteria crosses the human blood-brain barrier with the help of secretory factors like endotoxins, glycopeptides, and collagenase. Translocation from the blood to the brain can be via paracellular or transcellular mechanisms or, in some situations, via destruction of the brain's microvascular endothelium (Huang *et al*. 2000; Iversen and Forsythe 2003; Kim, 2008). In addition, dendritic cells can damage brain tissue in an *in vitro* necrotising enterocolitis model (Emami *et al*. 2011).

Several studies have demonstrated a link between *Cronobacter* spp. pathogenicity and outer membrane protein A (OmpA). OmpA expression is responsible for *Cronobacter* invasion of human brain microvascular epithelial cells (HBMECs) *in vitro* (Mohan Nair and Venkitanarayanan 2006; Singamsetty *et al*. 2008). Another study by Mittal *et al*. (2009) reports that the OmpA and OmpX proteins play roles in meningitis in aiding penetration of the blood-brain barrier. In addition, *ompA* helps initiate the invasion of HBMECs through the blood-brain barrier in *Cronobacter* spp. via fibronectin-binding proteins (Mohan Nair *et al*. 2009).

In 2007, Kothary and co-workers identified a gene encoding zinc-containing metalloprotease (*zpx*) gene that caused necrosis and cellular damage in NEC in infants. This enzyme might facilitate penetration of the blood-brain barrier and cause meningitis. Also, *Cronobacter* spp. encode a number of haemolysis genes (Joseph *et al.* 2012a).

A genomic study of *Cronobacter* spp. described ten fimbriae clusters (Joseph *et al.* 2012). Fimbriae are appendages that help bacteria adhere to the surfaces of host cells, thereby facilitating colonisation (Soto and Hultgren, 1999). Genomic analysis showed that only *C. sakazakii* has β-fimbriae and also that *C. sakazakii* lacks the curli fimbriae that are found in other species. This observation indicates that unlike *E. coli* pathovars, *C. sakazakii* pathogenicity does not require curli fimbriae (Joseph *et al.* 2012).

A study by Franco *et al*. (2011) demonstrated the important role of the plasminogen activator *cpa* in the survival of *C. sakazakii* in human serum. Notably, *cpa* is an outer membrane protease that is encoded on a plasmid. The genomic analysis showed that the *cpa* sequences in *C. sakazakii* are highly similar to those of the *Pla* subfamily of omptins, which was identified as a virulence factor in many Enterobacteriaceae (Franco *et al*. 2011). Proteins in the *Pla* subfamily help degrade many serum proteins, thus providing a protective effect against complement-dependent serum killing (Franco *et al.* 2011). Townsend *et al.* (2007) showed that the superoxide *sodA* gene, which plays a role in macrophage survival, may also be involved in bacterial resistance to oxidative stress. However, the *cpa* gene and the *sodA* gene are not consistently found in *Cronobacter* isolates (Joseph *et al.* 2012b).

Use of the open-access PubMLST database, which lists all 214 genomes of the known *Cronobacter* species, is helping researchers analyse genomic data to study the detailed ecology, taxonomy, and virulence traits of the organism. For example, BLAST analysis showed that *C. sakazakii* strains (n = 72 genomes) had the *cpa* gene and that this gene was absent from *C. malonaticus* (n = 14 genomes). This might explain, at least in part, the difference in host susceptibility (Forsythe *et al.* 2014).

A plasmid-borne Type VI secretion system, T6SS, was described recently that may contribute to bacterial virulence by influencing bacterial attachment, growth inside macrophages, cytotoxicity, host tissue invasion, and survival inside the host. In *Cronobacter*, there are 5 putative T6SS clusters (Kucerova *et al.* 2010; Joseph *et al.* 2012a). Zhou *et al.* (2012) described the association of T6SS with *E. coli* K1 and penetration of the human blood-brain barrier.

Genomic studies of *Cronobacter* also revealed the presence of a number of genes related to heavy metal resistance (i.e. resistance to copper, silver, zinc, and tellurite) (Kucerova *et al.* 2010; Grim *et al*. 2012). Several iron adaptation mechanisms have been demonstrated in different species of *Cronobacter* (Kucerova *et al.* 2010; Grim *et al*. 2012). Two iron acquisition systems have been found in *Cronobacter* that are located in plasmids pESA3 and pCTU1: a siderophore iron acquisition system (*iucABCD-iutA*) and an ATP-containing cassette transport- facilitated iron uptake system (*eitCBAD*) (Kucerova *et al.* 2010; Franco *et al.* 2011a; Joseph *et al.* 2012a). Recent analysis by Grim *et al*. (2013)

found that all *Cronobacter* species have genes for enterobactin synthesis (*entABCDEFS*) transport gene and for an enterobactin receptor gene (*fepABCDEG*). In addition, most *Cronobacter* species have the aerobactin synthesis operon *iucABCD* as well as the gene for its receptor, *iutA,* except *C. muytjensii* (Grim *et al*. 2013)

The *nanAKT* gene cluster encodes proteins that are involved in utilising exogenous sialic acid as a carbon source. These genes were recently identified as virulence factors by genomic analysis (Joseph *et al.* 2012b; Joseph *et al.* 2013; Forsythe, 2015). Notably, this gene cluster has only been found in *C. sakazakii* and in some *C. turicensis* strains. Sialic acid is found in the intestinal tract in mucin, and gangliosides are components of the human brain and breast milk. Accordingly, utilization of sialic acid by *Cronobacter* as an alternative source of carbon might allow bacteria to survive and attack the brain, leading to brain damage. This could be considered an important evolutionary adaptation to the host environment. In addition, sialic acid is a component of PIF because it is involved in brain development (Joseph *et al.* 2013). On the other hand, *C. sakazakii* cannot use malonic acid as a carbon source, but other species of *Cronobacter* can (Forsythe *et al.* 2014). As malonic acid is found in plant tissues, the ability of *Cronobacter* to use malonic acid reflects its association with plants. Genomic analysis has revealed that the genes encoding malonate decarboxylase, which allow the organism to use malonate, are near the auxin efflux transporter gene. The loss of the ability of *C. sakazakii* to use malonate and its acquisition of the ability to use sialic acid can be seen as an adaptation of *C. sakazakii* to a new environment and is therefore probably clinically relevant.

1.6 Sources of the *Cronobacter* genus (reservoirs)

Cronobacter spp. are prevalent organisms that have been isolated from a variety of sources, including cereals, herbs, spices, ready-to-eat products, salads, confectioneries, cheese products, meat, and vegetables (Iversen and Forsythe, 2004; Friedemann, 2007; Baumgartner *et al*. 2009). A number of these products resemble or include the natural plant habitat of *Cronobacter* spp., and the raw materials are considered possible sources of contamination by the organism. In Barcelona, Spain, a *Cronobacter* strain was isolated from a marine environment (Agogue *et al*. 2005); elsewhere, the *C. universalis* NCTC 9529^{T} strain was isolated from freshwater (Iversen *et al*. 2007). Other *Cronobacter*

natural environmental sources include soil, grass silage, and thermal mineral water springs, to name a few (Neelam *et al*. 1987; Mosso *et al.* 1994; Van Os *et al*. 1996). According to a study conducted by Kandhai *et al.* (2004), *Cronobacter* is present in domestic habitats, and was isolated from 5 of 16 vacuum-cleaner bags that were tested. Furthermore, *Cronobacter* spp. have been isolated from the floors, roofs, tanker bays, drying towers, roller dryers, conveyors, and air filters of industrial units, as well as from other manufacturing, processing, and non-processing areas in multiple milk powder factories (Hein *et al*. 2009; Craven *et al*. 2010; Jacobs *et al*. 2011).

Cronobacter strains have been isolated from animal sources as well: from the nostril of a stable horse (Holy *et al*. 2011); from wild house flies (*Musca domestica*); from the guts of the Mexican fruit fly, *Anastrepha ludens*; from stable fly larvae, *Stomoxys calcitrans*; and from filth flies (Kuzina *et al*. 2001; Mramba *et al*. 2006; Butler *et al*. 2010; Pava-Ripoll *et al*. 2012). These findings suggest that flies might serve as vectors for *Cronobacter* spp.

Cronobacter spp. have been isolated from many types of clinical specimens, including blood, cerebrospinal fluid (CSF), sputum, bone marrow, urine, faeces, and wound infections (Farmer *et al*. 1980; Muytjens *et al*. 1983; Gallagher and Ball, 1991; Iversen *et al*. 2006; Caubilla-Barron *et al*. 2007). *C. malonaticus* LMG 23826^{T} was isolated from a breast abscess (Joseph *et al.* 2012a). As mentioned previously, this organism has also been isolated from neonatal enteral feeding tubes in ICUs (Hurrell *et al*. 2009b).

However, the most well-known isolation source for this organism is baby food, most notably PIF but also follow-up formula and weaning foods (Chap *et al*. 2009). Breast milk is often replaced by PIF products, which are non-sterile, and these products are frequently contaminated with members of the *Enterobacteriaceae* family, especially *Cronobacter*. Once *Enterobacteriaceae* was found in PIF, additional testing showed that this organism could be found in PIF products worldwide (Muytjens *et al*. 1988; Nazarowec-White and Farber 1997; Iversen and Forsythe, 2004).

The *Cronobacter* MLST database includes records for more than a thousand isolates, including the source of the isolate, and these data reflect the geographic and temporal diversity of the genus (Forsythe *et al*. 2014). The oldest isolate, *C. sakazakii* NCIMB 8272, was isolated from dried milk powder in 1950. More recently, the genome of this isolate was sequenced and the sequence was added

to the PubMLST database (Masood *et al*. 2013b). Furthermore, various *Cronobacter* strains have been isolated from 36 different countries, and the various sources include the following: environment (35.15%), infant formula (21.6%), clinical specimens (20.4%), food and related items (14.2%), and water and other sources (4%) (Forsythe *et al*. 2014). Past studies reported the isolation of *Cronobacter* species from a variety of food items and plants, such as herbs, rice, spices, and wheat (Iversen *et al.* 2004).

1.6.1 Powdered infant formula (PIF) as a transmission source

The contamination of PIF with bacteria could occur during the manufacturing and packing processes or during reconstitution due to the use of non-sterile utensils like contaminated spoons. Multiple *Cronobacter* infections and outbreaks can be tracked back to PIF (Muytjens *et al.* 1983; Van Acker *et al*. 2001; Himelright *et al*. 2002).

Contamination involving food-borne pathogens, such as *Cronobacter*, can cause severe illness in newborns (Iversen and Forsythe, 2003; Caubilla-Barron et al. 2007; Joseph and Forsythe, 2011). *Cronobacter* spp. can withstand the desiccated conditions of PIF for at least two years, and upon PIF reconstitution, they can grow rapidly (Caubilla-Barron *et al.* 2007; Osaili and Forsythe, 2009). In the last two to three decades, concerns about PIF contamination with *Cronobacter* has prompted efforts to research and monitor this organism, which causes illness and death in newborns (Caubilla-Barron *et al*. 2007). Accordingly, it is worthwhile to briefly describe how PIF is produced and how it can become contaminated. Outbreaks and clinical cases associated with formula-fed infants have led to PIF product recalls due to *Cronobacter* contamination. The first recall was in response to the 2001 Tennessee outbreak (Himelright *et al*. 2002). More recently, in December 2011, Wal-Mart recalled a PIF product in response to the shocking death of an infant in Missouri, USA (CDC, 2011).

The United States Department of Food and Drug Administration regulates the nutrient content of PIF, and PIF components vary in different formula types. However, all types of formula have fats, proteins, and carbohydrates, as well as minerals, such as iron, zinc, sulphur, copper, sodium, phosphate, potassium, chloride, calcium, magnesium, and iodine. Babies' bodies require high levels of iron to maintain normal growth, which is why iron is considered a crucial constituent of PIF. Vitamins are

also added to PIF, including vitamin A, B12, C, D, E, thiamine, riboflavin, pyridoxine, niacin, pantothenate, and folacin.

1.6.2 PIF manufacturing

The main constituent of infant formula is bovine milk. Since cow's milk has higher levels of proteins, fats, and minerals than human milk, dilution and skimming help it to better resemble natural human milk (Koletzko *et al*. 2005). Some infant formula uses soy as the main ingredient; however, it is advised that its use be restricted due to the possible negative consequences of phytoestrogens on newborns (Bhatia and Greer, 2008; Badger *et al*. 2009).

European and American industrial experts note that manufacturing PIF involves mixing the ingredients, processing them, and packaging them. During the drying process, a liquid mixture is heated to 82°C and then pumped through high-pressure spray nozzles. The hot air causes the liquid mixture to dry rapidly, and the resulting powder falls to the bottom of the drying chamber, where it is collected. The final product, which is the result of all of the drying and blending steps, is transferred from storage to the filling machinery, where the final powdered product is packaged, including sealing, coding, and labelling. The final version of the product is not released unless it passes a final examination and evaluation process, which includes microbiological and nutrient analyses.

1.6.3 Possible contamination points during milk powder manufacturing

According to joint FAO/WHO meetings (2004), PIF can become contaminated by different organisms during the addition of heat-sensitive ingredients, such as amino acids, vitamins, and fatty acids, or during the dry-mixing and combined-mixing processes. There are three processes that can be used during milk powder manufacturing: a dry-mixing process, a wet-mixing process, and a combined-mixing process. The dry-mixing process does not include heat treatment that might kill bacteria in the finished project. Hence, if one or more ingredients are contaminated by a minimum number of bacteria, then their presence in the finished product is more likely to be detected. In the wet-mixing process, a spray-drying technique is used that involves processing equipment, such as a spray dryer and fluidized bed, that must be cleaned frequently. Frequent wet cleaning may result in a moist

environment that is suitable for bacterial growth and dissemination. If bacterial growth is not controlled, it can eventually lead to product contamination.

Environmental contamination of PIF can occur during all three of these manufacturing processes, especially after thermal processing during PIF drying or packaging. Notably, in the dry-mixing process, the ingredients added to PIF are not raw materials; rather, they are processed by the suppliers. Consequently, PIF contamination might occur during post-heat treatment. Contamination of the ingredients added to PIF depends on the ingredient itself. For example, oils have a lower risk than starches of becoming contaminated with PIF(Agostoni *et al.* 2004).

The major microorganisms that are found in contaminated PIF were identified by joint FAO/WHO meetings (2004 and 2006) and include the following: *Cronobacter* spp., *Citrobacter koseri*, *Salmonella* Enteritidis, *Citrobacter freundii*, *Enterobacter cloacae*, *Klebsiella oxytoca*, *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia* sp., *Acinetobacter* sp., *Listeria monocytogenes, Bacillus cereus*, and *Staphylococcus* spp. Any of these organisms can grow and multiply after reconstitution, as milk products are excellent media for such growth. This can occur regardless of whether the PIF itself is contaminated or the organisms are introduced during reconstitution (Agostoni *et al.* 2004).

1.7 Epidemiology

Cronobacter spp. have been recognized as causative agents of multiple diseases that affect a broad range of age groups, and some of the diseases can be life-threatening, such as NEC, meningitis, septicaemia, and pneumonia (Muytjens *et al*. 1983; Caubilla-Barron *et al*. 2007). *Cronobacter* spp. cases have been reported in infants, which are a group at very high risk of infection (Lai, 2001). *Cronobacter* spp. have also been found in pre-term, low-birth weight infants that weighed less than 2500 g and were less than 28 days old (Lai, 2001). The symptoms and diseases that are associated with *Cronobacter* infections include bacteraemia or sepsis, meningitis, abscess, digestive problems, enterocolitis, necrotising conjunctivitis, and tonsillitis (Lai, 2001). Among these, meningitis cases have been reported most frequently, followed by bacteraemia and NEC (Healy *et al*. 2009). The Foodborne Diseases Active Surveillance Network (FoodNet) conducted a survey in 2002 and found that the incidence of *Cronobacter* meningitis or bacteraemia was about 1 case for every 100,000

infants yearly (Bowen and Braden, 2008). On the other hand, additional reports indicated that 40%–80% of the infants infected by meningitis *Cronobacter* died, whereas those with NEC due to *Cronobacter* had a 10%–55% fatality rate (Iversen and Forsythe, 2003; Lai, 2001). Many cases that were traced back to multiple neonatal *Cronobacter* spp. infections were linked to PIF contamination (Muytjens *et al* 1983; Van Acker *et al*. 2001; Himelright *et al*. 2002).

Cronobacter, as a causative agent, may be best known for its link with neonatal infections through its presence in PIF. However, it can affect individuals of all ages, especially the elderly or immunocompromised adults, and it has been associated with bacteraemia cases as well as with sepsis, pneumonia, and infected wounds. Most adult infections are reported in those >50 years old (Lai, 2001). Some adult *Cronobacter* infections have also arisen as secondary infections that are the result of other hidden underlying problems, such as urinary tract infections, malignancies, infections from contaminated medical tools, or person-to-person infections (Friedemann, 2009; Flores *et al*. 2011). To date, there have been no reported meningitis cases in adults that were specifically caused by *Cronobacter* spp. (Friedemann, 2009).

1.8 Outbreaks related to contaminated infant formula

- Muytjens *et al*. (1983) reported *Cronobacter* infections in 8 infants in the Netherlands, 5 in one hospital and 3 in three other hospitals. The infants were diagnosed with neonatal meningitis, and 6 of them died. This is considered to be the first reported *Cronobacter* outbreak, and it was associated with contaminated infant formula (Muytjens *et al*. 1983). A study conducted by Joseph and Forsythe in 2012 characterized strain 558, which was isolated in 1983 in the Netherlands by Muytjens *et al*. This characterisation showed that the strain was an ST4 strain.
- In 1994, a *Cronobacter* spp. outbreak occurred in a NICU in France. Caubilla-Barron *et al*. (2007) analysed the phenotypes and genotypes of 31 *Cronobacter* spp. strains that were isolated from 16 infected infants, 3 of whom died, as well as from prepared and unprepared infant formula. Their PFGE analysis categorized these strains into four pulsetypes; notably, strains taken from the 3 infants who died all belonged to pulsetype 2. Strains with pulsetypes 1 to 3 were obtained from the other infants and from opened formula, while pulsetype 4 came from unopened formula.

Cluster 2 isolates, which involved the largest cluster, were associated with all three casualties (Caubilla-Barron *et al.* 2007). More importantly, all of the cluster 2 isolates belonged to clonal complex 4 (CC4). A recent genomic analysis of these strains was conducted, and the *C. sakazakii* strains were categorised into four distinct clusters (Masood *et al.* 2015). Each cluster belonged to different sequence types, such as cluster 1:ST12, cluster 2:CC4, cluster 3:CC13, and cluster 4:CC1 (Masood *et al*. 2015). The findings of the cluster analysis were in accordance with those of a previously conducted PFGE profile analysis (Caubilla-Barron *et al*. 2007).

- Van Acker *et al*. (2001) reported that 12 infants in a NICU in Belgium were involved in a *Cronobacter*-associated NEC outbreak. All of the affected infants were pre-term, low birth weight, and formula-fed, and 2 of them died. According to Van Acker *et al.* (2001), this was the first reported incident that linked *Cronobacter* to NEC. Two of the *C. sakazakii* isolates involved in this outbreak, *C. sakazakii* 2106 (ST21) and 2107 (ST12), were sequenced recently by our group and analysed by E. Jackson as well as part of her PhD project.
- In 2001, Himelright and co-workers reported an outbreak of *Cronobacter* spp. in a hospital in Tennessee. An infant died from meningitis, prompting the testing of 49 infants who were hospitalized at the time. Among them, 8 were positive for *Cronobacter* (Himelright *et al*. 2002). Two had respiratory illnesses, and 6 were asymptomatic. Further investigation linked the outbreak with infant formula, which eventually led the manufacturers to recall the product. This isolate was obtained from a formula product not recommended for use by neonates (Himelright *et al.* 2002).
- Block *et al.* (2002) reported another *Cronobacter* outbreak in a hospital in Jerusalem. *Cronobacter* strains were isolated from 5 infants, 3 of whom had bacteraemia and meningitis and 2 of whom were asymptomatic. Our group obtained the meningitis isolate, *C. sakazakii* 8399 (*C. sakazakii* 1587, ST109-CC4), and sequenced its genome (Masood *et al*. 2013c).
- In December 2011, the Center for Disease Control and Prevention (CDC) reported cases of *Cronobacter* spp. infections that occurred in the following 4 states in the USA: Florida, Illinois, Missouri, and Oklahoma. Three infants in Missouri and Florida had meningitis, 2 of whom died. Importantly, all of the infants had been fed PIF, but the cases were not linked to a particular

manufacturer or brand. After one of the deaths was investigated, Wal-Mart immediately recalled infant formula from its supermarket shelves. In Missouri, the organism was found in an opened PIF tin, in prepared PIF, and in an opened nursery water bottle that had been used to prepare PIF. These isolates were studied further by our group (Hariri *et al.* 2013). Specifically, we analysed 15 *Cronobacter* isolates from the CDC and found that all 5 CSF isolates were either ST4 or within the ST4 complex (variants at one or two loci); these isolates comprise the ST4 complex, which is also called CC4.

1.9 Genomic studies

Genomic studies of *Cronobacter* spp. have been ongoing in recent years. The first genome to be sequenced was that of *C. sakazakii*, strain BAA-894, which was sequenced by the Genome Center at Washington University, MO, USA (Kucerova *et al*. 2010). This strain was isolated from a formula tin associated with the Tennessee *Cronobacter* NICU outbreak. The genome included a 4.4-Mb chromosome and three plasmids, two of which, pESA2 and pESA3, are 31 kb and 131 kb in size, respectively (accession no. NC_009778–80). Kucerova *et al*. (2010) published this genome, which was based on a whole-genome microarray study of *Cronobacter* genus diversity. The genomic sequence of *C. sakazakii* strain BAA-894 was also published based on work utilizing a 384,030 probe oligonucleotide tiling DNA microarray, which was applied to 10 strains belonging to 5 species in the genus: *C. turicensis, C. sakazakii, C. malonaticus, C. muytjensii,* and *C. dublinensis* (Kucerova *et al*. 2010). The results of the microarray study showed a 55% core genome for *C. sakazakii* and a 43% core genome for the *Cronobacter* genus; it also identified mobile elements, including prophage regions, that are responsible for the species' extensive diversity. Two studies, by Joseph *et al*. (2012) and Kucerova *et al*. (2011), reported the abundance of multiple virulence factors in the genome that were related to the iron acquisition systems, fimbrial clusters, and type VI secretion systems.

Later, a *C. turicensis* strain z3032 genome was sequenced by the Institute for Food Safety and Hygiene, in Zurich, Switzerland (Stephan *et al*. 2011). *C. turicensis* strain z3032 was isolated in 2005 from a deadly case at Children's Hospital in Zurich. The organism's chromosome was 4.38 kb, and it had three plasmids that were 22 kb, 53 kb, and 138 kb in size (accession nos. NC_013282–85). A study conducted by Franco *et al*. (2011) compared larger plasmids, pESA3 and pCTU1, which are

131 kb and 138 kb, respectively. The researchers reported that the plasmids had similar content and called them virulence plasmids since they harboured critical regions, such as type VI secretion systems, plasminogen activator, iron acquisition systems, and filamentous haemagglutinin genes. Additionally, whole genome shotgun assembly sequencing of the *C. sakazakii* strain E899 genome (accession no. AFMO00000000) was conducted by Chen *et al*. (2011b). No plasmid sequences are available for this genome. Joseph *et al.* (2012) performed an extensive comparative genomics study of *Cronobacter* that involved 14 *Cronobacter* genomes from 7 species (Joseph *et al*. 2012b). The researchers reported a larger pan-genome, comprising over 6000 genes, plus a small core genome of *Cronobacter* that comprised 2000 genes (Joseph *et al*. 2012b). There was a significant degree of variation in terms of virulence, which is associated with adhesions genes, T6SS genes, and heavy metal resistance genes, which are related to resistance against tellurite, copper, and silver. The authors showed that *C. sakazakii* genomes were unique compared to other members of the genus in the sense that they alone harboured genes that are essential for the utilisation of exogenous sialic acid (Joseph *et al*. 2012b).

1.10 Neonatal meningitis and *C. sakazakii* ST4 (CC4)

The *Cronobacter* MLST scheme defined more than 300 STs for *Cronobacter* (Forsythe *et al.* 2014). *C. sakazakii* ST4, for example, was discovered to be the predominant ST within CC4, which is associated with neonatal meningitis and has often been isolated from CSF (Joseph and Forsythe, 2011). A previous study by Joseph and Forsythe (2011) suggested a strong association between *C. sakazakii* ST4 and meningitis in newborn infants. They analysed 41 *Cronobacter* strains, almost half (20/41) of which were *C. sakazakii* ST4 (Joseph and Forsythe, 2011). Furthermore, 9 of 12 meningitis isolates were *C. sakazakii* ST4. Thus, the study showed a strong relationship between *C. sakazakii* ST4 and neonatal meningitis and, moreover, presented *C. sakazakii* ST4 as a clonal lineage, since the isolates were collected from 7 countries over a 50-year period (Joseph and Forsythe, 2011). Our findings are supported by those from a study by Hariri *et al.* (2013), which analysed 15 *Cronobacter* isolates from the CDC and found that 5 CSF isolates belonged to CC4. Following up on the work of Joseph and Forsythe (2011), Hariri and colleagues (2013) introduced the concept of *C. sakazakii* CC4, which was determined to be a clonal lineage made up predominantly of ST4 strains and strains that

varied in only 1 or 2 MLST loci. Neonatal infections, especially neonatal meningitis, have been associated with CC4 isolates (Joseph and Forsythe, 2011; Hariri *et al.* 2013).

1.11 Public health significance and future directions

Although *Cronobacter* infections are not common, there is still a need to determine the potential health risks to the general public so that adequate measures can be implemented to control and restrict the growth of the organism. Since the pathogen's target population in the past has most often been infants, with PIF as the causal agent, extra caution needs to be taken and extensive awareness needs to be raised to keep infant formula and similar products safe from microbiological contamination. PIF manufacturing, handling, and reconstitution must be monitored and performed with caution to ensure that it is safe for consumption. Since manufactured PIF is not sterile, it is the manufacturers' responsibility to ensure minimal or no contamination of the product during processing.

Incidents associated with *Cronobacter* infection have led to changes being implemented by the FAO/WHO (2004), which determines the microbiological criteria for manufactured PIF. Such initiatives were discussed during risk assessment meetings that acknowledged *Cronobacter* spp. and *Salmonella enterica* as the major bacterial risk factors to infants consuming reconstituted PIF. Accordingly, these efforts highlight the importance of safe practices during formula reconstitution (FAO/WHO, 2004, 2006, 2008; CAC 2008).

WHO strongly advocates breast-feeding infants who are under 6 months of age. However, breast-feeding is not always possible. In such cases, product-reconstitution and formula-product standards need to be adhered to when such foods are introduced to non-breastfed infants. The individuals who prepare the reconstituted infant formula, such as mothers, caregivers, and medical personnel, should be trained and informed of possible contamination hazards. WHO recommends maintaining aseptic conditions during preparation and recommends taking measures to prevent post-preparation temperature fluctuations that could allow the organism to grow. The recommended temperature of the water for reconstitution is >70°C in order to kill contaminating bacteria. The prepared formula should be consumed within 30 minutes, and if it is stored, it should be refrigerated for 24 hours maximum (WHO, 2001; Iversen and Forsythe, 2003; WHO, 2007; Turck, 2012).

Despite the FAO/WHO risk assessment meetings in 2004, the microbiological criteria of the Codex Alimentarius Commission were not altered until 2008. These criteria now apply to PIF intended for consumption by infants with a target age of up to 6 months. However, the criteria were not applied to what is commonly known as 'follow-up formula,' which is used at the weaning stage for infants less than 6 months old. Although there is microbiological evidence of the frequent isolation of *Cronobacter* spp. from follow-up formula and weaning foods, the epidemiological evidence presented was not reliable enough for manufacturers to pursue additional microbiological testing (FAO/WHO, 2008). The FAO/WHO (2004) committee also highlighted the need for additional research into the organism to gain a better understanding into its taxonomy and virulence. The literature review presented here reflects the research conducted in the past decades and shows that there is still a fairly long way to go.

1.12 Aims and objective

Although *Cronobacter* spp. infections are rare, they are of great interest to researchers and clinicians because of the severity of the infections caused by the organism and because of the young age of the neonates that are most affected by them. In the last two decades, researchers have focused their attention on *Cronobacter sakazakii* and on the clonal complex 4 (CC4) lineage in particular. Our group at NTU found previously that this is the lineage that is associated with neonatal meningitis. Numerous outbreaks of this emerging food-borne pathogen have claimed the lives of babies, yet the environmental fitness and genetic features that most influence the virulence potential of this important lineage of *Cronobacter* in neonates are not known. *Cronobacter*, formerly termed *E. sakazakii*, is frequently isolated from powdered infant formula (PIF) processing plants, from PIF itself, and from milk powder factories in countries such as Germany and Australia (Muytjens *et al.*1983; Mullane *et al.* 2007; Proudy *et al.* 2008; Craven *et al.* 2010; Jacobs *et al*. 2011). *Cronobacter* in contaminated PIF has been linked to infant deaths.

The overall aim of this research project was to identify the physiological determinants of the neonatal meningitis bacterium *C. sakazakii* CC4 that are associated with its environmental persistence. To address this aim, three studies were conducted:

- **Investigation of the molecular epidemiology and clonality of environmental *Cronobacter* isolates with an emphasis on *C. sakazakii* CC4**

  This study had the following objectives:

  - Identification of environmental *Cronobacter* spp. isolates using multilocus sequence typing (MLST) with a focus on CC4
  - MLST analysis of three collections of environmental strains isolated from PIF, milk powder factory environments, and roller and spray dryers that had not been profiled previously by MLST
  - Determination of the dominant sequence type (ST) in the environment
  - Correlation of STs and pulsotypes

  The results of these analyses are presented in Chapter 3.

- **Determination of the physiological and virulence-related factors that affect the persistence of neonatal meningitis *C. sakazakii* strains in different environments and hosts**

This study had the following objectives:

- Characterisation of *C. sakazakii* CC4 and non-CC4 isolates using physiological assays such as heat tolerance, desiccation resistance, and acid resistance assays
- Investigation of the genes associated with specific physiological functions in sequenced *C. sakazakii* CC4 and non-CC4 genomes using *Cronobacter* BLAST

The results of these analyses are presented in Chapter 4.

- **Profiling of bacterial outer membrane proteins (OMPs) using genomic determination and SDS-PAGE analysis**

This study had the following objectives:

- *In silico* analysis of selected OMPs that were identified previously in the *E. coli*-mediated bacterial adaptation response to the host environment using the NCBI database and *Cronobacter* BLAST
- Investigation of the OMP profiles of *C. sakazakii* CC4 and non-CC4 isolates cultured at pH 6 versus pH 3.5 using SDS-PAGE analysis
- Correlation of OMP profiles and serotypes

The results of these analyses are presented in Chapter 5.

CHAPTER 2: Materials and methods

2.1 Safety considerations

All experiments in this study were carried out according to microbiology level 2 health and safety codes. All materials and protocols were assessed carefully, and the suitable COSHH forms were completed. Good laboratory practices were followed while operating laboratory equipment and while handling microbes, chemicals, and media. All waste materials were disposed according to the recommendations of the material safety data sheets.

2.2 Bacterial strains

All bacterial strains used in this study were obtained from the Nottingham Trent University (NTU) *Cronobacter* spp. culture collection or from international culture collections or collaborators. *Cronobacter* spp. strains from Australia and Germany were obtained from collaborators at the CSIRO Animal, Food and Health Sciences, Werribee, Victoria, Australia. *Cronobacter* spp. strains from Australia were subject to a Material Transfer Agreement that limited the analysis to MLST analysis. *Citrobacter koseri* strain 48 (BAA-895), which belongs to the Enterobacteriaceae family, was used in this study as a comparative control in the physiological experiments and as an outlier when constructing the MLST phylogenetic tree as it is the genus that is most related to *Cronobacter* spp. and is also associated with neonatal meningitis (Joseph *et al.* 2012). Additional strain details are listed in **Table 2.1**.

Table 2. 1The *Cronobacter* strains used in this study.

Species	NTU ID (International culture collection code)	Country of origin	Source	Year of isolation	A	B	C	D
C. sakazakii	1	USA	Clinical (throat)	1980	x		x	x
C. sakazakii	4	Canada	Clinical	1990	x		x	
C. sakazakii	5	Canada	Clinical	1990	x		x	
C. sakazakii	6	Canada	Clinical	2003	x		x	
C. sakazakii	20	CR	Clinical (faeces)	2004	x		x	
C. sakazakii	140	Unknown	Non-clinical (spice)	2005	x		x	
C. sakazakii	150	Korea	Non-clinical (spice)	2005	x		x	
C. sakazakii	377	UK	Milk powder	1950	x		x	
C. sakazakii	520	CR	Clinical	1983	x		x	
C. sakazakii	553	Netherlands	Clinical	1977	x		x	
C. sakazakii	555	Netherlands	Clinical	1979	x		x	
C. sakazakii	557	CR	Clinical	1979	x		x	
C. sakazakii	558	The Netherlands	Clinical	1983	x		x	
C. sakazakii	541	The Netherlands	Infant formula	1988		x		
C. sakazakii	543	The Netherlands	Infant formula	1988		x		
C. sakazakii	537	Russia	Infant formula	1988		x		
C. sakazakii	528	Belgium	Infant formula	1988		x		
C. sakazakii	545	The Netherlands	Infant formula	1988		x		
C. sakazakii	529	Canada	Infant formula	1988		x		
C. sakazakii	538	Russia	Infant formula	1988		x		
C. sakazakii	540	The Netherlands	Infant formula	1988		x		
C. sakazakii	544	The Netherlands	Infant formula	1988		x		
C. sakazakii	548	West Germany	Infant formula	1988		x		
C. sakazakii	FSL F6-032 (1921HPB-3284)	Uruguay	Infant formula	1988		x		
C. sakazakii	539	The Netherlands	Infant formula	1988		x		
C. sakazakii	532	East Germany	Infant formula	1988		x		
C. sakazakii	536	Russia	Infant formula	1988		x		

C. sakazakii	547	USA	Infant formula	1988		x		
C. sakazakii	531	Denmark	Infant formula	1988		x		
C. sakazakii	533	France	Infant formula	1988		x		
C. sakazakii	658 (ATCC BAA-894)	USA	Non-infant formula	2001	x		x	
C. sakazakii	680	USA	Clinical	1977	x		x	
C. sakazakii	696	France	Clinical	1994	x		x	
C. sakazakii	694	France	Clinical	1994	x			
C. sakazakii	701	France	Clinical	1994	x		x	
C. sakazakii	721	USA	Clinical (CSF)	2003	x		x	
C. sakazakii	767	France	Clinical (trachea)	1994	x		x	
C. sakazakii	730	France	Clinical	1994			x	
C. sakazakii	978	UK	Clinical (internal feeding tube)	2007	x		x	
C. sakazakii	984	UK	Clinical (internal feeding tube)	2007	x		x	
C. sakazakii	1218	USA	Clinical (CSF)	2001	x		x	
C. sakazakii	1219	USA	Clinical (CSF)	2002	x		x	
C. sakazakii	1220	USA	Clinical (CSF)	2003	x		x	
C. sakazakii	1221	USA	Clinical (CSF)	2003	x		x	
C. sakazakii	1225	USA	Clinical (blood)	2007	x		x	
C. sakazakii	1231	NZ	Clinical (faeces)	2004	x		x	
C. sakazakii	1240	USA	Clinical (CSF)	2008	x		x	
C. sakazakii	1249	UK	Clinical	2009	x		x	
C. sakazakii	HPB5174	Ireland	Powdered infant formula factory	Unknown	x		x	
C. sakazakii	SP291	Ireland	Powdered infant formula factory	2012	x		x	
C. sakazakii	ES15	Korea	Whole grain	Unknown	x		x	
C. sakazakii	1466	Australia	Environment	2007		x		
C. sakazakii	1467	Australia	Environment	2007		x		
C. sakazakii	1470	Australia	Environment	2007		x		
C. sakazakii	1471	Australia	Environment	2007		x		
C. sakazakii	1472	Australia	Environment	2007		x		
C. sakazakii	1473	Australia	Environment	2007		x		
C. sakazakii	1474	Australia	Environment	2007		x		
C. sakazakii	1475	Australia	Environment	2007		x		
C. sakazakii	1476	Australia	Environment	2007		x		
C. sakazakii	1477	Australia	Environment	2007		x		
C. sakazakii	1479	Australia	Environment	2007		x		

C. sakazakii	1480	Australia	Environment	2007		x		
C. sakazakii	1481	Australia	Environment	2007		x		
C. sakazakii	1482	Australia	Environment	2007		x		
C. sakazakii	1483	Australia	Environment	2007		x		
C. sakazakii	1484	Australia	Environment	2007		x		
C. sakazakii	1485	Australia	Environment	2007		x		
C. sakazakii	1486	Australia	Environment	2007		x		
C. sakazakii	1487	Australia	Environment	2007		x		
C. sakazakii	1488	Australia	Environment	2007		x		
C. sakazakii	1489	Australia	Environment	2007		x		
C. sakazakii	1490	Australia	Environment	2007		x		
C. sakazakii	1491	Australia	Environment	2007		x		
C. sakazakii	1492	Australia	Environment	2007		x		
C. sakazakii	1493	Australia	Environment	2007		x		
C. sakazakii	1494	Australia	Environment	2007		x		
C. sakazakii	1495	Australia	Environment	2007		x		
C. sakazakii	1496	Australia	Environment	2007		x		
C. sakazakii	1497	Australia	Environment	2007		x		
C. sakazakii	1498	Australia	Environment	2007		x		
C. sakazakii	1499	Australia	Environment	2007		x		
C. sakazakii	1500	Australia	Environment	2007		x		
C. sakazakii	1501	Australia	Environment	2007		x		
C. sakazakii	1502	Australia	Environment	2007		x		
C. sakazakii	1503	Australia	Environment	2007		x		
C. sakazakii	1504	Australia	Environment	2007		x		
C. sakazakii	1505	Australia	Environment	2007		x		
C. sakazakii	1506	Australia	Environment	2007		x		
C. sakazakii	1507	Australia	Environment	2007		x		
C. sakazakii	1508	Australia	Environment	2007		x		
C. sakazakii	1529	Germany	Environment	2006		x		
C. sakazakii	1530	Germany	Environment	2006		x		
C. sakazakii	1531	Germany	Environment	2006		x		
C. sakazakii	1532	Germany	Environment	2006		x		
C. sakazakii	1533	Germany	Environment	2006		x	x	
C. sakazakii	1534	Germany	Environment	2006		x		
C. sakazakii	1535	Germany	Environment	2006		x		
C. sakazakii	1536	Germany	Environment	2009		x	x	
C. sakazakii	1537	Germany	Environment	2009		x	x	

C. sakazakii	1538	Germany	Environment	2009		x		
C. sakazakii	1539	Germany	Unknown	Unknown		x		
C. sakazakii	1540	Germany	Environment	2009		x		
C. sakazakii	1542	Germany	Environment	2009		x	x	
C. sakazakii	1587	Israel	Clinical	2000			x	
C. sakazakii	1899	Australia	Environment	2007		x		
C. sakazakii	1900	Australia	Environment	2007		x		
C. muytjensii	530	Denmark	Infant formula	1988		x		
C. turicensis	1211 (LMG23827^T)	Switzerland	Blood	2005				x
C. turicensis	1468	Australia	Environment	2007		x		
C. turicensis	1469	Australia	Environment	2007		x		
C. turicensis	1478	Australia	Environment	2007		x		
C. turicensis	1509	Australia	Environment	2007		x		
C. turicensis	1510	Australia	Environment	2007		x		
C. turicensis	1511	Australia	Environment	2007		x		
C. turicensis	1512	Australia	Environment	2007		x		
C. turicensis	1513	Australia	Environment	2007		x		
C. turicensis	1898	Australia	Environment	2007		x		
C. malonaticus	681 (LMG23826^T)	USA	Clinical	1977				x
C. malonaticus	1514	Australia	Environment	2007		x		
C. malonaticus	507	CR	Clinical	1984				
C. malonaticus	527	Australia	Infant formula	1988		x		
C. malonaticus	535	NZ	Infant formula	1988		x		
C. condimenti	1330 (LMG26250^T)	Slovakia	Food	2010				x
C. universalis	581 (NCTC9529^T)	UK	Water	1956				
C. dublinensis	582 (NCTC9844 ^{T})	UK	Unknown	Unknown				
C. dublinensis	1210 (LMG23823 ^{T})	Ireland	Environment	2007		x		x
Citrobacter koseri	48 (CKSMT319)	USA	Clinical (CSF)	Unknown		x		x
Citrobacter koseri	BAA-895	USA	Unknown	Unknown			x	
Siccibacter turicensis	1974 (LMG23730^T)	Switzerland	Fruit powder	2007				x
Franconibacter pulveris	1976 (LMG24057^T)	Switzerland	Fruit powder	2008				x

Franconibacter pulveris	1978 (LMG24059 ^{T})	Switzerland	Infant formula	2008				x
Franconibacter helveticus	1975 (LMG23732^{T})	Switzerland	Fruit powder	2007				x
Siccibacter colletis	1383 (LMG28204^{T})	UK	Ingredients	2011				x
E. coli K12	MG1655	Unknown	Unknown	Unknown			x	
Staphylococcus aureus	NCIMB 6571	Unknown	Unknown	Unknown			x	
Streptococcus pyogenes	NCTC 9994	Unknown	Unknown	Unknown			x	
Salmonella enterica serovar Enteritidis	358	Unknown	Unknown	Unknown			x	

Comments:
x: Indicates the use of this strain.
T: Species type.
A: Strains that were used in the MLST laboratory experiments; the sequence data were analysed as part of this thesis.
B: Strains that were used in these physiological assays: heat tolerance, acid stress tolerance, desiccation assay, serum resistance assay, capsule production assay, haemolysin assay and outer membrane protein extraction and profile as a part of this thesis.
C: Strains that were used in the genomic analyses as a part of this thesis.
D: Strains that were used in the phylogenetic analyses of the *Cronobacter* spp.
CR: Czech Republic; **NZ**: New Zealand; **UK**: United Kingdom; **USA**: United States of America

2.3 Bacterial storage and culture

All bacteria were stored at -80°C in tryptone soya broth (TSB) (Oxoid Thermo Scientific, UK) plus 20% (v/v) glycerol (Fisher Scientific, UK). Before performing the experiments, each strain was cultured overnight on tryptone soya agar (TSA) plates by aerobic incubation at 37°C and checked for purity.

2.4 Sterilization

All media, buffers, and solutions were sterilised by autoclaving at 121°C for 15 min and/or by filtration through a 0.2-µm filter as indicated below. All equipment was washed with 70% alcohol prior to use.

2.5 Stock reagents and buffers

2.5.1 Saline solution

One saline tablet (BR0053G, Oxoid Thermo Scientific) was dissolved in 500 ml of distilled water and autoclaved at 121°C for 15 minutes.

2.5.2 Phosphate buffered saline (PBS)

One tablet of phosphate buffered saline (PBS) (D8662, Sigma-Aldrich, Ltd.) was dissolved in 200 ml distilled water and autoclaved at 121°C for 15 minutes.

2.5.3 Hydrochloric acid (HCl)

A 1M solution of HCl (Fisher Scientific) was used to adjust the pH of solutions and/or media as indicated. A 100-ml HCl solution was prepared by mixing 8.6 ml of concentrated 1M HCl with 91.4 ml of distilled water followed by sterilization using 0.2-µm filters.

2.5.4 HEPES [4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid] buffer

HEPES buffer (10 mM, pH 7.4) was used in the outer membrane protein (OMP) extraction method. In order to make 1 litre of 10 mM HEPES buffer, a 1 M HEPES buffer solution (83264; Sigma-Aldrich, Ltd.) needed to be diluted 1:100. Accordingly, 10 ml of 1M HEPES buffer solution was added to 990 ml of distilled water, then the pH was adjusted to pH 7.4 with 1 M HCl and autoclaved at 121°C for 15 minutes.

2.5.5 Phenylmethyl sulphonyl fluoride (PMSF)

Phenylmethyl sulphonyl fluoride (P7626; Sigma) was used for the OMP extraction. To prepare this solution, 0.174 g of PMSF was dissolved in 10 ml of 100% ethanol on ice. This was added to HEPES buffer to a final concentration of 100 mM.

2.5.6 2% IGEPAL with 10 mM HEPES buffer

The OMP extraction method used a 2% IGEPAL solution that was prepared by adding 4 µl of IGEPAL (CA-630; Sigma-Aldrich, Ltd.) to 200 µl of 10 mM HEPES buffer.

2.5.7 10X SDS- PAGE running buffer

To prepare this buffer 100ml of 10X Tris/glycine sodium dodecyl sulfate polyacrylamide gel - electrophoresis (SDS- PAGE) running buffer (national diagnostic) diluted in 900ml of deionized water.

2.6 Culture media

2.6.1 Tryptone soya agar (TSA)

To prepare TSA culture plates, 40 g of TSA (CM0131, Oxoid Thermo Scientific) were added to 1000 ml of distilled water followed by autoclaving at 121°C for 15 minutes. The agar was allowed to cool to 55°C, and approximately 20 ml was poured into each sterile petri dish. The plates were stored at 4°C in the refrigerator for a maximum of three weeks.

2.6.2 Tryptone soya broth (TSB)

To make TSB, 30 g of TSB powder (CM0129, Oxoid Thermo Scientific) were added to 1000 ml of distilled water followed by autoclaving at 121°C for 15 minutes. The broth was then allowed to cool to 55°C and stored at room temperature.

2.6.3 Luria-Bertani agar (LBA)

To prepare LBA culture plates, 20 g of LB (L3022, Merck, Germany) were added to 1 litre of distilled water. This was autoclaved at 121°C for 15 minutes. The agar was then allowed to cool to 55°C. Approximately 20 ml was poured into each sterile petri dish. The plates were stored at 4°C in the fridge for a maximum of three weeks.

2.6.4 Luria-Bertani broth (LB)

To prepare LB broth, 25 g of LB broth (1102830, Sigma-Aldrich, Ltd.) were added to 1 litre of distilled water. This was autoclaved at 121°C for 15 minutes. The broth was then allowed to cool to 55°C and stored at room temperature.

2.6.5 Brain-heart infusion (BHI) broth

To prepare BHI broth, 37 g of BHI broth (CM1135, Oxoid Thermo Scientific) were added to 1 litre of distilled water. The mixture was heated with mixing until the BHI was completely dissolved, then it was dispensed into universal bottles in 10 ml aliquots and autoclaved at 121°C for 15 minutes. The bottles were stored in the refrigerator at 4°C until use.

2.6.6 Milk agar

Milk agar was used in the desiccation study and in the capsule production assay to mimic the growth conditions in infant formula as described by Caubilla-Barron and Forsythe (2007). To prepare milk agar, 3 g of agar No. 1 (LP0011, Oxoid Thermo Scientific) and 0.4 g of ammonium sulphate were dissolved in 40 ml of distilled water. After autoclaving at 121°C for 15 min, the mixture was combined with 200 ml of warm (55°C) liquid infant formula (Cow & Gate Premium 1 [whey-based], Trowbridge, Wiltshire, UK) and dispensed into sterile petri dishes. The plates were stored at 4°C in the refrigerator for a maximum of three weeks.

2.6.7 Blood agar

Blood agar was used for the haemolysis assay. To prepare blood agar plates, 25 ml of sheep blood (SF0051B, Oxoid Thermo Scientific, UK) or 25 ml of horse blood (SR0050B, Oxoid Thermo Scientific) were added to 500 ml of TSA. Approximately 20 ml was poured into each sterile petri dish. The plates were stored at 4°C in the refrigerator for a maximum of three weeks.

2.6.8 Violet red bile glucose agar (VRBGA)

VRBGA is selective for Enterobacteriaceae. To prepare VRBGA plates, 38.5 g of VRBG agar (CM0485, Oxoid Thermo Scientific) were suspended in 1 litre of distilled water and the mixture was boiled until the VRBG dissolved completely. The agar was then allowed to cool to 55°C, and approximately 20 ml was dispensed into each sterile petri dish. The plates were stored at 4°C in the refrigerator for a maximum of three weeks.

2.6.9 Motility medium

Motility medium agar was used for the motility assay. To prepare the agar, 25 g of LB broth (1102830, Sigma-Aldrich, Ltd.) were added to 1 litre of distilled water along with 0.4% agar and 5 ml of a 1% TTC solution (2.3.5-triphenyl-tetrazolium chloride)(17779, Sigma-Aldrich, Ltd). This was autoclaved at 121°C for 15 minutes. The agar was then allowed to cool to 55°C and dispensed into tubes in 5-ml aliquots. The tubes were stored at 4°C in the refrigerator for a maximum of three weeks.

2.7 Multilocus Sequence Typing (MLST)

An MLST scheme was used for fast and reliable identification of organisms in different strain collections.

2.7.1 Bacterial strains used in this study

MLST was used to analyse 85 strains in this study: 20 strains from powder infant milk (PIF) (Muytjens *et al.* 1988); 13 strains from a powder milk factory in Germany (2006–2009) (Jacobs *et al.* 2011); and 52 strains from 5 powder milk factories in Australia (Craven *et al.* 2010). The latter two strain sets were representative of the pulsetypes described in the original publications. Additional strain details are given in **Table 2.1.**

In this project, MLST PCR, sequencing, and sequence analyses were performed on 72 of the 85 strains. The 12 remaining strains were subjected to sequencing and sequence analyses previously by another researcher in our group, Dr. Susan Joseph. The details of the final strain, *C. sakazakii* HPB-3284, was deposited into the database by other collaborators.

The strain IDs, the isolate details, the sources, the dates of isolation, and the MLST sequence profile details of all the strains in this study are available online in the *Cronobacter* MLST database at http://www.pubmlst.org/cronobacter, which is freely available and located by the University of Oxford. The *Citrobacter koseri* BAA-895 genome (GenBank accession number: CP000822) was used as an outlier when the phylogenetic tree was constructed for the strains in this study as previously described by Baldwin *et al.* (2009).

2.7.2 Genomic DNA extraction

A 1.5-ml culture grown overnight in TSB was used for genomic DNA extraction using the GenElute™ Bacterial Genomic DNA Kit (Oxoid Thermo Scientific) according to the manufacturer's protocol. The DNA was then checked for purity and concentration using a Nanodrop 2000 (Thermo Scientific). DNA samples with values of ~1.8 at 260/280 nm and ~ 2 at 260/230 nm were used for the analyses.

2.7.3 MLST PCR amplification

MLST PCR amplification was performed as described previously by Baldwin *et al.* (2007) on 7 housekeeping genes: *atpD*, *fusA*, *glnS*, *gltB*, *gyrB*, *infB*, and *ppsA*. The amplification and sequencing primers were designed by Baldwin *et al.* (2009) and were manufactured by Eurofins MWG Operon. The primers are listed in **Table 2.2**. The templates for the PCR reactions (i.e. the genomic DNA) were prepared using the GenElute™ Bacterial Genomic DNA extraction kit (Oxoid Thermo Scientific). Each PCR reaction was carried out in a 25-µl reaction volume that included 5 µl of 10X PCR buffer (Promega, UK), 2 µl of 15 mM $MgCl_2$ (Promega), 1.0 µl of dNTP solution (Promega, 10 mM each dNTP), 2.5 µl of the forward and reverse primers (10 pmol/µl for each primer), 0.25 µl of Taq DNA polymerase (Promega; 5 Units/µl), and 10.75 µl of PCR-grade water. The reaction conditions were as follows: initial denaturation at 96°C for 1 min, followed by 30 cycles of denaturation at 96°C for 1 min, primer annealing at 58°C for 1 min, extension at 72°C for 2 min, and a final extension step at 72°C for 5 min. The PCR products were held at 4°C after the reactions were complete. The PCR reactions were performed using a TC-4000 thermal cycler (Techne, Ltd).

Table 2. 2 The primers used to amplify and sequence 7 MLST genes in *Cronobacter* spp.

MLST gene	GenBank accession number[a]	Allele size (bp)	Direction	Amplification primers	Sequence primers	Function
atpD	ESA_04006	390	Forward	CGACATGAAAGGCGACAT	CGAAATGACCGACTCCAA	ATP synthase β chain
			Reverse	TTAAAGCCACGGATGGTG	GGATGGCGATGATGTCTT	
fusA	ESA_04401	438	Forward	GAAACCGTATGGCGTCAG	GCTGGATGCGGTAATTGA	Elongation factor
			Reverse	AGAACCGAAGTGCAGACG	CCCATACCAGCGATGATG	
glnS	ESA_02658	363	Forward	GCATCTACCCGATGTACG	GGGTGCTGGATAACATCA	Glutaminyl-tRNA synthetase
			Reverse	TTGGCACGCTGAACAGAC	CTTGTTGGCTTCTTCACG	
gltB	ESA_03606	507	Forward	CATCTCGACCATCGCTTC	GCGAATACCACGCCTACA	Glutamate synthase large subunit
			Reverse	CAGCACTTCCACCAGCTC	GCGTATTTCACGGAGGAG	
gyrB	ESA_03973	402	Forward	TGCACCACATGGTATTCG	CTCGCGGGTCACTGTAAA	DNA gyrase β subunit
			Reverse	CACCGGTCACAAACTCGT	ACGCCGATACCGTCTTTT	
infB	ESA_03561	441	Forward	GAAGAAGCGGTAATGAGC	TGACCACGGTAAAACCTC	Translation initiation factor IF-2
			Reverse	CGATACCACATTCCATGC	GGACCACGACCTTTATCC	
ppsA	ESA_02102	495	Forward	GTCCAACAATGGCTCGTC	ACCCTGACGAATTCTACG	Phosphoenolpyruvate synthase
			Reverse	CAGACTCAGCCAGGTTTG	CAGATCCGGCATGGTATC	

a GenBank accession numbers of the 7 housekeeping genes in the *C. sakazakii* BAA-894 genome (Kucerova *et al*. 2010)

2.7.4 DNA analysis by agarose gel electrophoresis

The PCR samples were visualised on 1% agarose gel that was prepared by adding 1 g of molecular agarose (Fisher Scientific) to 100 ml of 1X TAE and heating the mixture in the microwave until the agarose dissolved completely. The solution was allowed to cool, then 10 µl (0.01%) of SYBR® SafeTM gel stain (Invitrogen, UK) was added and mixed. The agarose gel was poured into a gel tray with a comb (Geneflow, UK) and allowed to set. The gel was then placed in an electrophoresis tank filled with 1X TAE, and 5 µl of PCR product was loaded into each lane. An appropriate DNA ladder was included on each gel (either a 100-bp or a 1-kb ladder; Promega). The DNA was electrophoresed

at 90V for 35 minutes, and the DNA bands on the gel were visualised under ultraviolet (UV) light using the InGenius® gel documentation system (Syngene, UK).

2.7.5 Purification of the PCR products

The amplification products on the electrophoresis gel were purified using the QIAquick PCR Purification Kit (Qiagen, UK) following the manufacturer's instructions. A Nanodrop 2000 (Thermo Scientific) was used to check the purity and concentration of the DNA samples before they were sent for sequencing.

2.7.6 Sequencing of the PCR product

Each purified PCR product (10 ng/µl) was sequenced by Source BioScience (Nottingham, UK). Both forward and reverse nucleotide sequences were determined using the sequencing primers. GENtle software, designed by Magnus Manske (2006), was used to view the sequence chromatograms for quality control. The sequences of each locus of each isolate were aligned then trimmed to the appropriated allele length using ClustalW software (http://www.ebi.ac.uk/clustalw) (Larkin *et al.* 2007).

2.7.7 Determination of the alleles and sequence type

Each new allele that was identified for a particular locus was given a number according to the MLST database order; these are called allelic profiles. The sequence type (ST) for a specific strain is defined by the 7 allelic codes determined by sequencing; allele numbers are assigned to each of the 7 loci of the isolate (e.g. ST1; Baldwin *et al.* 2009).

2.7.8 Phylogenetic analyses of DNA sequences

Phylogenetic analyses were performed on the strains in this study using the concatenated sequences of the 7 loci (a total of 3036 nt) with the maximum likelihood algorithm in the Molecular Evolutionary Genetics Analysis (MEGA) version 5.2 software using 1000 bootstrap replicates (Tamura *et al.* 2011).

2.8 Physiological experiments

2.8.1 Bacterial strains for physiological experiments

A total of 14 bacterial strains were selected for the physiological experiments. After MLST analysis of the environmental samples, 3 *C. sakazakii* (ST4) strains and 1 *C. sakazakii* (ST1) strain were chosen for further analysis from the German study to represent the environmental strains. In addition, 4 clinical strains, 3 *C. sakazakii* (ST4) strains, and 1 *C. sakazakii* (ST1) strain were selected for comparison purposes from the MLST database. Lastly, we included 2 *C. sakazakii* strains, an ST8 and an ST12 strain were included. The strain collection from Australia was subject to a Material Transfer Agreement that limited the analysis to MLST analysis. Strain details are listed in **Table 1.1.**

2.8.2 Capsule production assayed on milk agar

The capsule formation of the bacteria strains was assayed on milk agar plates to examine the effects of infant formula milk components (whey-based) on capsule formation. *Cronobacter* spp. strains were cultured on milk agar and incubated overnight at 37°C. Capsule production was assessed by visual evaluation of colony morphology using a qualitative scale: high capsule formation, ++; low capsule formation, + ; or no capsule formation - .

2.8.3 Motility assay

Bacterial motility was assayed by stabbing a single colony from overnight culture from each strain using a sterile needle and inoculating it into 3 ml LB broth tubes supplemented with 0.4% agar and TTC as described in section **2.6.9**. This was incubated at 37°C for 24 h. The motility assays were performed twice for each strain. The motility was determined by the visual evaluation of diffused spread of bacterial growth beyond the inoculation line using a qualitative scale: +++: high motility, ++: medium motility, + low motility and -: showed no motility. As organisms grow, they reduce the TTC, which produces a diffuse red colour in the medium. When there is a turbid red zone spreading away from the inoculation site, the result is considered positive. In contrast, red growth of the bacteria along the inoculation line is considered a negative result.

2.8.4 Haemolysis assay on blood agar

The haemolysis assay was performed by streaking a single colony from overnight culture of each strain onto sheep and horse blood agar plates that were prepared as described in section **2.6.7**. The plates were incubated for 24 h at 37°C. *Staphylococcus aureus* NCIMB 6571 was used as a positive control for α-haemolysis, and *Streptococcus pyogenes* NCTC 9994 was used as a positive control for β-haemolysis. The colonies were scored as positive for α-haemolysis if there was a green-brown zone surrounding the colony, as positive for β-haemolysis if there was a clear zone surrounding the colony, and as positive for gamma-haemolysis if there was no haemolysis.

2.8.5 Determination of sublethal injury during *C. sakazakii* desiccation

For this assay, *C. sakazakii* strains were cultured on milk agar plates at 37°C for 48 h as described in section **2.6.6**. Cells were harvested into 200 µl of sterile liquid infant formula (Cow & Gate Premium 1), and the cell density was about 10^{10} CFU/ml. Next, 0.2 ml of the suspension were transferred into a well in a six-well plates and placed in a class II cabinet at 25°C for 18 h to be air-dried as described by Caubilla-Barron and Forsythe (2007). Infant formula without inoculation was used as a negative control. The bacteria were resuspended in 2 ml of sterile water, and viable counts were determined in triplicate on TSA (CM0131, Oxoid Thermo Scientific, Ltd.) and on VRBGA (CM0485, Oxoid, Ltd.) The plates were incubated at 37°C for 24 h. The desiccated stressed cells were referred to as sublethally injured cells. Viable counts were determined in triplicate in at least two independent experiments

2.8.6 Effects of desiccation on the tolerance of *C. sakazakii* to dry heat

The effects of desiccation on dry heat tolerance in *C. sakazakii* was determined as described by Gruzdev *et al.* (2011) with a few modifications. Cultures were prepared by inoculation of a single colony grown on TSA agar into 5 ml of TSB broth and incubation overnight at 37°C with aeration. The cells were washed three times in sterile saline by centrifugation (5000 rpm, 7 min) at room temperature (25°C) using a Harrier 18/80R centrifuge (Harrier, UK), and the final pellet was re-suspended in 10 ml PBS to a concentration of about 10^9 CFU/ml. A 50-µl aliquot containing ~10^8 CFU of washed bacteria was placed in a capped, flat-bottom glass vial and air dried in a class II

biosafety cabinet for 24 h at 25°C. To determine the viable cell count following desiccation, the bacteria were counted after re-suspension of the cells in 50 µl of PBS for 5 minutes at room temperature (25°C). Bacterial suspensions were serially diluted (1:10), plated on TSA agar in triplicate using the Miles and Misra technique (Miles *et al.* 1938), cultured for 24 h at 37°C, and the number of colonies was recorded. For heat challenge experiments, desiccated bacteria in tightly capped flat-bottom glass vials were exposed to dry heat (60°C, 80°C, and 100°C) for 1 h and 30 min in an oven (Memmert heating cabinet, UK). The desiccated bacteria were resuspended in 50 µl of saline, and the viable cell count was determined in triplicate independently.

2.8.7 Acid resistance assay

Acid resistance was determined at pH 3.5 according to the methods of Edelson-Mammel *et al.* (2006). This pH was used to mimic the pH of a baby's stomach (Hurrell *et al.* 2009). A single colony from a TSA plate was used to inoculate 5 ml of TSB, and this was incubated overnight at 37°C with shaking at 200 rpm. The pH of sterile (whey-based) liquid infant formula (Cow & Gate Premium 1) was adjusted to pH 3.5 with 1M HCl that was re-sterilized by filtration (0.2 µm pore size), then 100 µl of the overnight culture were added to 10 ml of the acidified infant formula, distributed into sterile tubes, and incubated in a water bath at 37°C. Viable cells were counted after 0, 10, 15, 30, 45, 60, 90, and 120 minutes. At each time point, and according to the Miles and Misra technique, 20 µl of culture was transferred from each tube, diluted into normal saline, and plated in triplicate on TSA plates. The TSA plates were incubated at 37°C for 24 h before counting the cells. Two independent experiments were performed from separate overnight cultures, and each was plated in triplicate. The data were analysed for each time point.

2.8.8 Serum resistance

The resistance of the bacteria to human serum was assayed as described by Hughes *et al.* (1982) with a few modifications. A single colony from a TSA plate was used to inoculate 5 ml of TSB, and this was incubated overnight at 37°C with shaking at 200 rpm. The TSB from the overnight culture was then centrifuged for 10 min at 6000 rpm using a Harrier 18/80R centrifuge (Harrier, UK). The pellet was collected and diluted to 10^8 CFU/ml in 5 ml of PBS, then an inoculum of 0.5 ml was added to 1.5

ml of undiluted human serum (Sigma, UK) for a total volume of 2 ml. Viable cells were counted on TSA plates that were incubated at 37°C. *Salmonella enterica* serovar Enteritidis strain 358 (NCTC3046) was used as a positive control for serum resistance, and *Escherichia coli* K12 (MG1655) was used as a negative control strain. The experiment was carried out in triplicate independently.

2.8.9 Statistical analysis

Statistical analysis was performed using Microsoft Excel^{TM} software. Data were analysed using the unpaired t-test, and $P < 0.05$ was considered statistically significant.

2.9 Outer Membrane Protein (OMP) study

2.9.1 Bacterial strains

OMPs were profiled for 13 *C. sakazakii* strains and 1 *Citrobacter koseri* strain as a comparison strain (**Table 1.1**).

2.9.2 Outer membrane proteins (OMP) extraction and profile

The extraction of the outer membrane proteins from bacteria cultured at neutral pH (pH 6) and at acidic pH (pH 3.5) was conducted as described by Kim *et al.* (2010a) with some modifications. Bacterial cultures were grown on TSA overnight at 37°C. A single colony was picked from the TSA plate, inoculated into 10 ml BHI broth at pH 6 and pH 3.5 (CM1032, Oxoid Thermo Scientific), and incubated aerobically for 18 h at 37°C with shaking at 200 rpm. Then, 50 ml of BHI broth was inoculated with 5 ml of the overnight culture and incubated aerobically with shaking at 200 rpm for approximately 3 h at 37°C to achieve an optical density of 0.9–1.3 at 600 nm. A 25-ml culture of *C. sakazakii* or *Citrobacter koseri* was harvested by centrifugation (Oxoid Thermo Scientific) at 17,000 rpm for 20 min at 4°C. The bacterial pellet was re-suspended in 10 ml of 10 mM HEPES buffer at pH 7.4 (Sigma) and centrifuged at 20,000xg for 20 min. The pellet was washed with a total volume of 10 ml of 10 mM HEPES. PMSF (P-7626, Sigma-Aldrich Ltd.) was added to a final concentration of 100 mM and mixed. The bacterial suspension was then disrupted by sonication (2 x 2 minutes with a 15-minute interval on crushed ice in between) using an ultrasonic homogenizer (MSE-Soniprep-150,

Labexchange, Germany). After centrifugation (Harrier 18/80R; 1,700xg for 20 min at 4°C) to remove cell debris, the supernatant was removed carefully and centrifuged at 100,000xg for 60 minutes at 4°C (Beckman-Coulter Centrifuge; Optima L100XP). The isolated OMP pellet was re-suspended in 200 µl of RIPA (radioimmunoprecipitation assay) buffer (R0278, Sigma-Aldrich, Ltd.) plus an equal volume of 10 mM HEPES with 2% IGEPAL CA-630 (13021, Sigma-Aldrich, Ltd.) The samples were then incubated at 25°C for 30 minutes, then harvested by centrifugation using an Optima™ TLX ultra-centrifuge (Beckman-Coulter, L100XP, Ireland) at 100,000xg for 60 minutes at 4°C. The supernatants were stored at -20°C.

2.9.3 Protein concentration determination

The protein concentration was determined in samples using the QuantiPro™ BCA Assay Kit (QPBC; Sigma-Aldrich, Ltd.) following the manufacturer's instructions. In brief, BSA protein standards were prepared using the same buffer as the unknown samples at concentrations of 200, 400, 600, 800, and 1000 µg/ml (w/v), and a blank (buffer only) sample was prepared as well. These were used to estimate the amount of protein in the tested samples. The QuantiPro™ BCA working reagent is prepared by mixing 25 parts of reagent QA with 25 parts of reagent QB. Once reagent QA and QB have been combined, 1 part reagent QC (copper (II) sulphate) was added and mixed until the solution was uniform in colour. Next, 5 µl of the protein sample was mixed with 15 µl of RIPA buffer (R0278, Sigma-Aldrich, Ltd.). The assay was carried out in duplicate for each sample by mixing 20 µl of the protein/RIPA buffer sample (or standard protein/RIPA buffer sample) with 200 µl of the prepared working reagent in 96-well plates and incubating the plates at 60°C for 1 h. After incubation, the optical density at 600 nm was obtained, and the calibration graph was calculated using Excel™ software and data from the BSA standards. The protein concentration for the unknown sample was then calculated using the equation derived from the QuantiPro BCA standard curve.

2.9.4 SDS-PAGE

2.9.4.1 **12% Resolving gel**

The 12% resolving gel stock solution (10 ml) was prepared according to the manufacturer's instructions. A 4 ml acrylamide solution (ProtoGel 30%; EC-890; National Diagnostics; Atlanta, GA, USA) was mixed with 2.5 ml of resolving buffer (EC-892, National Diagnostics), 0.1 ml of 10% ammonium persulphate (APS) (National Diagnostics), 0.004 ml of N,N,N'N'-tetramethyl-ethylenedamine (TEMED) (T3100, Melford) and 3.3 ml of deionized water.

2.9.4.2 **2% Stacking gel**

The 2% stacking gel (2 ml) was prepared by mixing 0.33 ml of acrylamide solution (ProtoGel 30%; (EC-890; National Diagnostics), 0.25 ml of stacking buffer (EC-893, National Diagnostics), 0.002ml of N,N,N'N'-tetramethyl-ethylenedamine (TEMED) and 0.02 ml of 10% ammonium persulphate (APS) (National Diagnostics) and 1.4 ml of deionized water.

2.9.4.3 **Preparing the gel for SDS-PAGE analysis**

To prepare SDS-PAGE, the BioRad protean II apparatus was used according to the manufacturer's instructions. Ten ml of the 12% resolving gel was poured gently between two glass plates and allowed to solidify completely. About 2 cm space was left at the top of the glass plate. The top of the glass plate was covered with a layer of deionized water and allowed to set for around 30 minutes. The water was removed and 2 ml of the 2% prepared stacking gel mixture was poured on top of the resolving gel between the glass plates. A comb was placed in the stacking gel between the two plates, and the stacking gel was allowed to solidify for 30 minutes at room temperature. Finally, the comb was removed and the solidified gel was placed in an electrophoresis running apparatus, and 1X running buffer, Tris-glycine electrophoresis buffer TGS (EC-870; National Diagnostics) was added to the chambers.

2.9.4.4 **Preparation of protein samples for SDS-PAGE**

Ten microgram per millilitre (µg/ml) of each protein sample were mixed with an equal volume of 2X Laemmli sample buffer. The mixture was incubated at 95°C for 5 minutes.

2.9.4.5 **Gel electrophoresis of OMP samples**

The 5 µl of BLUeye pre-stained protein ladder (S6-0024; Geneflow, Ltd) and 10 µg/ml protein/sample was loaded per lane. The proteins were then separated by SDS-PAGE (2% stacking gel; 12% resolving gel). The gel was run at 170 V for around 45 minutes until the front dye reached the bottom of the gel. The gels were stained overnight using Coomassie brilliant blue stain (R250; BioRad) to visualise the OMP bands.

2.9.4.6 **Analysis of the protein band patterns**

The stained gels were analysed using GeneSnap software and the InGenius machine (Syngene, Cambridge, UK). Images of the gel were saved as TIFF files and in the GeneSnap format for further analysis. The GeneTools (Syngene) program was used for band matching and for calculating the molecular weight using the protein standards (BLUeye pre-stained protein ladder; S6-0024; Geneflow, Ltd). Cluster analysis of the different OMP profiles was performed using BioNumerics software, version 6. The Dice coefficient and unweighted pair group method with arithmetic mean (UPGMA) were used for cluster analysis.

2.10 Genomic study of genes related to physiological processes

2.10.1 Bacterial strains used in the genomic analysis

Thirty-eight *C. sakazakii* genomes were analysed for the presence or absence of physiologically important genes. The strain details are shown in **Table 1.1**.

2.10.2 Genomic analysis to determine the presence or absence of genes

To determine the presence or absence of key physiological genes including capsule genes, serum resistance genes, osmotolerance genes, and heat tolerance genes, genomic comparisons were

performed using BLAST tools in BIGSdb at http://pubmlst.org/perl/bigsdb/bigsdb.pl?page=plugin&name=BLAST&db=pubmlst_cronobacter_isolates. To predict similarities in OMP function, the amino acid sequences of selected well characterised OMP proteins of *C. sakazakii* and *E.coli* that are known to be associated with virulence and physiologic adaptation were used in a BLASTp search to predict amino acid sequences encoded by *C. sakazakii*.

CHAPTER 3: The molecular epidemiology and clonality of environmental *Cronobacter* isolates as determined by multilocus sequence typing

3.1 Introduction

3.1.1 Multilocus sequence typing (MLST)

MLST is a molecular typing technique which involves the identification and clustering of bacterial isolates that are based on the sequence analysis of multiple housekeeping genes, ideally scattered across the genome of the organism.

It was basically developed to modify the earlier typing tool, multilocus enzyme electrophoresis (MLEE). MLEE involved the identification of variations within multiple core metabolic or 'housekeeping' genes on the basis of the gene products' differing electrophoretic mobilities (Maiden *et al*. 1998). While MLST is a sequence-based technique, it has an advantage that even a single nucleotide change will be considered, to enhance discriminating between isolates. Therefore, MLST manages to provide greater resolution than the MLEE technique. The use of multiple genes for typing has many advantages. The gene targets for MLST are housekeeping genes, which encode for extremely essential proteins to the organism's survival. As a result, they would be expected to be free of the influence of selective pressures. The combination of the multiple sites on the genome would also provide increased informative characters and better discrimination than a single gene could provide.

This would also help to overcome the effect of any recombination events that could have occurred at a single locus (Maiden *et al*. 1998; Enright and Spratt 1999). In 1998, MLST was first applied and validated on a collection of *Neisseria meningitidis* strains by Maiden *et al*. Initially the MLST schemes were based on six loci but today most of the schemes are seven loci based, increasing its resolution and making it more discriminatory. The procedure for MLST is fairly uncomplicated. The gene targets are first amplified and then sequenced; using either the same set of primers or nested primers (outer primers for PCR amplification and inner primers for nucleotide sequencing **(Figure 3.1)**.

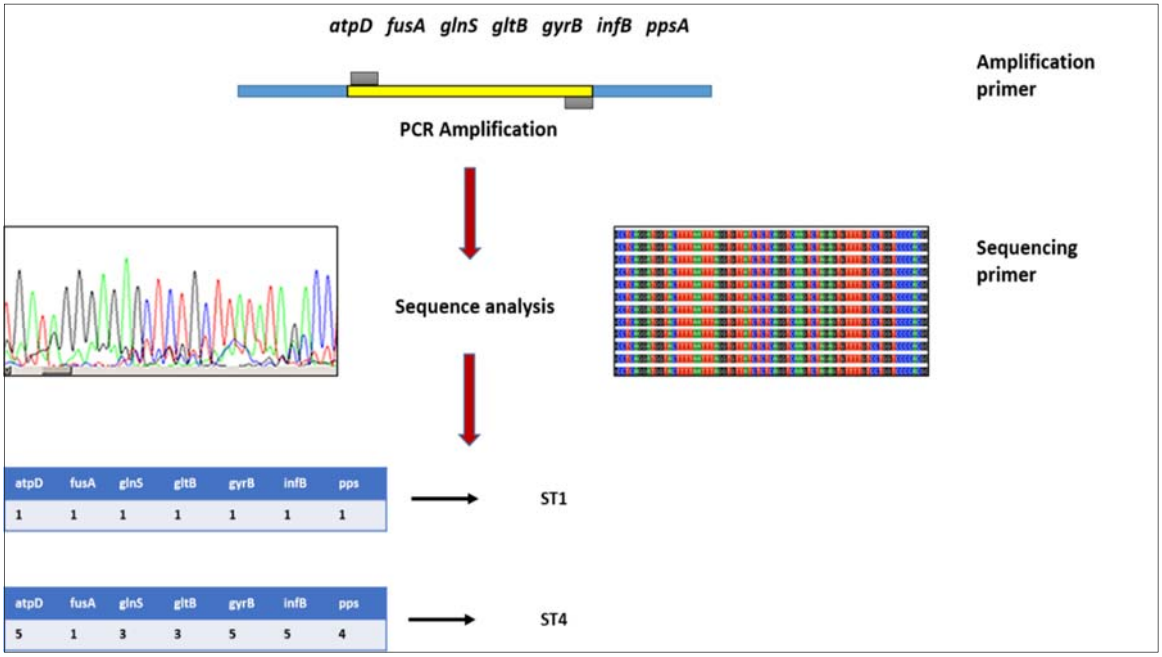


Figure 3. 1 The principle steps of Multi Locus Sequence Typing (MLST) method.

Generally, a 400-500 bp long target region will be followed up with the trimming and analysis, relative to each locus. The numerous alleles at each gene locus are aligned in parallel positions and compared with each other, and every variant allele is designated a unique allele number for that locus. These numbers are assigned arbitrarily and do not reflect any sort of relationship. Thus, in a 7-loci typing scheme, every isolate obtains a combination of seven allele numbers. A unique allelic profile is formed by the combination of seven loci numbers, which is denoted by a sequence type (ST) for the relative isolate. The strains with identical STs are considered to be isogenic strains or clones of each other since they cannot be distinguished at all seven loci. The whole of this sequence information can be made available on central database which can be retrieved from anywhere in the world (Maiden *et al*. 1998). Today, this technique is being used on a wide range of prokaryotic populations, with the sequence data being hosted on many centralised databases. These databases get support from the central analytical facilities and contain a lot of information on the typed strains including isolation history as well as sequencing data. They also provide access to publication links that the strains have been used in, thus presenting maximum information to the user in one place. The whole of these data is made freely available online, allowing convenient downloading without any registrations, and it

also enables comparative analysis for researchers, who are working with the said organism in any part of the world. The fact that every one of these databases is strictly curated also ensures the reliability and quality of the data that is stored by them. These gateways also facilitate the sequence analysis such as comparing sequence types, alleles and construction of phylogenetic trees to determine the relationship between different sequence types. There are embedded BLAST tools for similarity searches of the loci as well as options to download the profiles of the sequence types identified by the scheme. Some of the databases, such as PubMLST, have a "Locus Explorer" tool which enables the visualisation of the mutable positions in each locus, and predicts the translated protein sequence as well. Since these are housekeeping genes, the common nucleotide differences are usually in the third 'wobble' base position and so do not affect the resultant amino acid sequence and protein structure (called 'synonymous substitution'). Although non-synonymous substitutions may also occur in housekeeping genes, their numbers are significantly lower than the synonymous substitutions, which does not influence the selective pressure of the gene.

3.1.2 MLST analysis of *Cronobacter*

The MLST database for *Cronobacter* is part of the PubMLST group of databases and is available online at http://www.pubmlst.org/cronobacter. These databases were developed by Professor Keith Jolley and the *Cronobacter* database is curated by Professor Stephen Forsythe. PubMLST comprises more than 40 additional MLST scheme databases for other organisms, including *Campylobacter* spp., *Aeromonas* spp., *Neisseria* spp., *Helicobacter pylori, Pseudomonas aeruginosa*, *Clostridium difficile*, and *Yersinia* spp. Each database has two sections. One section introduces the MLST scheme, describes the protocols, and lists the amplification and sequencing primers that can be used for each locus. The second section contains all of the sequencing data, including MLST profiles and allele sequences, and the isolate details. Sequencing data obtained by the MLST method has many important applications, such as sequence typing, speciation determination, and population genetics. The MLST technique is widely used for studying pathogens and for analysing environmental samples (Brady *et al.* 2008).

The ubiquitous nature of *Cronobacter* infections in both neonates and in immunocompromised individuals prompted the development of an MLST approach for rapid and reliable organism identification. An MLST scheme has been established for the seven *Cronobacter* species and is curated in an open access database that is available at http://www.pubMLST.org/cronobacter (Baldwin *et al.* 2009; Joseph and Forsythe, 2012; Joseph *et al.* 2012a). It is important to note that the MLST scheme is based on seven housekeeping loci, namely *atp*D, *fus*A, *gln*S, *glt*B, *gyr*B, *inf*B, and *pps*A, which have a concatenated length of 3036 nucleotides (Baldwin *et al.* 2009). As noted above, the *Cronobacter* MLST scheme utilises the partial sequences of 7 housekeeping genes (Baldwin *et al*. 2009). These loci are distributed throughout the *Cronobacter* genome and are not co-inherited. In this method, a comparison of the DNA sequences of the loci with sequences in the *Cronobacter* MLST reference database (http://www.pubMLST.org/cronobacter) generates a 7-digit allele code and identifies the ST of the strain. When the 7-allele sequences are concatenated, they form a 3036-bp sequence that can be used for MLSA and phylogenetic analysis. The 7 loci used for the MLST profiles define the STs, and the relatedness of the STs defines them as clonal complexes (CCs) if there are 1 to 3 differences in the seven loci profile (Joseph and Forsythe, 2014). The MLST scheme has been applied to more than 1007 *Cronobacter* strains that are distributed widely in terms of geography, source, and time, having been collected over a 50-year period. Currently there are 410 defined STs covering all *Cronobacter* species. Some of these STs are stable clones; for example, for *C. sakazakii* there are a total of 195 isolates that belong to CC4, 87/195 of which are clinical isolates and 33/195 of which are environmental isolates (Joseph and Forsythe, 2011; Joseph *et al.* 2012a; Forsythe *et al.* 2014).

There are significant associations between STs as determined by MLST and the isolation source. For example, *C. sakazakii* ST1 is found predominantly in infant formula and in clinical sources, while *C. sakazakii* ST8 is isolated mainly from clinical sources. Interestingly, *C. sakazakii* ST4 appears to be associated more with neonatal meningitis (Joseph and Forsythe, 2011), while *C. malonaticus* ST7 is linked with adult infections, although the isolation source has not been identified (Joseph and Forsythe, 2011). To date, only strains from *C. sakazakii, C. malonaticus,* and *C. turicensis* have been

associated with neonatal infections, whereas *C. malonaticus* ST7 strains have been associated with adult infections (Joseph and Forsythe, 2011; Forsythe *et al.* 2014).

3.1.3 MLST clonality analysis

Over the past seven years, several studies have used whole genome analysis and MLST genotyping of *Cronobacter* spp. to improve our understanding of these bacteria and their epidemiological associations with infection (Baldwin *et al.* 2009; Joseph and Forsythe, 2012; Joseph *et al* .2012a; Joseph *et al.* 2012b). The seven housekeeping genes used for MLST help identify the linkage and the clonal relationships of the species (Joseph *et al.* 2012b). Two software programs, eBURST and goeBURST, were used to determine clonality. GoeBURST is a newer version of the globally optimised eBURST algorithm that was developed by Francisco *et al.* (2009) and that was used in this study via the Phyloviz program at http://goeburst.phyloviz.net/ (Francisco *et al*. 2012). eBURST and goeBURST use an algorithm that analyses data from the 7 MLST allelic profiles as well as ST data.

The results obtained with goeBURST differ from the results obtained from phylogenetic tree analysis of the nucleotide sequence of the seven MLST allelic profiles (Feil *et al.* 2004; Francisco *et al*. 2009). The goeBURST algorithm identifies the ST that appears most frequently in the bacterial population; this ST is linked to the greatest number of isolates and is known as the "founder clone" (Feil *et al.* 2004; Francisco *et al.* 2009). If the founder clone is connected to the closest possible clone, which in the context of MLST data is called an ST, the ST is referred to as a single locus variant (SLV) of the main founder. An SLV therefore diverges from the founder ST at one of the seven MLST alleles. STs within a cluster of closely related groups of STs in a bacterial population are called "clonal complexes" (CCs) (Feil *et al.* 2004). A double locus variant (DLV) diverges from the founder ST at two of the seven MLST alleles. If a strains diverges from the founder ST at three alleles, it is termed a triple locus variant (TLV). The results of MLST analysis are presented as a snapshot image of the MLST profile data. Specifically, the STs are displayed in a circle, and the diameter of the circle is an indicator of the frequency of the STs in the bacterial population (Feil *et al.* 2004). The CC description depends on the founder type in the PubMLST database. The results presented later in this chapter will included

the DLVs, TLVs, and QLVs (quarter locus variants) in the analysis of the CC relationship of the environmental populations.

3.1.4 Pulsed-Field Gel Electrophoresis (PFGE)

PFGE was considered the "gold standard" subtyping method for many years. It has been used in epidemiologic studies that investigated outbreaks of foodborne and infectious diseases as well as for tracking the source of infectious organisms and for organism surveillance (Nazarowec-White and Farber 1997; Swaminathan *et al*. 2001; Caubilla-Barron *et al.* 2007; Craven *et al.* 2010; CDC 2011). In addition, PFGE has been used to genotype *Cronobacter* strains isolated from milk protein factories, milk powder, and infant formula factories (Mullane *et al*. 2008a; Craven *et al.* 2010; Jacobs *et al.* 2011). PGFE is performed according to approved protocols from Pulsenet for *Salmonella* (Swaminathan *et al.* 2001), and it is highly discriminatory and reproducible (Olive and Bean, 1999). PFGE is based on the digestion of genomic DNA by restriction enzymes such as *XbaI*, *SpeI*, *ApaI*, *SmaI*, or *NotI*; the digestion reaction produces a DNA banding pattern on agarose gel electrophoresis that is unique to each species and enables better discrimination among bacterial species (Tenover *et al* .1995). Notably, the PFGE DNA banding patterns can be analysed using various software programs, such as BioNumerics and GelCompar software. PulseNet provide the standard PFGE protocols for foodborne pathogens (Swaminathan *et al.* 2001).

Several studies that used PFGE analysis of *Cronobacter* spp. strains revealed that this method shows high discrimination at the genomic level (Caubilla-Barron *et al.* 2007; Craven *et al.* 2010; CDC 2011). There are some disadvantages to PFGE; namely, it is time consuming, and the typing results are difficult to report (Durmuz *et al.* 2009).

3.1.5 PIF as a route of transmission

C. sakazakii has been associated with neonatal outbreaks and with contaminated PIF that was linked to many cases of illness and death (Muytjens *et al.*1983; Biering *et al.* 1989; Van Acker *et al.* 2001; Himelright *et al*. 2002). The association of PIF with some outbreaks and with neonatal cases led to market recalls of PIF products contaminated with *Cronobacter*. The first recall of a PIF product

occurred after the Tennessee outbreak in 2001 (Himelright *et al.* 2002), and the recent recall of a PIF product occurred at the Wal-Mart supermarket chain in December of 2011 after the death of an infant in Missouri, USA (CDC, 2011).

PIF is one of the probable reservoirs of infectious organisms to neonates and infants. PIF is widely recognized as not being a sterile product, and neonates are usually fed reconstituted PIF when they are not breast-feeding. This potential route of infection has been the focus of many studies that aim to reduce infection risk in neonates (Mullane *et al*. 2007, 2008; Proudy *et al.* 2008; Craven *et al.* 2010; Kucerova *et al*. 2011).

The term PIF refers to dairy products such as breast milk fortifiers and breast milk substitutes given to immune-deficient neonates and to premature babies less than 6 months old (CAC, 2008). Several studies conducted over many years showed that *Cronobacter* spp. could be isolated from PIF products from different countries (Muytjens *et al.*1988; Nazarowec-White and Farber, 1997; Iversen and Forsythe, 2004). Nazarowec-White and Farber (1997) analysed 120 infant formula brands in Canada and found that three *Cronobacter* spp. made up 6.7% of the isolates. In addition, a study by Muytjens *et al.* (1988) of Enterobacteriaceae in PIF found that about a third of Enterobacteriaceae strains were *Cronobacter* spp.. The *Cronobacter* spp. strains were isolated from 20 of 50 types of PIF purchased from countries across the world (Muytjens *et al.* 1988). Milk powder, which is the main ingredient of PIF, is another probable reservoir of infection. Specifically, many studies show that milk protein and milk powder manufacturing facilities can be contaminated with *E. sakazakii* strains (Mullane *et al.* 2007, 2008; Proudy *et al.* 2008; Craven *et al* .2010). The ingredients in PIF, including starch, rice flour, oat flour, and maltodextrin, may also contain *Cronobacter* spp. (FAO-WHO, 2006; Fu *et al.* 2011; Cetinkaya *et al.* 2012)

A clonal investigation of 129 *Cronobacter* spp. strains isolated from five factories in Australia using PFGE genotyping categorized the isolates into 49 pulsotypes (**Figure 3. 2)** (Craven *et al.* 2010). Most of the *Cronobacter* spp. strains identified were isolated from an air treatment region located above the spray driers (on the roof) and in areas with frequent foot traffic (**Figure 3. 2)** (Craven *et al.* 2010). Jacobs *et al*. (2011) studied 81 *Cronobacter* isolates from a factory environment and in finished

products. These strains were isolated from 2006 to 2009 in a milk powder factory in Germany; specifically, they were isolated from the spray-drying site and the roller-drying site at the factory. These strains were clustered into 13 pulsotypes by PFGE genotyping using two restriction enzymes, *XbaI* and *SpeI*. A 90% similarity index was used to determine the clonal identity of the PFGE DNA profile according to the criteria described by Tenover *et al.* (1995) as seen in **Figure 3.3** and **3.4** (Craven *et al.* 2010). Mullane *et al.* (2008) studied possible contamination routes in the PIF factories and suggested that air filters could be one source of product contamination. Craven *et al.* (2010) also studied the distribution of *Cronobacter* in milk powder manufacturing environments and found that it is widespread, most likely due to air movement, personnel movement, and movement of the milk powder product itself. However, none of these studies described the distribution of *Cronobacter* species within the factories.

This study applied MLST scheme on three strains collections including 85 *Cronobacter* strains that were described in previous publications (Muytjens *et al.* 1988; Craven *et al.* 2010; Jacobs *et al.*2011). The strain sets from the latter two studies are representative of the pulsotypes described in the original publications. It should be noted that the strain collection from Australia was subject to a Material Trust Agreement that limited the investigation to MLST analysis. As these strains are highly important for epidemiological and safety purposes, further analysis of the previously identified *E. sakazakii* strains is needed to re-identify the strains and their STs and to clarify whether they are *Cronobacter* isolates using MLST genotyping.

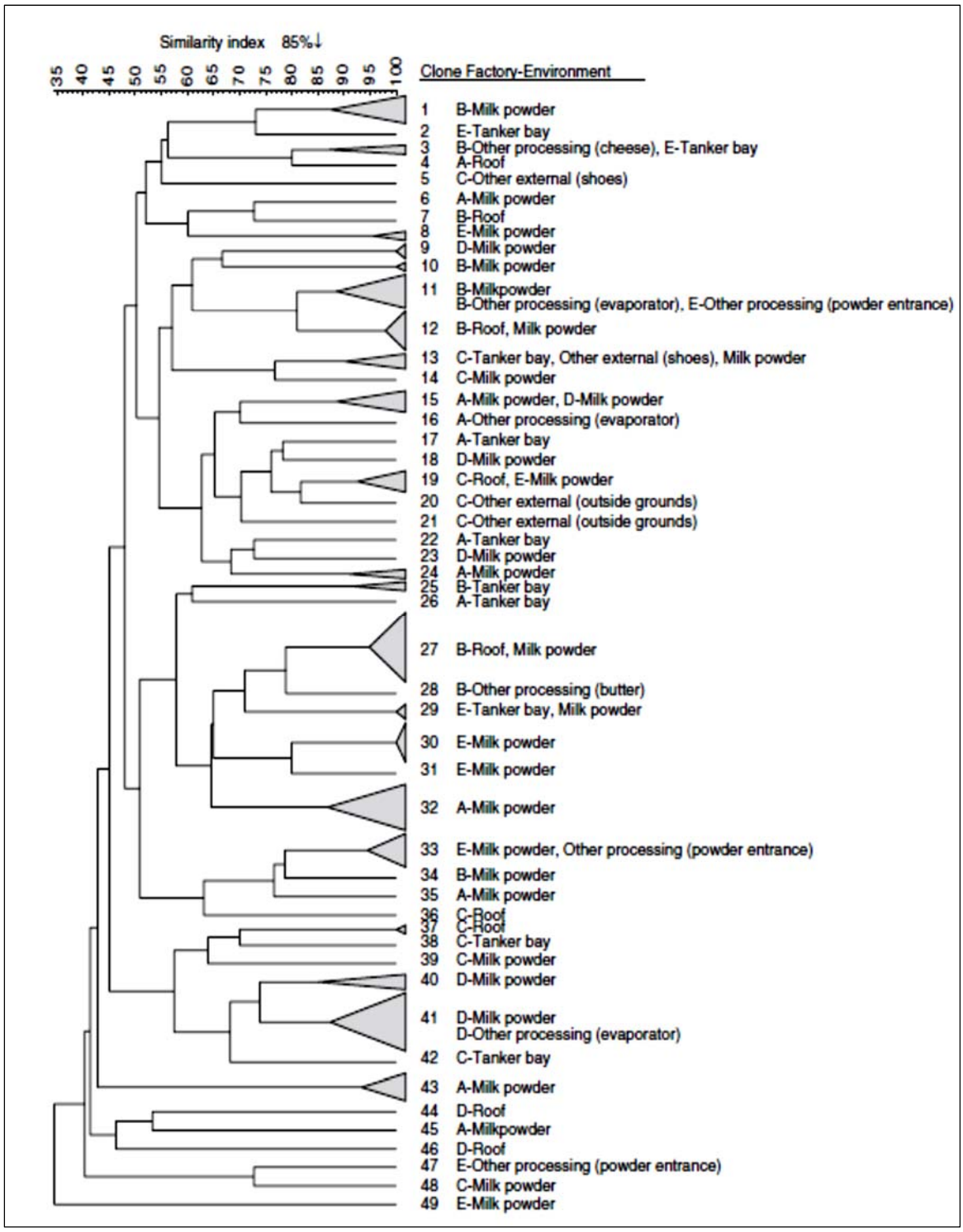


Figure 3. 2 Dendrogram of the pulsed-field gel electrophoresis DNA profiles of *Cronobacter* spp. from five milk powder factories in Australia by Craven *et al*. (2010).

The PFGE profile in **Figure 3. 2** was obtained using the *XbaI* restriction enzyme to digest *Cronobacter* isolated from five milk powder factories in Australia by Craven *et al.* (2010). The arrow at the top shows the 85% similarity cut-off used to differentiate DNA profile (clones) 1–49, and the letters on the right side (A–E) identify the five factories plus the isolation site as published by Craven *et al.* (2010).

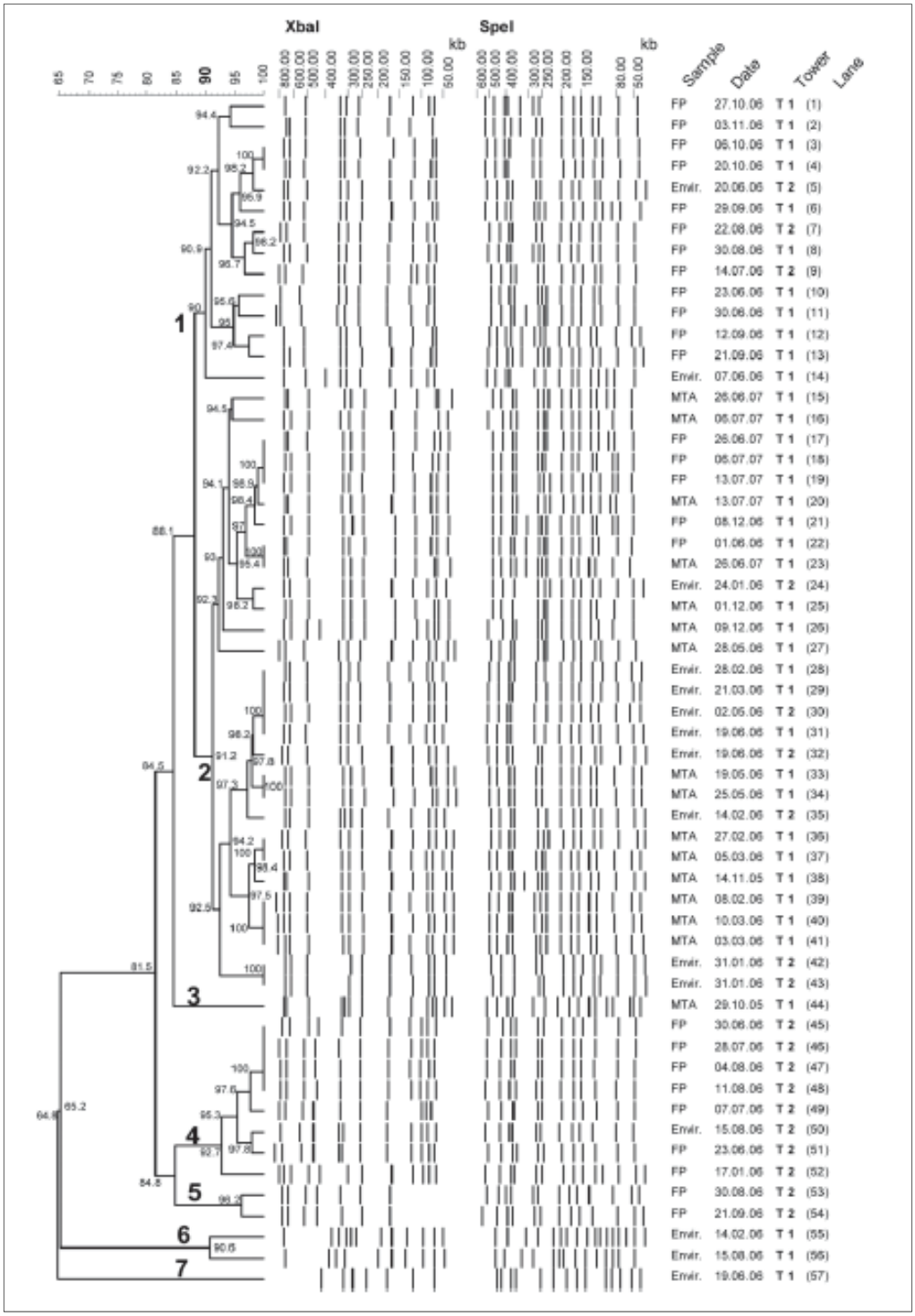


Figure 3. 3 Dendrogram of the pulsed-field gel electrophoresis DNA profiles of *Cronobacter* spp. from one milk powder factory in Germany in 2006 by Jacobs *et al.* (2011).

The PFGE profile in **Figure 3. 3** was obtained using two restriction enzymes (*XbaI* and *SpeI*) to digest *Cronobacter* isolated from one milk powder factory in Germany by Jacobs *et al.* (2011). The bold number at the top shows the 90% similarity cut-off used to differentiate DNA profile (clones) 1–7, and the details of the isolates on the right side identify the isolation site (FP: filter powder, MTA: microbiological trend analysis, and Envie: environment)and the date of isolates as published by Jacobs *et al.* (2011).

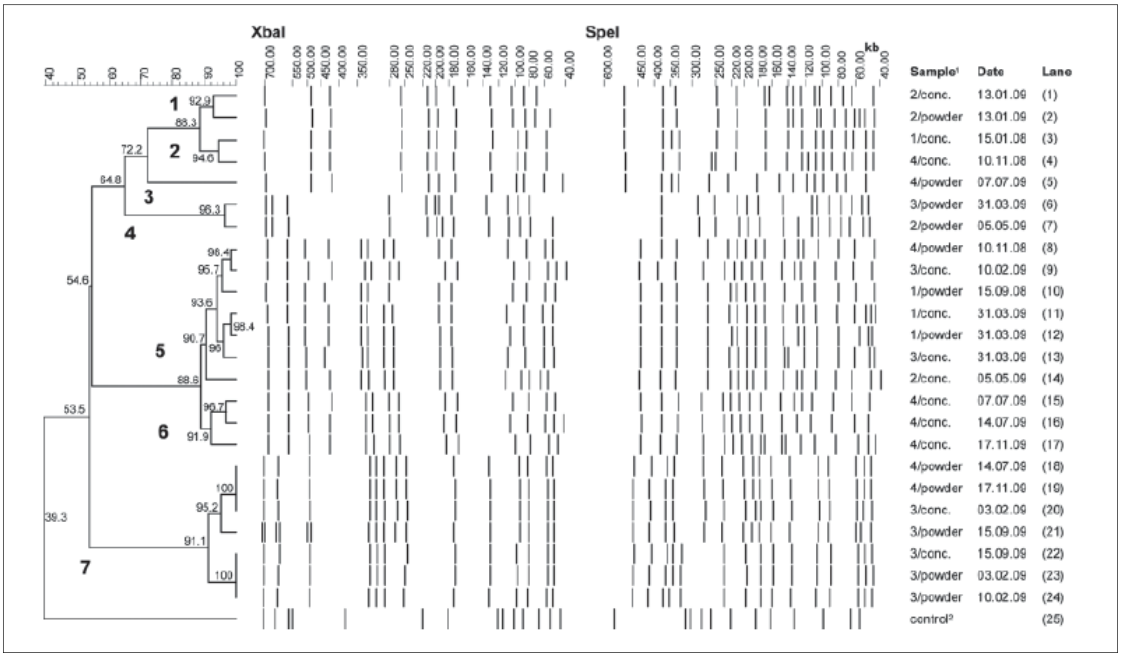


Figure 3. 4 Dendrogram of the pulsed-field gel electrophoresis DNA profiles of *Cronobacter* spp. from one milk powder factory in Germany in 2009 by Jacobs *et al.* (2011).

The PFGE profile in **Figure 3.4** was obtained using the *XbaI* and *SpeI* restriction enzyme to digest *Cronobacter* isolated from roller dryer in one milk powder factory in Germany by Jacobs *et al.* (2011). The bold number at the top shows the 90% similarity cut-off used to differentiate DNA profile (clones) 1–7, and the details of the isolates on the right side identify the isolation site (FP: filter powder, MTA: microbiological trend analysis, and Envie: eniviroment) and the date of isolates as published by Jacobs *et al.* (2011).

3.2 Aims

Multilocus sequence typing (MLST), which combines PCR amplification with sequence analysis to characterize seven housekeeping genes, is a robust and reliable technique for analysing bacteria of the *Cronobacter* genus (Baldwin *et al.*2009; Joseph *et al*. 2012b). Importantly, MLST shows a high level of discrimination between *Cronobacter* species (Joseph *et al.* 2012b). This chapter describes an MLST scheme that was used to analyse strains which had only been identified as *E. sakazakii* at milk powder manufacturing plants and environmental processing areas in Germany and Australia (Craven *et al.* 2010; Jacobs *et al.* 2011). MLST was also used to analyse strains isolated from powdered infant formula (PIF) purchased from thirteen countries and reported as *E. sakazakii* by Muytjens *et al.* (1988). In the work described in this chapter, the MLST scheme was applied to strains described in previous published studies to update the definitions of these strains at the species level and to define their sequence types (STs).

This chapter describes the bacterial species and sequence types that are present and persistent in milk powder and in the environments where milk powder is processed. It also reports the relationship between the various *Cronobacter* STs and how this information was used to investigate possible correlations between STs from the milk powder processing and storage environments versus STs from clinical sources. There was a focus on the source of these isolated environmental strains with the aim of identifying links between the STs identified in the milk powder and PIF processing environments and to study the physiological mechanisms that help the organism persist in these environments.

Lastly, this chapter includes related results that were published recently (Sonbol *et al.* 2013).

3.3 Materials and methods

The methods and details about DNA extraction and purification, primers, PCR conditions, and sequence analysis are described in Chapter 2, Section 2.7.

3.4 Bacterial strains used in this study

In this study, the MLST scheme was applied to three collections of environmental strains e.g: PIF, milk powder factories environments and milk powder processing equipment (spray drying tower and roller dryer), that had not been profiled previously**(Table 3.1).**

3.5 Results

3.5.1 MLST analysis of *Cronobacter* species

A total of 85 isolates from three strain collections that were previously identified as *E. sakazakii* was obtained and analysed by MLST in this study. The strains were originally isolated between 1988 and 2009 from 14 countries. The strain collection comprised 20 strains from Muytjens *et al.* (1988), 52 strains from Craven *et al.* (2010), and 13 strains from Jacobs *et al.* (2011). The German and Australian strain collections were selected as representing of each pulsotype and those which did not give banding patterns in PFGE. The strains used in this study are listed in **Tables 3.1.**

All 85 of these strains were genotyped by MLST, and the resulting data were submitted to the MLST database (www.pubMLST.org/cronobacter). Details of the *Cronobacter* spp. ST profiles are shown in **Tables 3.1, 3.2**, **3.3, 3.4** and are summarised in **Table 3.6**. The majority of the 85 strains were identified as *C. sakazakii* (n = 72), followed by *C. turicensis* (n = 9), *C. malonaticus* (n = 3), and *C. muytjensii* (n = 1). No *C. dublinensis, C. universalis*, or *C. condimenti* strains were identified. In addition, one strain from India was re-identified as *E. hormaechei.*

In this study, the alleles of the seven genes used for MLST were amplified and sequenced in all 85 strains using the primers listed in **Table 2.2, Section 2.7.3** , and 39 STs were identified across the *Cronobacter* genus **(Tables 3.1, 3.2, 3.3** and **3.4)**. The MLST profile of those strains are given in **Table 3.1.**

Table 3. 1 Details of the bacterial strains used in this study and their MLST profile for each strains.

NTU ID	Species	ST	CC	Country	Source	Year of isolation	atpD	fusA	glnS	gltB	gyrB	infB	pps
1529	*C. sakazakii*	99	99	Germany	Spray drying tower	2006	3	8	52	54	21	65	73
1530	*C. sakazakii*	101	99	Germany	Spray drying tower	2006	3	50	52	54	21	65	73
1531	*C. sakazakii*	99	99	Germany	Spray drying tower	2006	3	8	52	54	21	65	73
1532	*C. sakazakii*	99	99	Germany	Spray drying tower	2006	3	8	52	54	21	65	73
1534	*C. sakazakii*	33	-	Germany	Spray drying tower	2006	49	10	53	55	54	20	74
1535	*C. sakazakii*	99	99	Germany	Spray drying tower	2006	3	8	52	54	21	65	73
1533	*C. sakazakii*	4	4	Germany	Spray drying tower	2006	5	1	3	3	5	5	4
1538	*C. sakazakii*	1	1	Germany	Roller dryer	2009	1	1	1	1	1	1	1
1540	*C. sakazakii*	1	1	Germany	Roller dryer	2009	1	1	1	1	1	1	1
1541	*C. sakazakii*	1	1	Germany	Roller dryer	2009	1	1	1	1	1	1	1
1536	*C. sakazakii*	1	1	Germany	Roller dryer	2009	1	1	1	1	1	1	1
1537	*C. sakazakii*	4	4	Germany	Roller dryer	2009	5	1	3	3	5	5	4
1542	*C. sakazakii*	4	4	Germany	Roller dryer	2009	5	1	3	3	5	5	4
537	*C. sakazakii*	1	1	Russia	Infant formula	1988	1	1	1	1	1	1	1
541	*C. sakazakii*	1	1	Netherlands	Infant formula	1988	1	1	1	1	1	1	1
545	*C. sakazakii*	3	3	Netherlands	Infant formula	1988	3	3	3	5	3	3	3
538	*C. sakazakii*	4	4	Russia	Infant formula	1988	5	1	3	3	5	5	4
548	*C. sakazakii*	4	4	Germany	Infant formula	1988	5	1	3	3	5	5	4
532	*C. sakazakii*	13	13	Germany	Infant formula	1988	15	14	15	13	22	5	16
530	*C. muytjensii*	49	-	Denmark	Infant formula	1988	26	25	36	25	33	39	44
536	*C. sakazakii*	45	40	Russia	Infant formula	1988	3	15	28	22	5	21	19
547	*C. sakazakii*	65	83	USA	Infant formula	1988	19	16	19	41	19	14	23
531	*C. sakazakii*	57	1	Denmark	Infant formula	1988	3	1	3	1	1	1	1
528	*C. sakazakii*	3	3	Belgium	Infant formula	1988	3	3	3	5	3	3	3
529	*C. sakazakii*	4	4	Canada	Infant formula	1988	5	1	3	3	5	5	4
533	*C. sakazakii*	64	64	France	Infant formula	1988	16	8	13	40	15	15	10
539	*C. sakazakii*	124	8	Netherlands	Infant formula	1988	11	8	7	71	8	15	10
540	*C. sakazakii*	4	4	Netherlands	Infant formula	1988	5	1	3	3	5	5	4
543	*C. sakazakii*	1	1	Netherlands	Infant formula	1988	1	1	1	1	1	1	1
544	*C. sakazakii*	4	4	Netherlands	Infant formula	1988	5	1	3	3	5	5	4
HPB-3284	*C. sakazakii*	8	8	Uruquay	Infant formula	1988	11	8	7	5	8	15	10
527	*C. malonaticus*	62	62	Australia	Infant formula	1988	10	13	18	7	17	23	8
535	*C. malonaticus*	7	7	New Zealand	Infant formula	1988	10	7	6	7	9	14	9
1466	*C. sakazakii*	1	1	Australia	Environmental	2007	1	1	1	1	1	1	1
1467	*C. sakazakii*	37	-	Australia	Environmental	2007	44	15	3	5	5	38	59
1468	*C. turicensis*	114	-	Australia	Environmental	2007	45	22	54	56	56	6	60
1469	*C. turicensis*	114	-	Australia	Environmental	2007	45	22	54	56	56	6	60
1470	*C. sakazakii*	104	-	Australia	Environmental	2007	48	36	29	57	54	20	61
1471	*C. sakazakii*	40	40	Australia	Environmental	2007	3	15	28	22	5	38	19
1472	*C. sakazakii*	115	-	Australia	Environmental	2007	44	8	19	58	67	61	62
1473	*C. sakazakii*	64	64	Australia	Environmental	2007	16	8	13	40	15	15	10
1474	*C. sakazakii*	20	20	Australia	Environmental	2007	16	14	24	17	25	33	25
1475	*C. sakazakii*	100	100	Australia	Environmental	2007	10	17	30	59	57	66	63
1476	*C. sakazakii*	4	4	Australia	Environmental	2007	5	1	3	3	5	5	4

1477	*C. sakazakii*	4	4	Australia	Environmental	2007	5	1	3	3	5	5	4
1478	*C. turicensis*	116	24	Australia	Environmental	2007	22	22	57	16	55	18	64
1479	*C. sakazakii*	1	1	Australia	Environmental	2007	1	1	1	1	1	1	1
1480	*C. sakazakii*	4	4	Australia	Environmental	2007	5	1	3	3	5	5	4
1481	*C. sakazakii*	4	4	Australia	Environmental	2007	5	1	3	3	5	5	4
1482	*C. sakazakii*	4	4	Australia	Environmental	2007	5	1	3	3	5	5	4
1483	*C. sakazakii*	4	4	Australia	Environmental	2007	5	1	3	3	5	5	4
1484	*C. sakazakii*	4	4	Australia	Environmental	2007	5	1	3	3	5	5	4
1485	*C. sakazakii*	4	4	Australia	Environmental	2007	5	1	3	3	5	5	4
1486	*C. sakazakii*	4	4	Australia	Environmental	2007	5	1	3	3	5	5	4
1487	*C. sakazakii*	4	4	Australia	Environmental	2007	5	1	3	3	5	5	4
1488	*C. sakazakii*	4	4	Australia	Environmental	2007	5	1	3	3	5	5	4
1489	*C. sakazakii*	4	4	Australia	Environmental	2007	5	1	3	3	5	5	4
1490	*C. sakazakii*	97	4	Australia	Environmental	2007	5	1	3	60	5	5	4
1491	*C. sakazakii*	96	-	Australia	Environmental	2007	16	37	48	61	5	56	77
1492	*C. sakazakii*	1	1	Australia	Environmental	2007	1	1	1	1	1	1	1
1493	*C. sakazakii*	1	1	Australia	Environmental	2007	1	1	1	1	1	1	1
1494	*C. sakazakii*	1	1	Australia	Environmental	2007	1	1	1	1	1	1	1
1495	*C. sakazakii*	1	1	Australia	Environmental	2007	1	1	1	1	1	1	1
1496	*C. sakazakii*	1	1	Australia	Environmental	2007	1	1	1	1	1	1	1
1497	*C. sakazakii*	117		Australia	Environmental	2007	3	17	49	68	58	63	65
1498	*C. sakazakii*	83	83	Australia	Environmental	2007	19	16	19	41	19	15	23
1499	*C. sakazakii*	1	1	Australia	Environmental	2007	1	1	1	1	1	1	1
1500	*C. sakazakii*	83	83	Australia	Environmental	2007	19	16	19	41	19	15	23
1501	*C. sakazakii*	105	40	Australia	Environmental	2007	5	15	28	22	5	38	19
1502	*C. sakazakii*	1	1	Australia	Environmental	2007	1	1	1	1	1	1	1
1503	*C. sakazakii*	3	3	Australia	Environmental	2007	3	3	3	5	3	3	3
1504	*C. sakazakii*	46	-	Australia	Environmental	2007	34	36	10	44	43	48	51
1506	*C. sakazakii*	40	40	Australia	Environmental	2007	3	15	28	22	5	38	19
1507	*C. sakazakii*	40	40	Australia	Environmental	2007	3	15	28	22	5	38	19
1508	*C. sakazakii*	103	-	Australia	Environmental	2007	3	38	3	13	59	57	21
1505	*C. sakazakii*	40	40	Australia	Environmental	2007	3	15	28	22	5	38	19
1509	*C. turicensis*	126	-	Australia	Environmental	2007	46	39	4	62	60	49	66
1510	*C. turicensis*	127	-	Australia	Environmental	2007	47	39	60	63	60	49	67
1511	*C. turicensis*	118	-	Australia	Environmental	2007	6	5	58	64	66	58	68
1512	*C. turicensis*	119	119	Australia	Environmental	2007	36	39	27	65	46	18	69
1513	*C. turicensis*	120	119	Australia	Environmental	2007	36	39	56	65	46	18	69
1514	*C. malonaticus*	102	-	Australia	Environmental	2007	3	40	50	66	61	62	70
1898	*C. turicensis*	132	-	Australia	Environmental	2007	36	22	61	20	13	72	85
1899	*C. sakazakii*	3	3	Australia	Environmental	2007	3	3	3	5	3	3	3
1900	*C. sakazakii*	133	8	Australia	Environmental	2007	11	8	7	72	8	15	10

C. sakazakii

This study found that *C. sakazakii* was the predominant species, accounting for 72 of the 85 strains. Of these 72 *C. sakazakii* strains, 17 were from Muytjens *et al*. (1988), 42 were from Craven *et al.* (2010), and 13 were from Jacobs *et al*. (2011). ST4 was the predominant ST in this study (20/72 isolates), and ST1 was next (16/72 isolates). There were 27 STs identified in the *C. sakazakii* strains **(Table 3.6).** The predominant species was *C. sakazakii* and STs 4 and 1 were the most frequent STs in all three strain collections.

C. turicensis

Nine *C. turicensis* strains were found in the strain collection from Craven *et al.* (2010). Eight STs were identified in the milk powder factory samples: ST114, ST116, ST118, ST119, ST120, ST126, ST127, and ST132.

C. malonaticus

Three *C. malonaticus* strains were found in the strain collections from Muytijens *et al.* (1988) and Craven *et al.* (2010), and analysis identified ST7, ST62, and ST102 in these environmental samples.

C. muytjensii

The single *C. muytjensii* strain found in this study was from the Muytjens *et al*. (1988) strain collection, and it was identified as ST49. No *C. muytjensii* strains were found in the Craven *et al*. (2010) or Jacobs *et al.* (2011) strain collections.

3.5.2 MLST analysis of the PIF strain collections

Muytjens *et al.* (1988) previously investigated Enterobacteriaceae in a total of 141 PIF samples from 35 countries and identified 20 *E. sakazakii* strains. These strains were isolated before there was international concern about the transmission of neonatal infections via reconstituted infant formula. However given the unique data gathered by Muytjens *et al.* (1988), this study led to changes in the microbiological guidelines for PIF manufacturers by the Codex Alimentarius Commission (2008). MLST analysis of these 20 *E. sakazakii* strains showed that 17 were *C. sakazakii*, 2 were *C. malonaticus*, and 1 was *C. muytjensii* **(Table 3.2).** After MLST genotyping, these strains were re-

identified and classified at the species level. The strains were isolated from PIF purchased from 12 countries: Australia, Belgium, Canada, Denmark, France, West Germany, East Germany, New Zealand, Russia, The Netherlands, Uruguay, and USA.

A total of 13 STs and 10 CCs were identified in this collection of 20 strains from PIF. The STs that are most important in terms of their association with infections and illness were reported previously to be ST4, ST8, and ST3 for *C. sakazakii* and ST7 for *C. malonaticus* (Joseph and Forsythe, 2011; Joseph *et al*. 2012a). Among these 20 strains, the majority of the STs were ST4; 5/17 *C. sakazakii* strains that were isolated from PIF samples purchased in Canada, Russia, West Germany, and The Netherlands were ST4. There strains were isolated over a 26-year period (Muytjens *et al.* 1988). Three strains of *C. sakazakii* ST1 were isolated from PIF samples from The Netherlands and Russia. One strain belonged to CC1 (ST57), and two strains of *C. sakazakii* ST3 were found in PIF from Belgium and The Netherlands. There were two strains of *C. sakazakii* CC8, one was ST8 and one was ST124. One of the isolates previously known as *E. sakazakii* by Muytjens *et al*. (1988) was re-identified as *E. hormaechei* . This strain has been isolated from PIF purchased from India. Of an additional 7 STs identified in the PIF samples, ST45, ST65, ST57, and ST64 were identified in *C. sakazakii* strains found in PIF from Russia, the USA, Denmark, and France. Interestingly, ST57 (strain 531) belonged to CC1 and differed at two alleles, *atpD* and *glnS* from ST1. There were two nucleotide differences between *glnS*: 1 and *glnS*: 3, one at position 174 (G → A) and one at 297 (A → G). There was one nucleotide difference between a*tpD*: 1 and *atpD*: 3 at position 348 (C → T). Two STs, ST7 and ST62, were identified in *C. malonaticus* isolates 535 and 527, respectively. These STs were found in PIF samples purchased from New Zealand and Australia.

Table 3. 2 The MLST profiles of *Cronobacter* strains isolated from powder infant formula (Muytjens *et al.*1988).

Cronobacter species	ID	ST[a]	Clonal complex[b]	Country
C. sakazakii	541	1	1	The Netherlands
C. sakazakii	543	1	1	The Netherlands
C. sakazakii	537	1	1	Russia
C. sakazakii	531	57	1	Denmark
C. sakazakii	528	3	3	Belgium
C. sakazakii	545	3	3	The Netherlands
C. sakazakii	529	4	4	Canada
C. sakazakii	538	4	4	Russia
C. sakazakii	540	4	4	The Netherlands
C. sakazakii	544	4	4	The Netherlands
C. sakazakii	548	4	4	West Germany
C. sakazakii	HPB-3284[c]	8	8	Uruguay
C. sakazakii	539	124	8	The Netherlands
C. sakazakii	532	13	13	East Germany
C. sakazakii	536	45	45	Russia
C. sakazakii	547	65	83	USA
C. sakazakii	533	64	64	France
C. malonaticus	535	7	7	New Zealand
C. malonaticus	527	62	62	Australia
C. muytjensii	530	49	-	Denmark
Total		20		

Strains analysed by Dr. Susan Joseph this included: 527, 530, 531, 532, 535, 536, 537, 538, 541, 545, 547 and 548.
[a]ST, Sequence type.
[b]Clonal complexes with single locus variants.
[c]Information obtained from the MLST database.
ID, the strain identification numbers used in this study.

3.5.3 MLST profiles of isolates from five milk powder manufacturing plants in Australia

In the study by Craven *et al.* (2010), 129 *E. sakazakii* strains were identified by PFGE in processing and non-processing milk powder facilities. These strains were collected from November 2006 to March 2008. Craven *et al.*(2010) identified 49 pulsotypes by digestion with the *XbaI* restriction enzyme; these pulsotypes represented 126 samples from 100 locations in 5 milk powder factories in Australia. There were 3 strains that were not pulsotyped, giving the total of 129 strains.

At the time these data were published, the strains were not identified at the species level. MLST analysis of the 52 *E. sakazakii* strains (49 pulsotypes and 3 strains which were not pulsotyped) showed that these strains included *C. sakazakii* (42/52), *C. turicensis* (9/52), and *C. malonaticus* (1/52) **(Table 3.3).** These isolates included 24 STs; 13 of these belonged to *C. sakazakii* CC4, including 12 ST4 strains and 1 ST97 strain. Strain 1490 (ST97) was isolated from a tanker bay at a factory (B). ST97

differed from ST4 at one nucleotide, with an A → G difference between *gltB:* 60 and *gltB:* 3 at position 321. Thus, these environmental isolates included 18 of the 207 *C. sakazakii* STs that have been identified to date. Interestingly, some of these STs matched STs that had been identified in clinical isolates (Joseph and Forsythe, 2011).

It was of particular interest that of the isolates from these 5 factories, 32/129 belonged to CC4 and 33/129 were ST1. These particular STs merit special attention due to their association with neonatal infections (Joseph and Forsythe, 2011). Two isolates (2/129) were ST3, and both were isolated from the tanker bay at the same factory (factory C). Additional isolates (16/129) belonged to CC45; these were isolated from milk powder and from the tanker bay and roof at three factories (A, C, and D). ST83 (7/129) was isolated from milk powder and from other processing areas at two factories (A and E). The remaining 21/129 strains were isolated from milk powder and were categorised as ST117, ST20, ST46, ST64, ST100, and ST103. These STs were found in 5 factories (A, B, C, D and E). One strain ST104 was isolated from workers' shoes in factory C, and one strain that was ST115 was isolated from roof in factory B. In addition, two strains from tanker bays at two factories, E and A, were ST37 and ST96, respectively **(Table 3.3).** A single ST133 strain (1900) that belonged to CC8 was isolated from a floor in factory B; this isolate could not be pulsotyped.

Out of 52 *Cronobacter* strains (selected from the 49 pulsotype and 3 non-pulsotyped strains), 9 that were *C. turicensis* represented 12/129 samples analysed in the original study by Craven *et al*. (2010). There were two *C. turicensis* strains that were ST114, while two *C. turicensis* strains that were ST119 and ST120 belonged to CC119. Single strains that were ST116 (CC24), ST118, ST126, and ST127 were found in samples from the roof and from milk powder in two factories, A and D. For *C. turicensis,* strain 1478 represented 3/129 of the sampled strains, and this strain was ST116, which in turn belonged to CC24**(Table 3.3).** These strains were found in a tanker bay, on shoes, and in milk powder. One *C. malonaticus* strain (1514) (ST7) was found in this collection, and it was in milk powder at factory E. There were three strains that could not be pulsotyped; two *C. sakazakii* strains (1900 and 1899) that were ST133 (CC8) and ST3, respectively, while one *C. turicensis* strain (1898) was ST132. These were from the tanker bay and the floor and were sampled at factories C and B **(Table 3.3).**.

Table 3. 3 The MLST profiles of *Cronobacter* strains isolated from five milk powder manufacturing plants in Australia between 2006 and 2007 (Craven *et al.* 2010).

Cronobacter species	ID	ST[a]	CC[b]	Isolation environment	Pulsotype[c]	Number of isolates from Craven *et al.* (2010)	Factory[d]
C. sakazakii	1466	1	1	Milk powder	1	5	B
C. sakazakii	1479	1	1	Milk powder	14	1	C
C. sakazakii	1492	1	1	Roof, milk powder	27	12	B
C. sakazakii	1493	1	1	Other processing area (butter)	28	1	B
C. sakazakii	1494	1	1	Tanker bay, milk powder	29	3	E
C. sakazakii	1495	1	1	Milk powder	30	7	E
C. sakazakii	1496	1	1	Milk powder	31	1	E
C. sakazakii	1499	1	1	Milk powder	34	1	B
C. sakazakii	1502	1	1	Roof	37	2	C
Total n. of CC1 strain	**n=8**					**n=33**	
C. sakazakii	1497	117	-	Milk powder	32	8	A
C. sakazakii	1503	3	3	Tanker bay	38	1	C
C. sakazakii	1899	3	3	Tanker bay	NP[e]	1	C
C. sakazakii	1476	4	4	Milk powder, other processing (evaporator), other processing area(powder entrance)	11	6	B, E
C. sakazakii	1477	4	4	Roof, milk powder	12	7	B
C. sakazakii	1480	4	4	Milk powder	15	4	A, D
C. sakazakii	1481	4	4	Other processing area (evaporator)	16	1	A
C. sakazakii	1482	4	4	Tanker bay	17	1	A
C. sakazakii	1483	4	4	Milk powder	18	1	D
C. sakazakii	1484	4	4	Roof, milk powder	19	4	C, E
C. sakazakii	1485	4	4	Other external area (outside grounds)	20	1	C
C. sakazakii	1486	4	4	Other external area (outside grounds)	21	1	C
C. sakazakii	1487	4	4	Tanker bay	22	1	A
C. sakazakii	1488	4	4	Milk powder	23	1	D
C. sakazakii	1489	4	4	Milk powder	24	2	A
C. sakazakii	1490	97	4	Tanker bay	25	2	B
Total n. of CC4 strains	**n=13**					**n=32**	
C. sakazakii	1474	20	20	Milk powder	9	3	D
C. sakazakii	1467	37	-	Tanker bay	2	1	E
C. sakazakii	1471	40	45	Milk powder	6	1	A
C. sakazakii	1505	40	45	Milk powder	40	3	D
C. sakazakii	1506	40	45	Milk powder, other processing area (evaporator)	41	10	D
C. sakazakii	1507	40	45	Tanker bay	42	1	C
C. sakazakii	1501	105	45	Roof	36	1	C
Total n. of CC45 strains	**n=5**					**n=16**	

***Cronobacter* species**	**ID**	**ST[a]**	**CC[b]**	**Isolation environment**	**Pulsotype[c]**	**Number of isolates from Craven *et al.* (2010)**	**Factory[d]**
C. sakazakii	1504	46	-	Milk powder	39	1	C
C. sakazakii	1473	64	64	Milk powder	8	2	E
C. sakazakii	1498	83	83	Milk powder, other processing area	33	6	E
C. sakazakii	1500	83	83	Milk powder	35	1	A
C. sakazakii	1491	96	-	Tanker bay	26	1	A
C. sakazakii	1475	100	100	Milk powder	10	2	B
C. sakazakii	1508	103	-	Milk powder	43	5	A
C. sakazakii	1470	104	-	Other external area (shoes)	5	1	C
C. sakazakii	1472	115	-	Roof	7	1	B
C. sakazakii	1900	133	8	Floor	NP[e]	1	B
C. malonaticus	1514	102	-	Milk powder	49	1	E
C. turicensis	1468	114	-	Other processing area (cheese), tanker bay	3	2	B, E
C. turicensis	1469	114	-	Roof	4	1	A
C. turicensis	1478	116	24	Tanker bay, other external area (shoes), milk powder	13	3	C
C. turicensis	1511	118	-	Roof	46	1	D
C. turicensis	1512	119	119	Other processing area	47	1	E
C. turicensis	1513	120	119	Milk powder	48	1	C
C. turicensis	1509	126	-	Roof	44	1	D
C. turicensis	1510	127	-	Milk powder	45	1	A
C. turicensis	1898	132	-	Floor	NP[c]	1	B
Total		**n=52**				**n=129**	

[a] ST, Sequence type.
[b]CC, clonal complex.
[c]Pulsotype according to Craven *et al.* (2010).
[d]The five milk powder processing factories in Australia, with A, B, C, D, and E designations from Craven *et al*. (2010).
[e]NP, no profile found by PFGE analysis in Craven *et al.* (2010).
ID, the strain identification numbers used in this study.
n: Number of the strains.

3.5.4 MLST profiles of *Cronobacter* strains from a German manufacturing plant

Jacobs *et al*. (2011) isolated 81 *E. sakazakii* strains from a German manufacturing plant that they categorized into 13 pulsotypes covering all 81 isolates. In this study, all representative strains of these 13 pulsotypes were identified as *C. sakazakii* **(Table 3.4).** The 13 strains included 5 STs and 4 CCs. These strains were primarily ST1 (n = 4), ST4 (n = 3), and ST99 (n = 4). Of the 4 ST1 strains, 2 strains were from roller dryer powder, and 2 strains were from roller dryer concentrate. Similarly, 3/13 of the factory isolates were ST4. There were 3 ST4 strains, 2 of which were isolated from a roller dryer and 1 of which was isolated from the environment in drying tower 1. The *C. sakazakii* ST1 strains were isolated from samples taken from a roller dryer in 2009. The *C. sakazakii* ST4 strains

were isolated from samples taken from a roller dryer in 2009 and from samples taken from a drying tower in 2006. The four *C. sakazakii* strains that were ST99 were collected from filter powder and from routine samples taken from two spray drying towers in 2006. In addition, one strain (1530) was ST101, which belonged to CC99. This ST differs from ST99 by one nucleotide between *fusA* 8 and *fusA* 50 (378 G→ A). One strain that was ST33 was isolated from drying tower 1 in 2006 (Jacobs *et al.* 2011).

Jacobs *et al.* (2011) sampled 81 isolates and analysed them by PFGE according to the isolate collection year, i.e. there was a 2006 isolate group and a 2009 isolate group. With regard to PFGE analysis, 24/81 strains isolated in 2009 represented 6 pulsotypes, and 57 strains isolated in 2006 represented 7 pulsotypes (Jacobs *et al.*2011). The MLST analysis in the current study found that of the total number of 81 strains: 19 belonged to CC4, 7 belonged to ST1, and 54 belonged to CC99 **(Table 3.4).** In addition, 10/54 CC99 strains were ST101. As noted earlier, ST4 and ST1, which were found in this collection, merit attention because of their associations with neonatal infections (Joseph and Forsythe, 2011). The ST4 strains were isolated from a roller dryer and from drying tower 1 in 2006 and 2009, whereas the ST1 strains were from a roller dryer in 2009 **(Table 3.4).** One isolate (1/81) was ST3, which was isolated from spray drying tower 1. CC99 was isolated from spray drying tower 1 and 2 .

Table 3. 4 The MLST profiles of *Cronobacter* strains isolated from a milk powder manufacturing plant in Germany in 2006 and 2009 (Jacobs *et al.* 2011).

Year of isolates	*Cronobacter* species	ID	ST[a]	CC[b]	Source	Year	Pulsotype[c]	Number of isolates in the pulsotype[d]
2009	*C. sakazakii*	1536	1	1	Roller dryer	2009	2009-2	2
	C. sakazakii	1538	1	1	Roller dryer	2009	2009-1	2
	C. sakazakii	1540	1	1	Roller dryer	2009	2009-3	1
	C. sakazakii	1541	1	1	Roller dryer	2009	2009-4	2
	C. sakazakii	1537	4	4	Roller dryer	2009	2009-5	10
	C. sakazakii	1542	4	4	Roller dryer	2009	2009-6	7
	Total	**n = 6**						**n = 24**
2006	*C. sakazakii*	1533	4	4	Spray drying tower 1	2006	2006-6	2
	C. sakazakii	1534	33	-	Spray drying tower 1	2006	2006-7	1
	C. sakazakii	1529	99	99	Spray drying tower 1	2006	2006-3	1
	C. sakazakii	1531	99	99	Spray drying tower 1	2006	2006-1	14
	C. sakazakii	1532	99	99	Spray drying tower 2	2006	2006-5	2
	C. sakazakii	1535	99	99	Spray drying tower 1	2006	2006-2	29
	C. sakazakii	1530	101	99	Spray drying tower 2	2006	2006-4	8
	Total	**n = 7**						**Total = 57**
	Total No. of MLST strains	**13**						**81**

[a]ST, sequence type
[b]CC, clonal complex
[c]Some strains isolated in 2006 and 2009 have the same pulsotype numbers, but this does not reflect any similarities.
[d]The original number of isolates in Jacobs *et al*. (2011).
ID, the strain identification numbers used in the MLST study.

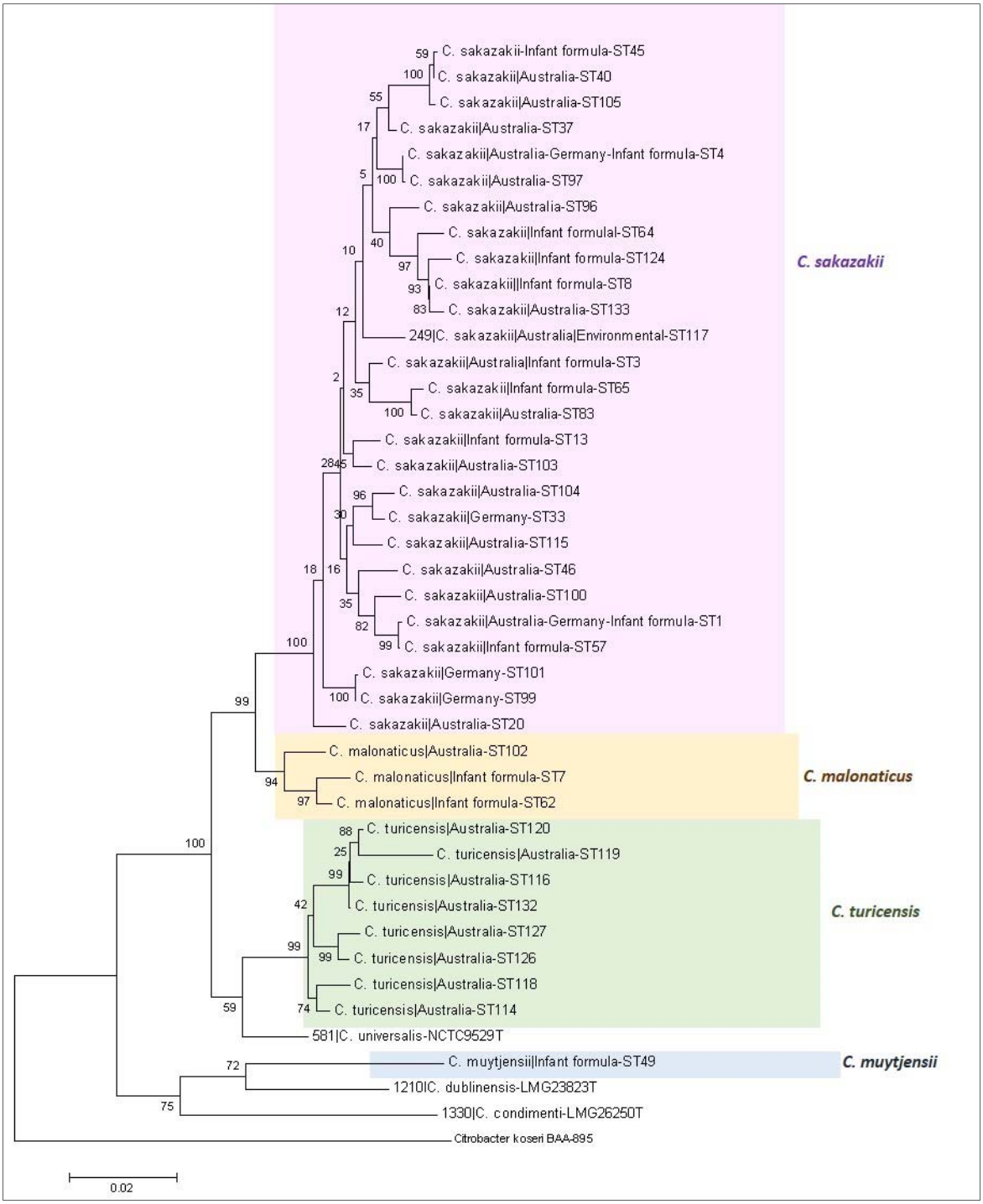
3.5.5 The phylogenetic relationships of the environmental strains of *Cronobacter* spp.

The two collections of strains from Australian and German powder milk factory samples, as well as strains isolated from PIF, were studied by Multilocus Sequence Analysis (MLSA) using the concatenated sequences of the MLST alleles (Muytjens *et al.* 1988; Craven *et al.* 2010; Jacobs *et al.* 2011). These were submitted to the MLST database (http://www.pubMLST.org/*Cronobacter*). The sequences were aligned using the ClustalW2 program (Thompson *et al*. 1994). MEGA (Molecular Evolutionary Genetics Analysis) software version 5.2 was used to construct the tree (Tamura *et al.* 2011) (**Section 2.7.8 and Figuer 3.5)**.

3.5.5.1 **The phylogenetic analysis of the STs in this study**

A phylogenetic tree based on the concatenated 7 MLST sequence alleles (3036 nucleotides) of the 39 STs was constructed using the maximum-likelihood method with MEGA software version 5.2 in order to study the phylogeny of the *Cronobacter* strains in environmental samples **(Figure 3.5).** The tree shows that unique STs are predominant in isolates from milk powder factories and from PIF. Constructing the phylogenetic tree revealed the predominant isolates and STs in the environmental samples from Australia and Germany. The tree also showed the relatedness of the STs and demonstrated the predominance of the *C. sakazakii* ST4 and ST1 strains (**Figures 3.5; Table 3.1).** The tree was rooted using the sequences of the concatenated 7 MLST loci and using a genus that is closely related to *Cronobacter* spp., *Citrobacter koseri* BAA-895, as an outlier. The type strains of *C. condimenti* LMG26250^T, *C. dublinensis* LMG23823^T, and *C. universalis* NCTC9529^T were used to show the diversity. The details of the *Cronobacter* spp. MLST profiles are given in **Tables 3.1, 3.2, 3.4** and are summarized in **Table 3.6.**

The phylogenetic tree in **Figure 3.5** is based on the concatenated MLST sequences of 7 loci of the 85 *Cronobacter* spp. strains and shows clear clustering across the *Cronobacter* genus in four of the seven species. It demonstrates that of the 85 *Cronobacter* spp. isolates, *C. sakazakii*, *C. turicensis*, *C. malonaticus*, and *C. muytjensii* were the predominant species in PIF and in milk powder manufacturing environments. Phylogenetic tree analysis shows that these four species are clustered closely together and are more genetically related based on the 7 MLST allele sequences compared to the other species (*C. condiment* , *C. dublinensis* and *C. universalis*), which were not found in this study.



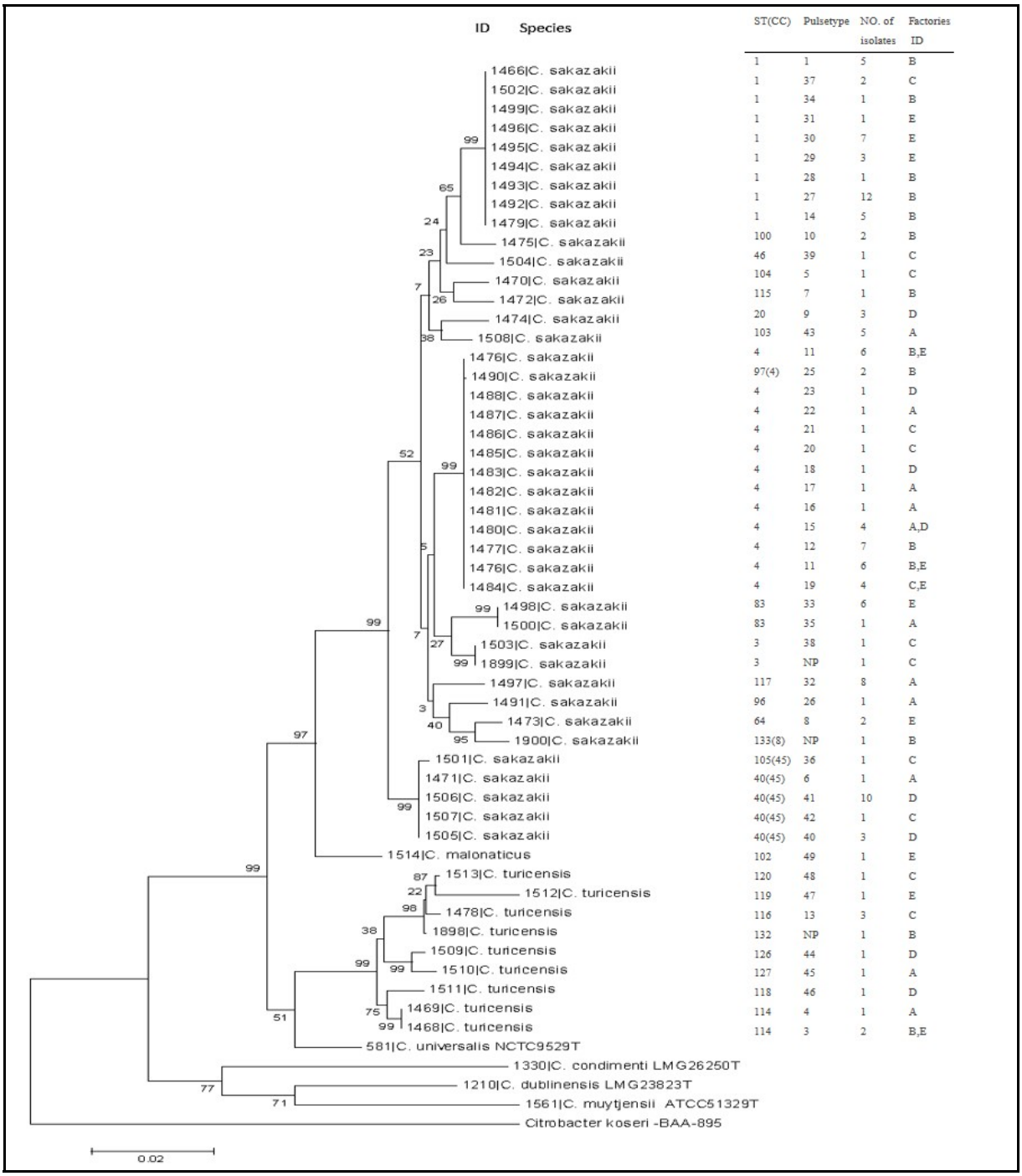
*CC, clonal complex.
Australia: Strain collection from Craven *et al.*(2010)
Germany: Strain collection from Jacobs *et al.* (2011)
Infant formula: Strain collection from Muytjens *et al.* (1988).

Figure 3. 5 The maximum likelihood tree for the seven MLST loci of 85 *Cronobacter* spp. strains identified in the 3 strain collections.

The tree was constructed using 7 MLST loci (3036 concatenated nucleotides) for 85 *Cronobacter* spp. strains shows the STs of the isolated strains and the type strains of the *Cronobacter* species that were identified in the 3 strain collections. The tree was drawn using MEGA v5.2 (http://www.megasoftware.net/) with 1000 bootstrap replicates. The type strains of *C. condimenti*, *C. dublinensis* and *C. universalis* in addition to *C. koseri* were included as an outlier. MLST tree analysis revealed clusters and subclusters based on the level of nucleotide sequence similarity. The number next to the branch point shows the bootstrap rate (1000 replicates). The scale bar shows the ratio of nucleotide substitution.

3.5.5.1.1 The phylogenetic analysis of the Australian isolates

As shown in **Figure 3.6**, the phylogenetic tree of the 7 MLST alleles of the strains from 5 Australian factories shows a clear distinction between 3 of the species, namely *C. sakazakii*, *C. turicensis*, and *C. malonaticus.* This distinction was not evident using the PFGE method as in the Craven *et al*. (2010) study. The tree also shows that the MLST scheme can distinguish among species with different STs, and it shows when a particular ST was found in a specific place in the factories. For example, ST4 and ST1 were the most common isolates from the 5 factories in Australia for 12 and 9 strains, respectively **(Table 3.3).**



a,CC, clonal complex,

b,Pulsetype as determined by PFGE (from Craven *et al*. 2010).

c,NO, the number of isolates (from Craven *et al*. 2010).

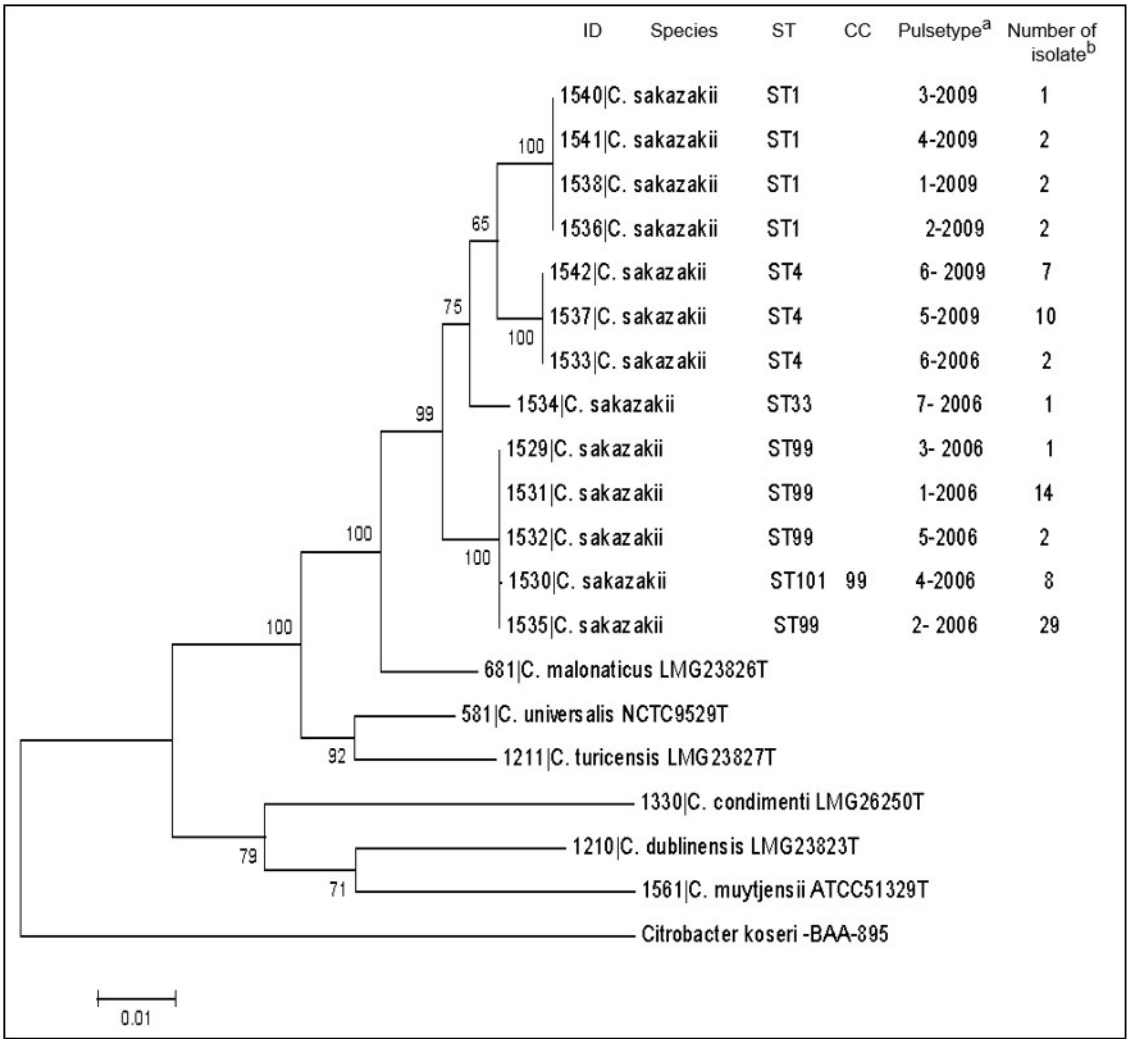
Figure 3. 6 Phylogenetic analysis of the concatenated sequences of the seven MLST loci for the Australian strains (Craven *et al* .2010).

The phylogenetic tree was constructed using the maximum-likelihood method in MEGA v.5.2 with 1000 bootstrap replicates. MLST tree analysis revealed clusters and subclusters based on the level of nucleotide sequence similarity. The number next to the branch point shows the bootstrap rate (1000 replicates). The scale bar shows the ratio of nucleotide substitution.

3.5.5.1.2 The phylogenetic analysis of the German isolates

In **Figure 3.7**, the phylogenetic tree for the *Cronobacter* strains from a German factory shows that *C. sakazakii* was the only species that was isolated from this collection. It also shows the predominance of some "clone" STs in this factory, namely ST4, ST1, ST99, and ST33. In addition, it demonstrates the persistence of ST4, which was collected on two occasions (periods), i.e. in 2006 and in 2009, from the roller dryer and drying tower areas **(Table 3.4)**.

It should be mentioned that the strains isolated from the German factory that were ST4, ST1, ST99, and ST33 could survive the extreme dryness and the high heat inherent to the roller dryer and spray-drying processes (Jacobs *et al.* 2011). The roller dryer is used in one of the processing steps of milk powder in the factories (Jacobs *et al*. 2011). According to Jacobs *et al*. (2011), these strains were exposed to different temperatures during the spray drying and roller drying process. In the spray-drying steps, the skim milk was initially pasteurized at high temperature e.g 72°C to 74°C for 15 to 20 seconds, followed by concentration at 74°C to 120°C for 5 minutes (Jacobs *et al.* 2011). There was an additional heat-treatment step of 70°C to 72°C for 30 second after the spray-dryer was preheated to 210°C. Moreover, in the roller dryer steps, these strains were exposed to 150°C to 160°C heat for 1.5 second, with the exact heating temperature depending on the rotation speed and the drying roll diameter.



CC, Clonal complex
ST, Sequence type.
CC, Clonal complex.
[a]Pulsotype from Jacobs *et al*. (2011).
[b] Number of isolates from Jacobs *et al*. (2011).

Figure 3. 7 Phylogenetic analysis of the concatenated sequences of the seven MLST loci for the German strains (Jacobs *et al.*2011).

The phylogenetic tree was constructed using the maximum-likelihood method in MEGA v5.2 with 1000 bootstrap replicates. MLST tree analysis revealed clusters and subclusters based on the level of nucleotide sequence similarity. The number next to the branch point shows the bootstrap rate (1000 replicates). The scale bar shows the ratio of nucleotide substitution.

3.5.6 Analysis of STs by source and pulsotype

MLST analysis of the strains in the processing and non-processing areas of milk powder manufacturing plants in Germany and Australia revealed the distribution of certain STs that were isolated at six factories (five Australian and one German; Craven *et al.* 2010; Jacobs *et al.* 2011) in 2006–2009 **(Figures 3.6** and **3.7).** These findings prompted us to study the correlation between the ST clustering of 64 *Cronobacter* spp. strains and the pulsotype data obtained previously (Craven *et al.* 2010). In the PFGE profiles, a 94% similarity cut-off was used to determine the strains clonality clustering ; this led to the generation of additional pulsotypes (Craven *et al.* 2010). PFGE analysis of 126 *Cronobacter* isolates identified 49 pulsotypes (Craven *et al.* 2010). The STs 4 and 1 were subdivided according to pulsotype as described below.

3.5.6.1 **STs analysis of the Australian pulsotypes isolates**

Of the *Cronobacter* spp. strains analysed , 9/52 strains were ST1, which indicates that 33/129 isolates in the Craven *et al*. (2010) study were ST1, since the analysed strains were representative of pulsotype . These strains were isolated from similar areas in 3 out of 5 milk powder factories **(Table 3.3** and **3.5).** MLST analysis showed that the majority of the isolates (116/129) from the 5 milk processing factories in Australia were *C. sakazakii* strains, and 32/129 belonged to CC4 according to the original number of the isolates in the Craven *et al.* (2010) study. Interestingly, the *C. sakazakii* species showed that some pulsotype clusters were associated with the same ST **(Table 3.5, Figure 3.6).** For instance: *C. sakazakii* ST1 was subdivided into 9 pulsotypes according to the PFGE profile results of Craven *et al.* (2010).

Table 3. 5 Sequence types of the Australian representative pulsotypes as determined by Craven *et al.* (2010).

Species	Strain	ST[a]	CC[b]	Pulsotype[c]
C. sakazakii	1476	4	4	11
C. sakazakii	1477	4	4	12
C. sakazakii	1480	4	4	15
C. sakazakii	1481	4	4	16
C. sakazakii	1482	4	4	17
C. sakazakii	1483	4	4	18
C. sakazakii	1484	4	4	19
C. sakazakii	1485	4	4	20
C. sakazakii	1486	4	4	21
C. sakazakii	1487	4	4	22
C. sakazakii	1488	4	4	23
C. sakazakii	1489	4	4	24
C. sakazakii	1490	97	4	25
C. sakazakii	1466	1	1	1
C. sakazakii	1479	1	1	14
C. sakazakii	1492	1	1	27
C. sakazakii	1493	1	1	28
C. sakazakii	1494	1	1	29
C. sakazakii	1495	1	1	30
C. sakazakii	1496	1	1	31
C. sakazakii	1499	1	1	34
C. sakazakii	1502	1	1	37
C. sakazakii	1506	40	45	40
C. sakazakii	1507	40	45	41
C. sakazakii	1505	40	45	42
C. sakazakii	1501	105	45	36
C. sakazakii	1471	40	45	6
C. sakazakii	1498	83	83	33
C. sakazakii	1500	83	83	35
C. turicensis	1511	118	-	46
C. turicensis	1512	119	119	47
C. turicensis	1513	120	119	48
C. turicensis	1509	126	-	44
C. turicensis	1510	127	-	45
C. turicensis	1898	132	-	NP

[a] Sequence type
[b] CC, clonal complex
[c] Pulsotype as determined by PFGE in Craven *et al.*2010
NP, no profile found by PFGE analysis in Craven *et al.*2010

It was found that 13/52 isolates belonged to CC4. Strains 1490, 1476, 1477 and 1480-1489 clustered together, as shown in the phylogenetic tree **(Figure 3.6)** and in **Table 3.5.** These 13 strains had the following pulsotype profile numbers: 25, 11, 12, 15, 16, 17, 18, 19, 20, 21, 22, 23, and 24, respectively, indicating that they had similar DNA profiles by PFGE (Craven *et al.* 2010). This analysis revealed considerable discrepancies between the two typing techniques since some STs included multiple pulsotypes.

The phylogenetic tree in **Figure 3.6** shows that a cluster of 5/52 *C. sakazakii* strains belonged to CC45, and 3 of these 5 were clustered according to their PFGE profiles as determined in by Craven *et al.* (2010) **(Figure 3.2)**. Specifically, strains 1506, 1507, and 1505 were pulsotypes 41, 42, and 40, respectively (Craven *et al.* 2010). In addition, two additional strains 1501 and 1471, were pulsotypes 36 and 6, respectively (Craven *et al.* 2010). This cluster represented 16/129 strains isolated from 3/5 factories in Australia (Craven *et al.* 2010).

In this study, two strains, 1498 and 1500, were *C. sakazakii* ST83 and had pulsotypes 33 and 35, respectively (Craven *et al*. 2010). These strains represented 7 isolates from 2/5 factories. In addition, *C. sakazakii* ST3 included two strains: strain 1503 had pulsotype 38, and strain 1899 which had no PFGE profile. These two strains represent 2/129 strains isolated from one factory. There was one *C. sakazakii* CC8 strain that could not be pulsotyped. The rest of the strains had pulsotypes 10, 39, 5, 7, 9, 43, 26, and 32 and belonged to the following *C. sakazakii* STs: 1475-ST100, 1504-ST46, 1472-ST115, 1474-ST20, 1508-ST103, 1491-ST96, 1473-ST64, and 1497-ST117, respectively.

Similarly, the MLST allele phylogenetic tree **(Figure 3.6)** revealed that the *C. turicensis* species showed five clusters, and 8 STs belonged to 9 *C. turicensis* strains. The first cluster included strains 1513 and 1512, which were ST120 and ST119 and pulsotypes 48 and 47, respectively **(Table 3.5)**. The second cluster included strains 1478 and 1898, which were ST116 and ST132. Strain 1478 had pulsotype 13, whereas strain 1898 could not be pulsotyped. The third cluster included strains 1509 and 1510, which were ST126 and ST127 and pulsotypes 44 and 45. The fourth cluster included strain 1511, which was ST118 and pulsotype 46. The fifth cluster included 2 strains, strains 1469 and 1468, both of which were ST114 and which were pulsotypes 4 and 3, respectively. Even though the MLST

analysis subdivided the *C. turicensis* strains into 5 clusters, all of the *C. turicensis* strains were clustered closely together according to MLST and pulsotype results **(Table 3.5).** One *C. malonaticus* strain was ST102 and pulsotype 49.

These findings verified that for species level identification of *Cronobacter* genus bacteria, the results of PFGE analysis are inconsistent with the results of MLST analysis. Using the pulsotypes determined using PFGE analysis with one restriction enzyme, *XbaI*, *C. sakazakii*, and *C. turicensis* were sometimes clustered together, although these are distinct species. For example, *C. turicensis* strains 1469 and 1468, which have pulsotypes 4 and 3 and are ST114, were clustered within the *C. sakazakii* clones in the PFGE analysis as seen from the pulsotype numbers in **Table 3.5.** as pulsotype 1 and 5 belong to *C. sakazakii* strains.

3.5.6.2 **STs analysis of the German pulsotypes isolates**

An original study by Jacobs *et al.* (2011) identified 81 *Cronobacter* spp. isolates from a single German milk powder manufacturing plant, and these were categorised into 13 pulsotypes. The strains were collected in two stages. The first strains, which represented 7 pulsotypes, were collected in 2006, while the second strains, which represented 6 pulsotypes, were collected in 2009. In this study, all representative strains of these pulsotypes were identified as *C. sakazakii* **(Table 3.4).** The 13 strains that represented the original pulsotypes (of a total of 81 strains; Jacobs *et al*. 2011), included 5 STs and 4 CCs; these strains were mainly ST1 (n = 4), ST4 (n = 3), and ST99 (n = 4).

MLST analysis revealed the identification of four *C. sakazakii* ST1 strains. These strains were isolated from a roller dryer that was sampled in 2009 (Jacobs *et al.* 2011). These 4 strains were pulsotypes 1-2009, 2-2009, 3-2009, and 4-2009. These 4 pulsotypes represent the 81 isolates from the original study by Jacobs *et al.* (2011), which included 7/81 ST1 strains that were isolated from roller dryer concentrate and from roller dryer powder. In addition, pulsotype 3-2009 represents 1/81 of the isolates and pulsotypes 4-2009 represents 2/81 of the isolates; these two pulsotypes were isolated from the roller dryer concentrate. Moreover, 2/81 isolates were pulsotype 1-2009, and 2/81 isolates were pulsotype 2-2009; these two pulsotype were isolated from the roller dryer powder.

In this study, three *C. sakazakii* ST4 strains were isolated from a roller dryer in 2009 and from a spray drying tower in 2006 (Jacobs *et al.* 2011). **Table 3.4** shows that ST4 strains 1537, 1542, and 1533, which represent pulsotypes 5-2009, 6-2009, and 6-2006, were collected in 2009 (1537 and 1542) and in 2006 (1533). These strains were isolated from different places: strains 1537 and 1542 were isolated from roller dryer powder and roller dryer concentrate, respectively, whereas strain 1533 was isolated from the environment of drying tower 1. Moreover, these pulsotypes represent the total number from the original study by Jacobs *et al.* (2011), which included pulsotype 5-2009 (10/81 isolates), pulsotype 6-2009 (7/81 isolates), and pulsotype 6-2006 (2/81 isolates), all of which are ST4.

All of the strains in the Jacobs *et al.* (2011) study were identified as *C. sakazakii*, and ST4 strains represented the most isolates (about 27/81 isolates). In addition, five C. *sakazakii* strains were identified as belonging to CC99. These strains were isolated by Jacobs *et al.* (2011) from filter powder and during routine testing of spray drying towers 1 and 2. The five C. *sakazakii* strains, 1529, 1531, 1532, 1535, and 1530 represented the following pulsotypes: 3-2006, 1-2006, 5-2006, 2-2006, and 4-2006, respectively. CC99 included the most strains isolated from the German factory, with 54/81 strains belonging to this ST, and the pulsotypes are clearly clustered **(Table 3.4).**

3.5.7 Clonality and predominant STs in the environmental *Cronobacter* strains

The goeBURST analysis of the MLST data of the 3 *Cronobacter* strain collections revealed the relatedness of the STs. There were 8 SLVs (CCs) in the 39 STs identified in the *Cronobacter* strains in this study **(Figure 3.8, Tables 3.2, 3.3,** and **3.4).** An SLV was the minimum requirement for forming a CC that was distinct from the main founder ST. Such an SLV shared 6 out of the 7 loci of a ST and is shown with a number 1 on the black lines in the goeBURST figures. A number of DLVs, TLVs, and QLVs were observed that showed the relatedness of the STs **(Figures 3.8** and **3.9).**

The goeBURST analysis in **Figure 3.8** illustrates the diversity of the *Cronobacter* STs according to the source and species of the strains isolated from PIF and milk powder factories. The diagram shows the predominate species in this isolates collection, namely *C. sakazakii*, *C. turicensis, C. malonaticus*, and *C. muytijensii.* It also shows the predominance of specific STs in *C. sakazakii* isolated from the milk powder factory environment and from PIF, which are ST4, ST1, ST40, and ST99.

Seven of eight of the SLV CCs that had single nucleotide difference in one allele were *C. sakazakii*: i.e. ST4 and ST97, ST99 and ST101, ST8 and ST124 and ST133, ST119 and ST120, ST40 and ST45 and ST105. There was just one SLV in CC4 of ST4 and ST97 **(Figures 3.8** and **3.9)** in the *gltB* allele, and there was one DLV between ST1 and ST57 in the *C. sakazakii* strains. There were 3 QLV linkages in two *C. sakazakii* strains, including a QLV between ST8 and ST64 and a QLV between ST40 and ST37.

Figure 3.8 shows the goeBURST analysis of the most important STs based on the source of isolation using different colours to represent the source of the strains (milk powder factories in Australia and Germany). Most of the isolates belonged to *C. sakazakii* CC4 and CC1, showing that these CCs were predominant in PIF purchased from 13 countries in 1988 and isolated between 2006 and 2009 from different areas in milk powder factories in two countries, including the roller dryer, drying tower, milk powder, roof, and tanker bay. **Figure 3.8** also shows the link between the STs found in PIF and the STs found in the processing environments of milk powder. For example, ST65 is one of the STs isolated from infant formula (strain 547) and it belongs to CC83. In addition, it was found that one strain 1500 has the same CC83 (ST83) was isolated from a milk powder factory in Australia.

CC40 is shown as an SLV in **Figures 3.8 and 3.9** and includes ST40, ST45, and ST105, with ST40 being the main founder clone for this group. CC40 included strains that were isolated at a milk powder processing facility in Australia (Craven *et al.*2010) plus one strain that was isolated from PIF purchased from Russia (Muytjens *et al.*1988).

CC99 included ST99 and ST101. **Figures 3.8 and 3.9** show that ST99 and ST101 are SLVs. They differ in the *ppsA* allele. Specifically, ST99 has the MLST profile 3-8-52-54-21-65-73, whereas ST101 has the MLST profile 3-50-52-54-21-65-73. CC99 was isolated from the drying tower of the milk powder factory in Germany.

There were 2 SLVs CC8 clusters that included ST124, and ST113. These were found in two isolates from PIF samples from two countries: Uruguay and The Netherlands. ST113 was isolated from the floor of a milk powder plant in Australia. This strain could not be pulsotyped (Craven *et al.*2010).

There was one SLV, CC119, among the 8 STs identified in *C. turicensis* strains. This CC included ST120 and ST119, which differ in the *glnS* allele. There was one QLV cluster in the two *C. turicensis* STs, ST126 and ST127. The QLV differed in the *atpA, glnS, gltB*, and *ppsA*, alleles.

Figure 3.9 shows that 4 STs, ST4, ST99, ST101, and ST33, were identified in strains isolated from a drying tower. Two STs, ST1 and ST4, were isolated from a roller dryer. The STs identified in PIF in 1988 included ST4, ST1, ST8, ST124, ST64, ST45, ST65, ST13, ST3, and ST57 for *C. sakazakii*; ST7 and ST62 for *C. malonaticus*; and ST49 for *C. muytjensii.* This reveals that the predominant *C. sakazakii* STs that were isolated from PIF purchased from 13 countries were also isolated from six milk powder factories in Australia and Germany.

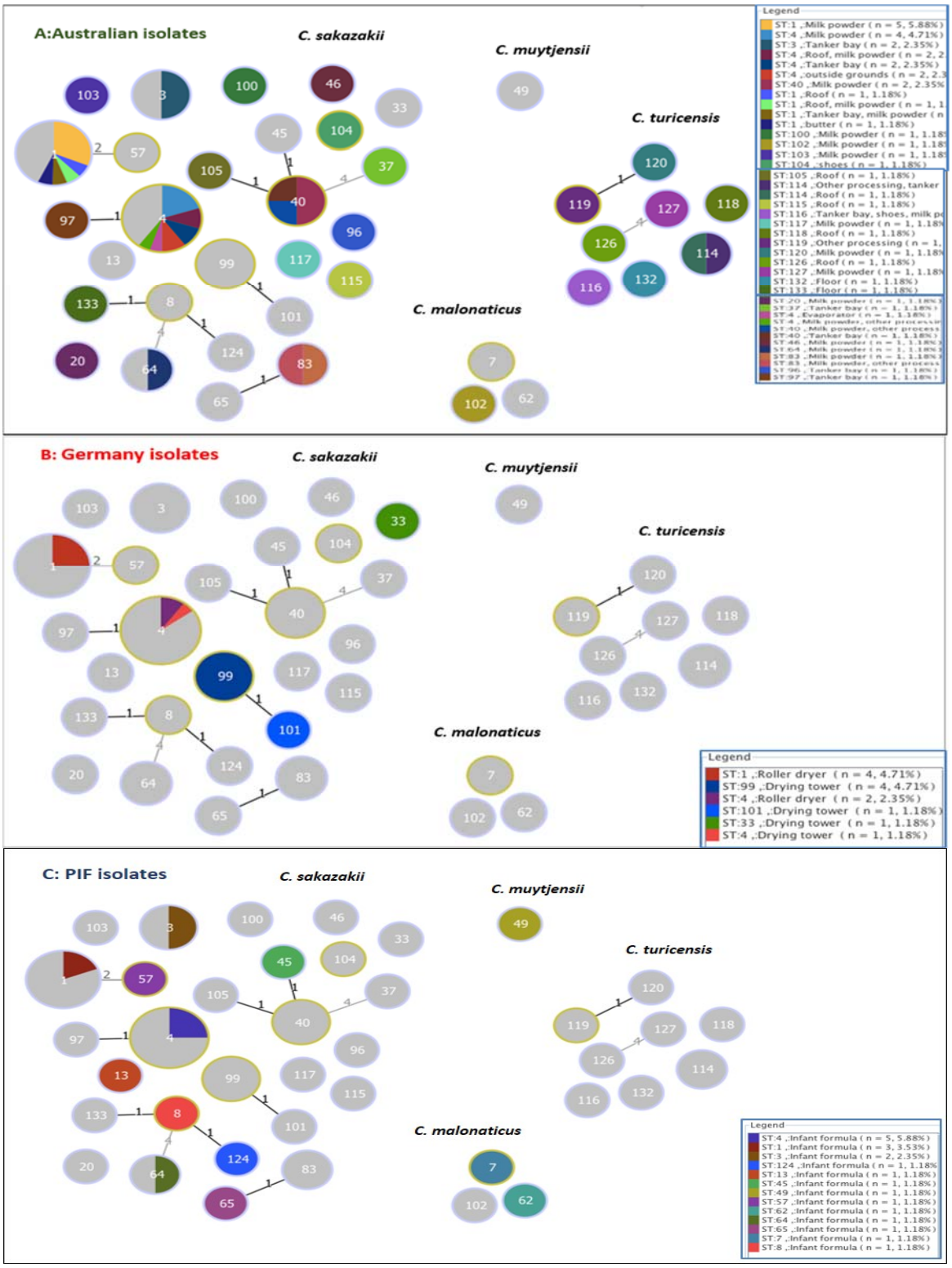


Figure 3. 8 A snapshot of the MLST data of 85 *Cronobacter* isolates generated using goeBURST software (Francisco *et al.* 2012).

The diagram shows the diversity of the sequence type (STs) based on the species and the source of the three collection strains (A, B, and C). The quadruple level was selected for the output for quarter locus variants (QLV). The dominant STs are denoted by circles with larger diameters and dark shade shows the stability of definite STs e.g. ST4 and ST1 complex. Clusters of linked isolates correspond to CCs as shown in **Tables 3.2, 3.3,** and **3.4.**

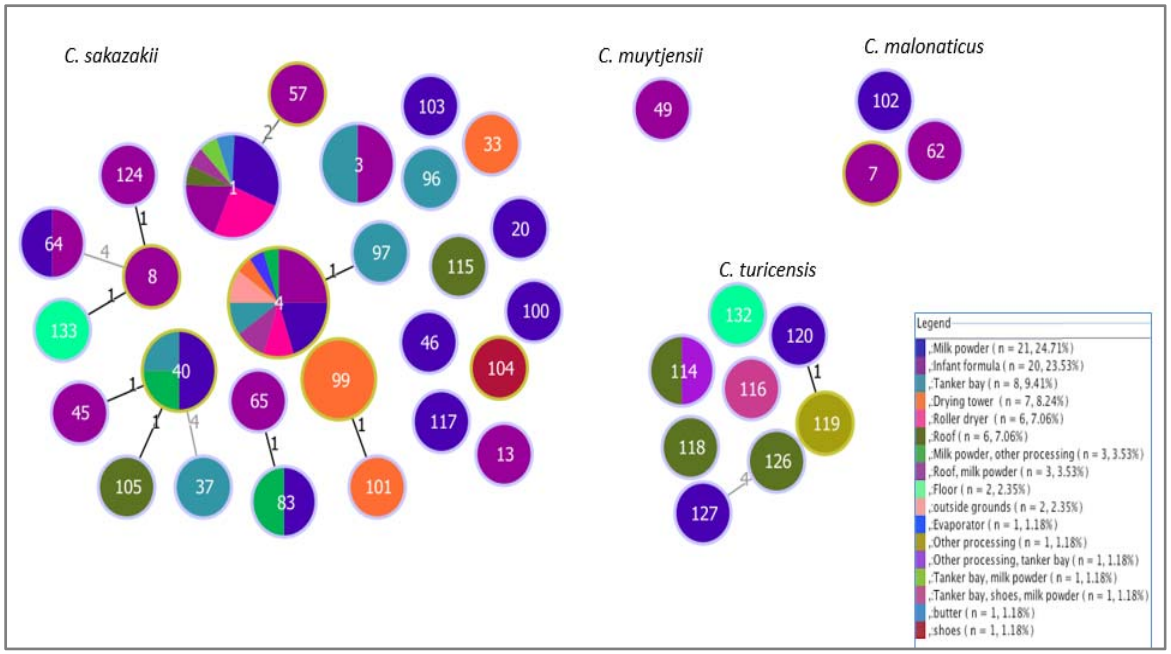


Figure 3. 9 A snapshot of the MLST data of 85 *Cronobacter* isolates generated using goeBURST software (Francisco *et al*. 2012).

The diagram shows the clonal complexes (CCs) and the source of the strains based on the species in the three collection strains. The quadruple level was selected for the output for quarter locus variants (QLV). The dominant STs are denoted by circles with larger diameters. Clusters of linked isolates correspond to CCs as shown in **Tables 3.2, 3.3,** and **3.4.**

Table 3. 6 The most frequently identified clonal complexes according to the MLST profiles of the 85 *Cronobacter* isolates in this study.

Bacterial species	Sequence type	Clonal complex	Number of isolates			Total number of isolates	Percentage (%)
			Muytjens *et al.* (1988)	Craven *et al.* (2010)	Jacobs *et al.* (2011)		
C. sakazakii	4	4	5	12	3	20	24
	96	4	0	1	0	1	1
	97	4	0	1	0	1	1
	1	1	3	9	4	16	19
	117	-	0	1	0	1	1
	40	45	0	4	0	4	5
	105	45	0	1	0	1	1
	99	99	0	0	4	4	5
	101	99	0	0	1	1	1
	3	3	2	2	0	4	5
	8	8	1	0	0	1	1
	124	8	1	0	0	1	1
	133	8	0	1	0	1	1
	83	83	0	2	0	2	3
	65	83	1	0	0	1	1
	45	45	1	0	0	1	1
	37	-	0	1	0	1	1
	13	13	1	0	0	1	1
	103	-	0	1	0	1	1
	115	-	0	1	0	1	1
	104	-	0	1	0	1	1
	33	-	0	0	1	1	1
	46	-	0	1	0	1	1
	57	1	1	0	0	1	1
	64	64	1	0	0	1	1
	20	0	0	1	0	1	1
	100	0	0	1	0	1	1
Total	24	17	17	43	13	72	85
C. turicensis	114	-	0	2	0	2	3
	116	-	0	1	0	1	1
	118	-	0	1	0	1	1
	119	-	0	1	0	1	1
	120	-	0	1	0	1	1
	126	-	0	1	0	1	1
	127	-	0	1	0	1	1
	132	-	0	1	0	1	1
Total	-	-	0	9	0	9	11
C. malonaticus	102	-	0	1	0	1	1
	7	7	1	0	0	1	1
	62	-	1	0	0	1	1
			2	1	0	3	4
C. muytjensii	49	-	1	0	0	1	1
Total	-	-	20	53	13	85	100

ST, sequence type
CC, clonal complex

3.6 Discussion

MLST is a fast and reliable technique that was applied recently for analysing the *Cronobacter* genus. Our MLST is based on an analysis of 7 housekeeping genes: *atpD, fusA, glnS*, *gltB*, *gyrB*, *infB*, and *ppsA* (Baldwin *et al.* 2009). MLST, and phylogenetic analysis using MLSA, has revealed the diversity of the *Cronobacter* genus and identified two new species, *C. universalis* and *C. condimenti* (Joseph *et al.* 2012b). A recent MLST study by Joseph and Forsythe (2011) revealed the association of *C. sakazakii* CC4 with neonatal meningitis.

In this chapter, MLST was used to analyse previously un-speciated *Cronobacter* strains (identified as *E. sakazakii* in previous publications) that were isolated from PIF and from milk powder processing plants. The species of these strains were determined and their STs were defined. This study also constructed a phylogenetic tree to analyse these strains based on the MLST data, and the tree revealed the diversity of species in the *Cronobacter* genus as well as the major STs found in PIF and milk powder processing plants. This was the first time that MLST has been used to analyse strains from PIF and milk powder facilities, and this analysis provides important insights that help us understand the predominant species and STs that are commonly found or are persistent in milk powder environments. The results may help develop and improve strategies that minimise contamination by these organisms in dairy production facilities.

3.6.1 General analysis of the strains collection in this study

A total of 85 isolates that were previously identified as *E. sakazakii* were obtained from three separate strain collections. The isolates, which were originally collected between 1988 and 2009 from 14 countries, were analysed using the MLST scheme. The strain collections included 20 available strains from Muytjens *et al*. (1988), 52 strains from Craven *et al*. (2010) (including 3 strains that could not be profiled using PFGE), and 13 strains from Jacobs *et al*. (2011). Three *Cronobacter* species were not isolated from these three collection strains, namely *C. condimenti, C. dublinensis*, and *C. universalis*.

Cronobacter spp. strains that were previously identified as *Enterobacter sakazakii* in PIF and milk powder factories included 39 STs in four out of seven species. The majority of the strains that were identified were *C. sakazakii* (85%), followed by *C. turicensis* (11%), *C. malonaticus* (4%), and *C. muytjensii* (1%) **(Section 3.5.1) (Table 3.6).** In terms of sequence type, the majority of the strains were *C. sakazakii* ST4 (20%) and ST1 (16%). *C. sakazakii* strains that were ST4 accounted for 20/72 isolates, with an additional strain that was ST97 (which belongs to CC4). Moreover, 16/72 *C. sakazakii* strains were ST1 **(Table 3.6). Table 3.6** shows that there were 24 STs that were *C. sakazakii*, 8 STs that were *C. turicensis*, 3 STs that were *C. malonaticus*, and one ST that was *C. muytjensii* **(Section 3.5.1)**. These results illustrate the persistence and appearance of *C. sakazakii* in industrial dairy environments and are in agreement with reports of the predominance of *C. sakazakii* in neonatal infections, with just a few cases associated with *C. turicensis* and *C. malonaticus* (Hariri *et al* .2012; Kucerova *et al.* 2011). Until now, only *C. sakazakii*, *C. malonaticus*, and *C. turicensis* were reported to be associated with neonatal infections (Joseph *et al.*2012b).

3.6.2 PIF isolate analysis

MLST was used to analyse 20/50 strains isolated from PIF cans; these strains were identified previously as *E. sakazakii* by Muytjens *et al.* (1988). We, again, found that the majority (17/20) of the strains were *C. sakazakii*, but 2/20 strains were *C. malonaticus*, and 1/20 was a *C. muytjensii* strain **(Table 3.2).** Muytjens *et al*. (1988) isolated 50 strains from PIF purchased from 35 different countries. These strains were characterized before there was international concern about neonatal infections from the preparation of infant formula, and they were extremely important as they were used as the basis of changes in the microbiological strategies for PIF manufacturers in the Codex Alimentarius Commission (2008). Moreover, the Muytjens *et al*. (1988) study was used in many FAO/WHO risk assessments about the presence of *E. sakazakii* in PIF (FAO/WHO, 2004, 2006, 2008). It should be mentioned that not all of the strains identified in this collection were *C. sakazakii* (Muytjens *et al.* 1988). One strain in this particular strain collection was *C. malonaticus* ST7, which was linked previously to adult infections and found as most common STs in *C. malonaticus* (Joseph *et al*. 2012a; Joseph and Forsythe, 2011; Forsythe *et al.* 2014). There was one *C. muytjensii* strain, strain 530

(ST49), that was isolated from PIF purchased in Denmark (**Section 3.5.2**). Until now, this species has not been associated with clinical infection. One strain that was isolated from PIF from India was re-identified as *E. hormaechei*. It is not the first time that these two species have been misidentified, as previous study re-identified *E. sakazakii* from PIF from The Netherlands as *E. hormaechei* (Townsend *et al.* 2008). Although the majority of the *C. sakazakii* isolates in PIF samples were *C. sakazakii* ST4(**Section 3.5.2) (Table 3.2)**, Muytjens *et al.* (1988) reported that the organism was not detected in any sample at levels greater than 1 cell g^{-1}. Consequently, the preparation of formula should follow good hygienic techniques to reduce the risk of infection and bacterial growth (FAO/WHO, 2006, 2008).

3.6.3 Australian and German isolates analysed by MLST

Fifty-two *Cronobacter* spp. strains were isolated from 5 milk powder manufacturing plants in Australia, and 13 *Cronobacter* spp. strains were isolated from one milk powder manufacturing plant in Germany between 2006 and 2009 **(Figures 3.6 and 3.7)** (Craven *et al*. 2010; Jacobs *et al.* 2011).

These sets of strains were analysed using MLST. Of the 27 STs found in the isolates from 5 milk powder factories in Australia, 17 STs belonged to *C. sakazakii* and 8 STs belonged to *C. turicensis* **(Figure 3.6).** In addition, 5 STs were identified in the strains from the milk powder factory in Germany. Three strains were ST4, and four strains were ST1. This result confirmed the dominance and persistence of these particular STs among the *Cronobacter* spp. strains. Moreover, this study demonstrated that the *C. sakazakii* ST4 in the environment of milk powder factories, such as in the tanker bay, on the roof, on workers' shoes, on the roller-dryer, in the spray-drying area, and in the milk powder itself, was the same lineage that was found during neonatal meningitis outbreaks (Joseph and Forsythe, 2012).

A survey conducted by Proudy *et al.* (2008) showed the persistence of *Cronobacter* in the processing environment area, with 78% of the *Cronobacter* being isolated from the surfaces near the dryer, on the floors of the packaging zones, and in the blender; 12% being isolated from the ingredients; and 10% isolated from the final products. A recent study by Mùller *et al*. (2013) used PFGE and MLST to analyse 19 *C. sakazakii* strains isolated from the raw materials, the PIF production environment,

and the finished products of a factory in Switzerland. They found that ST4 was the most dominant ST among these isolates, followed by ST1, ST83, and ST64. Another study by Fei *et al*. (2014) reported that *C. sakazakii* ST1was the main ST in PIF in China. That study found that specific STs were associated with specific aspects of PIF production: ST1 was isolated from spray-drying areas and from milk powder end products, and ST64 and ST4 were found in the milk powder end products. The results of the studies of Mùller *et al*. (2013) and Fei *et al*. (2014) further support the results presented in this chapter. ST4, ST1, ST64, ST99, ST3, and ST83 were the predominant STs that were identified and that were persistent in milk powder facilities and in PIF and milk pwdered production facilities in Germany and Australia. Power *et al.* (2013) found that one *C. sakazakii* ST4 strain (SP291), which was isolated from a PIF manufacturing plant, was persistent and thermo-tolerant. Yet another *C. sakazakii* strain, HPB5174, which belongs to CC45 has frequently been identified in food processing facilities and in PIF in Ireland (Pightling and Pagotto, 2014). The predominance of CC4 was confirmed by the recent US outbreak in 2011, as CC4 strains were associated with meningitis cases (Hariri *et al* .2013).

A recent study by Cui *et al*. (2015) used MLST to analyse strains collected from various food products and baby food in China between 2010 and 2012. Cui *et al*. (2015) identified 85 STs in 151 strains. The strains were isolated from drinking water, healthy individuals samples, PIF, follow-up formula, noodles, cookies, and vegetables. Most of the isolates were identified as *C. sakazakii* (80.2%) and *C. malonaticus* (16.8%). However, *C. dublinensis* (10.6%), *C. turicensis* (2.0%), and *C. muytjensii* (0.7%) were also identified. The strains belonged mainly to clonal complexes CC1, CC3, CC4, and CC7. Some of the sequence types, such as ST4, ST64, ST7, and ST1, have been reported previously in other countries (Mùller *et al*. 2013; Power *et al.* 2013; Fei *et al*. 2014; Pightling and Pagotto, 2014; Cui *et al*. 2015). This study confirmed our result of the incidences of some STs in the production facilities that might indicated some physiological properties theses STs have to persist in such harsh environments.

3.6.4 PIF contamination

PIF contamination can be due to ingredient contamination or due to contamination from the outside environment during product preparation or delivery (Holý and Forsythe, 2013). The ingredients of PIF include milk, milk products, carbohydrates, soy protein, minerals, fats, vitamins and additives such as fruit powder (Proudy *et al.* 2008: Mùller *et al.* 2013). The FAO/WHO (2004) states that PIF may contain ingredients that are contaminated with *Enterobacteriaceae*, such as starch, which is considered to be at high risk of contamination, as well as other ingredients, such as oils, that are at low risk of contamination. Drinking water is another possible source of PIF contamination. For example, *Cronobacter* spp. have been isolated from drinking water in China (Liu *et al*. 2013). Hariri *et al.* (2013) analysed samples isolated from PIF reconstituted with water that was linked with an incident in Illinois. This strain 1578 (ST111) differed from ST4 at 35/3036 nucleotides (Hariri *et al.* 2013).

MLST and PFGE are two independent genotyping methods that have been applied to *Cronobacter* spp. isolates for many years (Craven *et al.* 2010; Jacobs *et al.* 2011; Mùller *et al.* 2013; Fei *et al.* 2015). However, not all *Cronobacter* strains yield PFGE profiles (Craven *et al*.2010). Within the 7 alleles that are sequenced for MLST, there is no restriction site for *XbaI*; hence the MLST analysis is independent of the PFGE analysis used by Craven *et al*. (2010) and Jacobs *et al*. (2011). Although Craven *et al.* (2010) and Jacobs *et al*. (2011) isolated the strains before the taxonomic revision of the *Cronobacter* genus and used the PFGE technique to profile the strains from 6 milk powder production facilities, this MLST study identified these strains as the correct species and demonstrated the predominance of *C. sakazakii* ST4 among the isolates.

3.6.5 Evaluation study STs and pulsotype

MLST differentiates between strains that differ by just one nucleotide of the 3036 total concatenated nucleotides that are used to construct the phylogenetic tree (Joseph and Forsythe, 2012; Joseph *et al.* 2012b). **Figure 3.5** shows the diversity of all of the *Cronobacter* species and STs identified in this study**(Sections 3.5.6.1** and **3.5.6.2)**. most of the isolates are *C. sakazakii* and comprise just a few STs mostly ST4. PFGE can further distinguish among strains of *Cronobacter* spp. that have a common

ST pattern, as seen in **Figures 3.6** and **3.7.** PFGE is more suitable for epidemiological tracing, while it is less suitable for fast typing or for determining the species of the strains. In this study, MLST clearly separated the *Cronobacter* isolates at the species level and further divided the species into STs.

According to the criteria used to interpret the PFGE pattern, Tenover *et al.* (1995) noted that epidemiologically-related isolates have closely related or indistinguishable fragment patterns. The PFGE studies of the *Cronobacter* spp. strains by Craven *et al*. (2010) and Jacobs *et al*. (2011) used 95% cut-off of similarity of one restriction enzyme, *XbaI*, and 90% cut-off of similarity of two restriction enzymes, *XbaI* and *SpeI* respectively, in order to determine the clonal identity of the isolates. This demonstrates that the strains in this collection are very similar in terms of their PFGE patterns. This was observed in a few strain clusters in which some strains have similar PFGE patterns and also the same STs **(Figures 3.6** and **3.7).** This demonstrated that the MLST scheme is an effective, fast, and reliable typing method for identifying the correct species as well as the subtypes of strains according to STs. This MLST study further shows the value of these strain collections, which should be used by the scientific community and by public health experts to better understand the prevalence of specific clones and to help improve quality control of the final product in the milk powder industry. The results of the MLST analysis of the isolates, which are based on sequence relationships, cannot be obtained from a PFGE approach. PFGE and MLST analyses of bacterial DNA are completely different as there are no restriction enzyme sites for the *Xba*I within the 7 MLST loci (Hariri *et al.* 2013)

3.6.6 The goeBURST analysis of the environmental isolates

The goeBURST analysis in **Figures 3.8 and 3.9** shows that these environmental isolates included 39 STs; 27 of the STs were defined as *C. sakazakii*. It was particularly interesting that 14/27 STs represented 9 CCs (CC1, CC3, CC4, CC13, CC45, CC64, CC83, CC100, and CC8; Forsythe *et al.* 2014) that were the same as the CCs of clinical isolates in the MLST database. Fie *et al.* (2014) performed MLST analysis of samples from PIF and milk production facilities, and the major STs that were identified were ST4, ST1, ST8, ST45, ST64, and ST83 for *C. sakazakii* and ST7 for *C. malonaticus*. The current study identified 3 *C. malonaticus* strains, namely ST7, ST102, and ST62.

3.6.7 The incidence of ST4 in environmental isolates

Joseph *et al.* (2012) demonstrated, by whole genome sequencing, that the predominant STs, *C. sakazakii* ST1 and ST4, are not related, and Joseph and Forsythe (2011) reported that it is unclear why *C. sakazakii* ST4 is associated with neonatal infections. They hypothesized that the association could be due to physiological factors that resulted from increased neonatal exposure to the organism as well as to the presence of particular virulence traits. Importantly, the result of this study showed that some strains with certain STs are found both in the production facility and in the final products. This may occur if the air filter is the port of entry for contaminating the products in the facilities, as reported by Mullane *et al.* (2008) and by Jacobs *et al*. (2011), who studied possible contamination pathways in PIF facilities.

Many studies show that this organism can persist in these environments because of its ability to survive spray drying, desiccation, and osmotic stress (Breeuwer *et al.*2003; Arku *et al.*2008; Osaili and Forsythe, 2009). Arku *et al.* (2008) reported that *Cronobacter* strains could be detected after the spray-drying process at 160°C, although there was reduction in the viable count of the organism.

Jacobs *et al*. (2011) isolated *Cronobacter* strains from one German factory; to manufacture the powdered milk, the factory used a temperature of 160°C in the roller-drying step, 210°C to pre-heat the spray dryer and 120°C as the drying temperature.

Spray drying as part of the milk powder production is not considered as a sterilization step and the final product of PIF after spray drying is not a sterile product. This is supported by many studies that showed the use of spray drying to produce lactic acid bacterial cultures and dehydrated probiotic bacteria to ensure it survival and its recovery after desiccation (Riveros *et al*. 2009; Peighambardoust *et al*. 2011). Ananta *et al*. (2005) showed that in reconstituted skimmed milk, the microbial survival rate of *Lactobacillus rhamnosus* was 60% in the spray drying process. However, the effects of spray drying on the probiotic bacteria differed depending on various matters and differed among the same species in the population as stated in the following paragraphs.

The bacterial cells are exposed to heat stress, dehydration, oxygen exposure, and osmotic stress (Brennan *et al.*1986; Teixeira *et al.*1997; Meng *et al.* 2008). Spray drying can weaken the cell membrane and increase cell permeability, resulting in the leakage of intracellular components from the cell into the surrounding environment. It may also affect DNA and RNA and disrupt metabolic activity (Teixeira *et al.*1997; Meng *et al.*2008). Bacteria respond to changes in their immediate environment by metabolic preadaptation, which results in a cellular state that shows improved resistance to the particular change or stressor (Pichereau *et al*. 2000; Meng *et al.* 2008). Also, this might led to metabolic pre-adaptation that can cause cross-protection (Kim *et al*. 2001; Meng *et al.* 2008). Moreover, during the spray drying process drying medium experience a high temperatures in a short time, which lead to granules formation.

It has been found that bacteria used cross-protection mechanisms to protect themselves from environmental changes. It has been identified as either one of two defence systems which different bacteria use. The first system is induced by physical stress, such as heat shock or a sub-lethal dose of a chemical, and it allows the bacteria to survive that particular challenge (Pichereau *et al.* 2000; Desmond *et al.* 2001; Gouesbet *et al.* 2001; Meng *et al.* 2008). The second system is a more a general mechanism that allows the cells to survive very diverse ecological stresses without exposure of the cells to a particular stress (Pichereau *et al.* 2000; Desmond *et al.* 2001; Gouesbet *et al* .2001; Meng *et al.* 2008). This might explain the predominance of the *C. sakazakii* ST4 in both isolates from PIF and milk powder factories as well as in clinical isolates (Joseph and Forsythe, 2011; Hariri *et al.* 2013).

Many factors have been found to support bacterium resistance on the spray drying. In probiotics bacteria were found to be adapted to salt or heat which led to enhancing their heat resistance during the spray drying process. For instance, pre-adapted *L. paracasei* strains exposed to 0.3 M NaCl showed greater heat resistance to spray-drying temperatures (outlet temperatures of 95–100°C) compared to non-adapted control cells (33.46 ± 2.3% survival vs. 8.27 ± 4.42% survival, respectively) (Desmond *et al.* 2001; Meng *et al.* 2008). In addition, they found that the cell count of *L. paracasei* that were pre-adapted to heat was 18-fold greater than the control in reconstituted skimmed milk during spray drying (outlet temperatures of 95–105°C) (Desmond *et al*. 2001; Meng *et al.*2008).

However, the incidence of bacteria in spray and roller dryers may differ between various species and on their STs. Study by Gardiner *et al*. (2000) found that bacterial species differ with regard to their tolerance to spray drying. Illustrating this, *L. paracasei* survives better than *L. salivarius* in similar spray drying settings which suggested that this different might be due to the more heat tolerance of strain *L. paracasei* compared to that of *L. salivarius* (Gardiner *et al.* 2000; Meng *et al.* 2008).

Milk powder isolates from spray drying and roller drying are highly important as the strains` exposure to heat and dryness stress conditions during the milk powder production might increase their heat and desiccation tolerance after reconstitution of the milk powder formula. On the other hand , the concentration of the bacteria must be reduced in the PIF at low level avoiding any growth after reconstituting of the milk powder. According to FAO/WHO (2004), contaminated PIF with low levels of *Cronobacter* are considered hazardous given the probability that bacteria will grow during formula preparation and while the reconstituted formula sits before being fed to the infant. Notably, infant food manufacturing plants must state the methods they use to reduce the concentration and occurrence of *Cronobacter* in both the industrial environment and in PIF itself (FAO/WHO, 2004).

Cronobacter infections in neonates can have devastating consequences. Because such infections are associated with the consumption of contaminated powdered infant formula, the Food and Agriculture Organisation-World Health Organisation (2004 and 2006) recommended that molecular typing protocols be developed to better recognize *Cronobacter* species and to reduce or eliminate deadly outbreaks. All of the known *Cronobacter* species have been subjected to MLST analysis, and the *Cronobacter* PubMLST database now includes the MLST profiles of strains isolated from all known international outbreaks. Accordingly, laboratories can now efficiently analyse the interspecies diversity of *Cronobacter*, and strains can be characterized according to their source and according to virulence factors. Notably, once strains that were identified in the past are analysed by MLST, the PubMLST database enables surveying and re-analysis of historic outbreaks and cases. Incorrect taxonomic identification can cause loss of these strains (Forsythe *et al.* 2015).

Molecular typing of *Cronobacter* isolates has helped researchers and clinicians better understand the diversity of the genus and has been useful for developing additional MLST schemes. The initial scheme used 7 loci (3036 nt). In contrast, a newer MLST method termed ribosomal protein-MLST (rMLST) analyses 53 loci, while COG-cgMLST analyses about a third of the genome (1865 loci). In addition, researchers can develop novel MLST approaches that utilise the data in the *Cronobacter* PubMLST database (Forsythe *et al*. 2014). The use of whole genome sequencing and MLST analysis supports the formal recognition of the *Cronobacter* genus as one that includes 7 distinct species. Previously, these 7 species were considered a single species, *Enterobacter sakazakii*, based on an early typing method. Hence, MLSA analysis based on housekeeping genes, including the *fusA* gene, is a useful tool for the taxonomic analysis of Enterobacteriaceae and is more effective than phenotyping for *Cronobacter* speciation analysis (Joseph and Forsythe, 2012).

This study demonstrated that *C. sakazakii,* particularly ST4, is the predominant species in the milk powder production environment. The results have important implications for PIF and milk powder production facilities because they showed the number of *Cronobacter* spp. isolates from this PIF and the milk powder factories as well as the predominance of *C. sakazakii* ST4 among three collection which identified previously as meningitis signature (Joseph and Forsythe, 2011). It was found that ST4 was the most prevalent STs in this study which confirmed the previous observation of the predominance of this ST4 not just in the *C. sakazakii* isolates but also in the entire genus (Joseph *et al.* 2012a). This finding increases our understanding of the distribution of important strains of the organism and can help control the spread of pathogenic organisms in the dairy industry. To accomplish this goal, the infant food industry should use an effective environmental inspection programme and use techniques that identify Enterobacteriaceae rather than "coliform" as standard hygiene control practices in PIF factories (FAO/WHO, 2004).

Recently, increased knowledge of the genome studies has led to advances in genomic analysis of strains isolated from PIF and from manufacturing plants, and this analysis may provide insights into

the potential occurrence of resistance genes and the survival of these special STs. This will be discussed in Chapter 4, which focuses on the physiology and survival of this organism.

CHAPTER 4: Determination of the physiological and virulence-related factors that affect the persistence of neonatal meningitis *C. sakazakii* strains in different environments and hosts

4.1 Introduction

Many studies have found a direct association between *Cronobacter* spp. outbreaks in neonatal intensive care units and the use of reconstituted powdered infant formula (PIF). In these outbreaks, the PIF was contaminated by *Cronobacter* spp. that may have been introduced during PIF preparation or administration or even at the PIF factories themselves (Van Acker *et al.* 2001; Himelright *et al.* 2002; Caubilla-Barron *et al.* 2007; CDC, 2011). FAO/WHO (2008) stated that most reported *Cronobacter* infection cases are in adults; nevertheless, neonates and infants account for 40%–80% of the mortality tied to such infections, mainly due to meningitis, septicaemia, and necrotising enterocolitis (NEC) (Joseph and Forsythe, 2012). Townsend *et al.* (2007, 2008) found that *Cronobacter* can attack and invade intestinal cells and can survive in macrophage cells.

The ability of these organisms to persist in harsh environments is illustrated by their ability to survive the spray drying, osmotic stress, and desiccation steps involved in PIF processing factories (Breeuwer *et al.* 2003; Arku *et al.* 2008; Osaili and Forsythe, 2009). Specifically, *Cronobacter* show unusually high resistance to heat, drying, and acid stress compared to other Enterobacteriaceae members (Nazarowec-White and Farber, 1997; Breeuwer *et al.* 2003; Edelson-Mammel *et al*. 2005; Dancer *et al.* 2009; Jaradat *et al.* 2014). Many studies have reported the isolation of *C. sakazakii* from multiple milk powder and PIF manufacturing facilities (Mullane *et al.* 2007; Craven *et al*. 2010; Jacobs *et al.* 2011), and one study showed that *Cronobacter* spp. can survive more than two years in desiccated PIF (Caubilla-Barron and Forsythe, 2007). Many researchers have studied the recovery and persistence of *Cronobacter* spp. in infant formula after desiccation for different time periods and under many stress conditions (Caubilla-Barron and Forsythe, 2007; Al-Holy *et al.* 2008; Osaili *et al*. 2008).

Thermal tolerance is an important trait that can affect the survival of the organism in the environment. Studies have shown that heat resistance differs among *Cronobacter* strains (Breeuwer *et al*. 2003; Iversen *et al*. 2004b) and that there is a link between bacterial desiccation and thermal tolerance (Osaili *et al.* 2008; Shaker *et al.* 2008). *Cronobacter* spp. are the most heat-resistant Enterobacteriaceae in dairy products, as evidenced by their survival during the powder milk production procedure (Nazarowec-White and Farber, 1997). In many microorganisms, ABC transporters, including *yehZYXW*, *proP*, *proU*,

and *opuCABCD*, are involved in bacterial osmoprotection (Frossard *et al*. 2012; Finn *et al*. 2013; Yan *et al*. 2013). In addition, Feeny and Salter (2011) identified osmotolerance genes in *C. sakazakii* that play a significant role in the adaptation to environmental stress both in the host and in the environment. Gastric tract acidity is the first line of host defence against food-borne pathogens. After surviving desiccation and heat exposure in the production process, bacteria that are ingested via contaminated PIF are exposed to the highly acidic environment of the stomach. Notably, the intestinal flora in infants is influenced by feeding type (Hurrell *et al.* 2009). Hurrell *et al.* (2009) investigated the influence of neonatal stomach acidity on different Enterobacteriaceae spp. in breast milk and PIF. The gastric pH prior to breast-feeding was between pH 2.5 and pH 4.3, while it was between pH 3.5 and pH 4.3 for other feeding types, such as PIF.

Serum resistance refers to the ability of bacteria to resist (survive) the human complement system which is part of the innate adaptive immune system (Gondwe *et al*. 2010). Complement activity and the bactericidal effect of serum may protect hosts from the invasion of serum-sensitive bacterial pathogens (Gondwe *et al.* 2010). Franco *et al.* (2011) identified the gene that is responsible for serum resistance, termed *Cpa*, on the pESA3 plasmid in *C. sakazakii* strain BAA-894. This gene encodes an outer membrane protease. There are many additional factors that may mediate serum resistance, such as lipopolysaccharides and capsule proteins (Leying *et al.* 1990; Williams *et al.* 2001).*Cronobacter* spp. produce extracellular polymers that result in highly mucoid colony formation (Hurrell *et al*. 2009a; Hurrel *et al.* 2009b; Joseph *et al.* 2012b). Indeed, capsular formation on the bacterial cell surface may play a major role as a protective factor against environmental stress and bacterial pathogenicity, including protection from desiccation. Capsular formation may also mediate serum resistance (Williams *et al.* 2001; Caubilla Barron and Forsythe, 2007). Moreover, the capsulated *C. sakazakii* strains are more resistant to desiccation stress than are other Enterobacteriaceae (Caubilla Barron and Forsythe, 2007). The bacterial flagellum is an organelle that primarily aids motility. Joseph *et al.* (2012) found that the *C. sakazakii* genome encodes a number of flagellum genes (ESA_01248–61 and ESA_02264–77) . A recent study reported that *C. sakazakii* strains that possess flagella are involved in biofilm formation and in adhesion to epithelial cells via Caco-2 tissue culture assay (Hatmann *et al*. 2010).

4.2 Aims

Joseph and Forsythe (2011) found that meningitis isolates are frequently *C. sakazakii* ST4 strains. Their study demonstrated that *C. sakazakii* ST4 is a highly stable clone with considerable potential for causing neonatal meningitis. The reasons underlying the predominance of *C. sakazakii* ST4 were unclear, although they suggested that the environmental fitness of this organism might play a role. The MLST study in Chapter 3 characterized the predominant *Cronobacter* isolates in 6 milk powder processing manufacturing plants and in PIF as *C. sakazakii* ST4 and ST1 strains. The strains that were isolated from PIF and from the roof, roller dryers, and tanker bays of manufacturing plants were of special interest. Chapter 4 reports the results of experiments that investigated physiological traits that are related to the virulence traits of *C. sakazakii* CC4 and non-CC4 strains and that affect its persistence and adaptation to changes in the environment.

The following were investigated:

- **The effects of desiccation**. *C. sakazakii* can cause many neonatal infections, such as meningitis, septicemia, necrotizing enterocolitis, and bacteremia, especially in early-born babies **(**Van Acker *et al*. 2001; Himelright *et al*. 2002; Caubilla-Barron *et al*. 2007).PIF is a possible route of neonatal infection, and the predominance of *C. sakazakii* CC4 in PIF may be due to its ability to recover from desiccation. Accordingly, this study examined its persistence after desiccation and the recovery of sub-lethally injured cells of *C. sakazakii* CC4 strains in infant formula.
- **Heat tolerance after desiccation**. The milk-processing environment usually includes exposure to high-heat step or steps that could induce the expression of stress response proteins in the bacteria. Therefore, this study studied the effects of desiccation on thermal tolerance in *C. sakazakii* CC4 and non-CC4 strains. This experiment can help to understand *C. sakazakii* behaviour in stressful conditions and monitoring it in the powder milk factory processing environment.
- **Acid resistance**. To mimic the adaptation of bacteria to the acidic conditions of the neonatal gastrointestinal tract after milk consumption, in this study acid resistance assay was performed in which the bacteria were exposed to pH 3.5, which is representative of the pH of the neonatal

stomach (Hurrell *et al*. 2009a). Infant formula was adjusted to pH 3.5 with HCl, and the survival growth curves of *C. sakazakii* strains were determined at 37°C.

- **Serum resistance**. This is defined as the ability of bacteria to resist being killed by the innate immune system and the complement system (Gondwe *et al.* 2010). This study examined and compared the serum resistance of *C. sakazakii* strains in representative CC4 and non-CC4 isolates. In addition, the serum resistance gene, *cps* was analysed in available 38 *C. sakazakii* genome sequences.
- **Bacterial adaptation to environmental stress and host**. This study examined physiological traits for associations with bacterial adaptation to environmental stress. These traits included factors involved in the interactions of the cell wall with the environment, since they might affect the environmental fitness of the bacteria. Such factors are important due to their roles in mediating resistance to the environment and host. These factors include motility, haemolysis on blood agar and capsule production.
- **Genes related to physiological persistence.** This study used online tools to analyse and compare bacterial genomic data to determine the presence or absence of genes, such as carbon starvation genes, carotenoid genes, osmotolerance associated genes and capsule biosynthesis and regulator genes, that are related to physiological persistence. Specifically, the genomic analyses performed using BLAST in *Cronobacter* MLST database.

4.3 Materials and methods

The methods, and the culture media and culture conditions used in the experiments in this chapter are described in Chapter 2 Materials and Methods (**Section 2.5, 2.6** and **2.8).**

4.4 Bacterial strains used in this study

Thirteen *C. sakazakii* strains were used in the physiological assays which included: haemolysis assay, motility test, capsule production assay, determination of sub-lethal injury during desiccation, the effects of desiccation on tolerance to dry heat, acid resistance assay, and serum resistance assay.

Table 4. 1 Details of the *C. sakazakii* strains used in the physiological assays

Bacterial species	NTU ID	ST[b]	Clonal complex[a]	Source	Year	Country	Details
C. sakazakii	721	4	4	Clinical	2003	USA	Neonate aged 2 weeks; Meningitis
C. sakazakii	1221	4	4	Clinical	2003	USA	Neonate aged less than 1 month; meningitis adverse neurological outcome
C. sakazakii	701[c]	4	4	Clinical	1994	France	Peritoneal fluid isolate, fatal NECIII infant case.
C. sakazakii	1533[d]	4	4	Environment	2006	Germany	Milk powder drying tower
C. sakazakii	1537[d]	4	4	Environment	2009	Germany	Roller dryer powder
C. sakazakii	1542[d]	4	4	Environment	2009	Germany	Roller dryer
C. sakazakii	1587	109	4	Clinical	2000	Israel	baby girl, 36 week gestation, C-section. Fed infant formula. . CSF isolate
C. sakazakii	658	1	1	Environment	2001	USA	Non-infant formula
C. sakazakii	1536[d]	1	1	Environment	2009	Germany	Roller dryer
C. sakazakii	680	8	8	Clinical	1977	USA	Spinal fluid isolate
C. sakazakii	1	8	8	Clinical	1980	USA	Throat isolate from child (ATCC 29544)
C. sakazakii	520	12	12	Clinical	1983	Czech Republic	Details unknown
C. sakazakii	696	12	12	Clinical	1994	France	Faecal isolate. NECII infant
Citrobacter koseri	48	6	-	-	-	USA	Comparative control organism

a: Clonal complex (CC), this included ST4 and ST109.b: Sequence type (ST).c: French outbreak isolates (Caubilla-Barron *et al.* 2007)
d: Strains isolated from milk powdered factory in Germany (Jacobs *et al.* 2011), NECII: necrotizing enterocolitis. CSF: cerebrospinal fluid. USA: United States of America.-: not available.

Table 4. 2 Details of the *C. sakazakii* strains used in the genomic analysis.

NTU ID (International culture collection code)	ST[a]	Country of origin	Source	Year of isolation
1	8	USA	Clinical (throat)	1980
4	15(CC4)[b]	Canada	Clinical	1990
5	8	Canada	Clinical	1990
6	4	Canada	Clinical	2003
20	4	CR	Clinical (faeces)	2004
140	40	Unknown	Non-clinical (spice)	2005
150	16	Korea	Non-clinical (spice)	2005
377	4	UK	Milk powder	1950
520	12	Czech Republic	Clinical	1983
553	4	Netherlands	Clinical	1977
557	4	Czech Republic	Clinical	1979
558	4	Netherlands	Clinical	1983
658 (ATCC BAA-894)	1	USA	Non-infant formula	2001
680	8	USA	Clinical	1977
696	12	France	Clinical	1994
694	4	France	Clinical	1994
701	4	France	Clinical	1994
721	4	USA	Clinical (CSF)	2003
767	4	France	Clinical (trachea)	1994
730	4	France	Clinical	1994
978	3	UK	Clinical (internal feeding tube)	2007
984	3	UK	Clinical (internal feeding tube)	2007
1218	1	USA	Clinical (CSF)	2001
1219	4	USA	Clinical (CSF)	2002
1220	4	USA	Clinical (CSF)	2003
1221	4	USA	Clinical (CSF)	2003
1225	4	USA	Clinical (blood)	2007
1231	4	NZ	Clinical (faeces)	2004
1240	4	USA	Clinical (CSF)	2008
1249	31	UK	Clinical	2009
HPB5174	45	Ireland	Powdered infant formula factory	Unknown
SP291	4	Ireland	Powdered infant formula factory	2012
ES15	125	Korea	Whole grain	Unknown
1533	4	Germany	Environment(Drying milk powder tower)	2006
1536	1	Germany	Environment(Roller dryer)	2009
1537	4	Germany	Environment(Roller dryer)	2009
1542	4	Germany	Environment(roller dryer)	2009
1587	109(CC4)[b]	Israel	Clinical	2000

[a]:Sequence types (ST).
[b]:Clonlal complex 4 (CC4), this included ST4, ST15 and ST109.
CR: Czech Republic; NZ: New Zealand; UK: United Kingdom; USA: United States of America

4.5 Results

4.5.1 Determination of sub-lethal injury during *C. sakazakii* desiccation

A recovery assay was used to determine the effects of desiccation on 14 strains of bacteria from different sources, including 13 *C. sakazakii* strains plus *Citrobacter koseri* strain 48 (as a comparison control strain). Briefly, the bacteria were incubated overnight at room temperature (20–25°C), and cells were counted after 18 hours of culture at 37°C on VRBGA selective media and on TSA media as described in Chapter 2 (**Section**s **2. 8. 5**). All of the strains showed greater recovery on TSA ($P<0.01$) than on VRBGA (**Figure 4.1**). The differences in recovery after desiccation reflect the number of cells that were sublethally injured during the desiccation procedure. The viability of strains that were desiccated overnight in infant formula was reduced by up to 3.5 $\log_{10}$ CFU/ml on TSA and by up to 5.3 $\log_{10}$ CFU/ml on VRBGA.

4.5.1.1 Recovery of *C. sakazakii* CC4 and non-CC4 strains on TSA media after desiccation

After desiccation, all of the *C. sakazakii* CC4 and non-CC4 strains recovered on TSA media, showing decreases in viability of 0.66 to up to 3.58 $\log_{10}$ CFU/ml. There was no significant difference in the desiccation resistance of CC4 versus non-CC4 strains in terms of their recovery on TSA ($P>0.05$). However, four strains, 701 (CC4), 696 (ST12), 1221(CC4) and 1533 (CC4), showed better recovery on selective media suggesting higher resistance to desiccation (medium reduction) than the other strains, with 0.60, 0.74, 1.07 and 1.66 $\log_{10}$ CFU/ml reductions in viability, respectively (**Figure 4.1**). In addition, strain 658 (CC1) showed a greater reduction in viability after desiccation of 3.58 $\log_{10}$ CFU/ml. In general, most *C. sakazakii* strains, 721, 1537, 1542, 1587, 658, 1536, 680, and 520, showed highly reduced cell counts (sensitivity to desiccation) of 1.83 to 3.58 $\log_{10}$ CFU/ml. *C. koseri* strain 48 showed a reduction of viability of 1.40 $\log_{10}$ CFU/ml (**Figure 4.1**).

4.5.1.2 **Recovery of *C. sakazakii* CC4 and non-CC4 strains on VRBGA media after desiccation**

After desiccation, all of the *C. sakazakii* strains recovered on VRBGA media, showed decreases in viability of 2. 14 to 5. 23 $\log_{10}$ CFU/ml. There was no significant difference between the desiccation resistance of CC4 and non-CC4 strains in terms of their recovery on this medium (P>0. 05). However, strain 701 and strain 1533 showed better recovery on selective media suggesting higher resistance to desiccation than the other strains, with 2. 14 and 2. 33 log10 CFU/ml reductions in viability on VRBGA media (**Figure 4.1**). In addition, strain 1542, strain 1587, strain 658, strain 680, and strain 721 showed considerable sensitivity on VRBGA, with cell count reductions of 4. 34 to 5. 32 $\log_{10}$ CFU/ml (**Figure 4.1**).

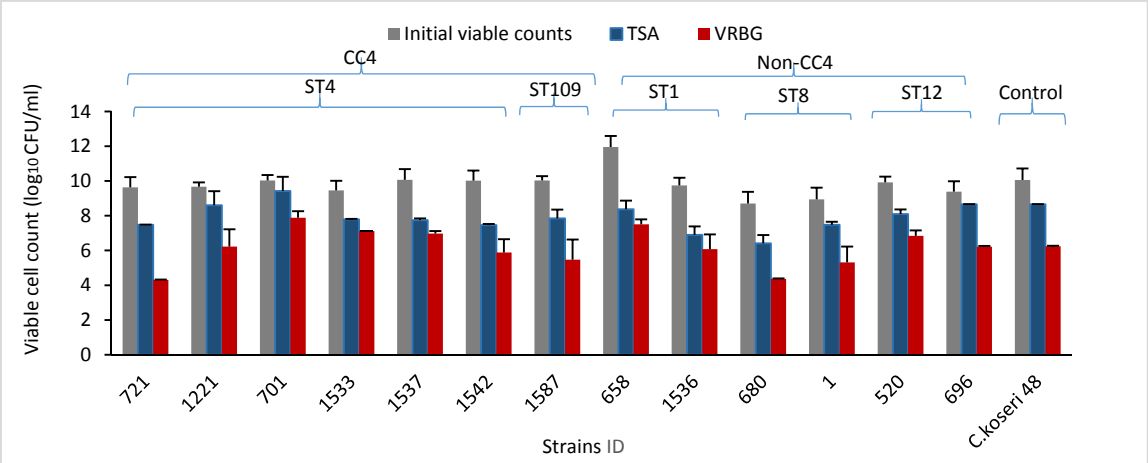
4.5.1.3 **Sub-lethal injury of cells after desiccation**

To determine the extent to which *C. sakazakii* cells were sublethally injured, the log difference was calculated (in CFU/ml) between cell recovery on TSA versus recovery on VRBGA. The number of sublethally injured cells varied in the different strains. There were no significant differences between CC4 and non-CC4 (ST1, ST8, and ST12) strains with regard to the number of sublethally injured cells recovered after desiccation (P>0.05) (**Figure 4.1**). Overall, most of the strains showed a range of sublethally injured cells of 0.66 to 2.55 $\log_{10}$ CFU/ml. However, strain 721 (ST4) showed the most sublethally injured cells, with a 3.16 $\log_{10}$ CFU/ml reduction in cell viability. In addition, strains 701, 1533, 1537, 658, 1536, 1542 and 520 showed fewer injured cells (i.e. they showed resistance or high resistance to desiccation; **Table 4.17**). This included all of the environmental strains but also some of the clinical strains. Specifically, there were reductions in viability of 1.54 $\log_{10}$ CFU/ml for strain 701(ST4), 0.67 $\log_{10}$ CFU/ml for strain 1533 (ST4), 0.77 $\log_{10}$ CFU/ml for strain 1537 (ST4), 0.87 $\log_{10}$ CFU/ml for strain 658 (ST1), 0.82 $\log_{10}$ CFU/ml for strain 1536 (ST1), 1.9 $\log_{10}$ CFU/ml for strain 1542(ST4) and 1.25 $\log_{10}$ CFU/ml for strain 520 (ST12). (**Figure 4.1).**

The number of injured cells was high (moderately sensitive or sensitive to desiccation) for *C. sakazakii* clinical isolate strains 721 (ST4), 1221 (ST4), 1587 (ST4), 680 (ST8), 1 (ST8), and 696 (ST12), which

had reductions in their viability (injured cells) of 3.16, 2.38, 2.38, 2.05, 2.17 and 2.44 $\log_{10}$ CFU/ml, respectively. The reduction in viability (injured cells) of *C. koseri* strain 48 was 2.40 $\log_{10}$ CFU/ml (**Figure 4.1**).

An unpaired student's t-test was performed to compare the effect of media type on the recovery of the cells after desiccation. There was a significant difference in the recovery of the strains on TSA medium versus VRBGA medium ($P<0.001$). Strains 721, 1537 and 1542 showed significant reductions in viability on VRBGA compared to TSA ($P<0.05$). On the other hand strains 1533 (ST4) and 696 (ST12) showed less reductions in the recovery of the strains on VRBGA compared to TSA ($P<0.001$). For the other strains, no differences were observed.



ST: sequence type
CC4: Part of clonal complex 4; which includes ST4 and ST109.

Figure 4. 1 Viability counts of sub-lethally injured *C. sakazakii* cells that recovered after desiccation.

Recovery was measured on TSA and VRBGA media at 37°C. *C. koseri* strain 48 was used as a comparison control. The data are presented as means ± standard deviation of triplicate counts of at least two independent experiments. The initial inoculum was ~10^{10} CFU/ml for the desiccated culture.

4.5.2 Gene responses in *C. sakazakii* desiccation survival

4.5.2.1 Genes involved in desiccation survival

Yan *et al.* (2013) identified desiccation-related genes that are encoded by yih operons in *C. sakazakii* SP291. These included the *yihUTRSQVO* gene cluster, which plays roles in metabolism and in the transport of carbohydrates and glucuronide (Grim *et al*. 2013), and the osmoprotectant ABC transporter

genes, including *yehZYXW*, which play roles in bacterial survival. BLAST tools were used to search the genomes of 38 *C. sakazakii* strains for genes that are associated with the osmotic stress response (Grim *et al.* 2013; Yan *et al.* 2013). These genes included the osmotolerance regulation genes *yiaD*, *osmY*, *ompA*, *aqpZ*, and *glpF*. The search also included genes associated with osmotic stress, namely *osmB* and *osmO*, *yciT*, *yciM*, and *pgpB*. Periplasmic glucan synthesis genes were also included; these genes, including *mdoC*, *mdoH*, *mdoG*, *mdoD*, *mdoB*, and *opgC*, are responsible for the regulation of osmotolerance.

The results of BLAST search of 38 sequenced *C. sakazakii* CC4 and non-CC4 strains are presented in **Table 4.3**. All of the CC4 strains had all of the genes, except for strain 557; this strain did not have 3 of the genes, namely *yehY*, *yihO*, and *mdoD*. Most of the non-CC4 strains also had all of the genes. Notably, ST8 strain 5 and strain 1 lacked just *yehX* or *yehY*, respectively, strain 696 (ST12) had all of the genes except *yehY*, and strain 978 (ST3) had all of the genes except for *yihO* and *osmB*.

Table 4. 3 The presence or absence of genes identified as being involved in desiccation survival in *C. sakazakii*

Gene description			Osmoprotectant ABC transporter genes				Desiccation-related genes										Osmotic stress related genes															
Species	**Strains**	**ST**	***yehX***	***yehW***	***yehY***	***yehZ***	***yihU***	***yihT***	***yihR***	***yihS***	***yihQ***	***yihV***	***yihW***	***yihP***	***yihA***	***yihO***	***osmY***	***osmB***	***mdoC***	***mdoH***	***mdoD***	***mdoG***	***mdoB***	***rpoS***	***ompA***	***yiaD***	***yciM***	***yciT***	***glpF***	***opgC***	***pgpB***
ST4	SP291	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	6	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	20	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	553	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	557	4	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
	558	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	721	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	767	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1219	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1220	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1221	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1225	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1231	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1240	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	701	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	694	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	730	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	377	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1533	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1537	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1542	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CC4	1587	109	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	4	15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Non-CC4	658	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1218	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1536	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	5	8	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	680	8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1	8	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	520	12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	696	12	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	140	40	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	150	16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	978	3	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	984	3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	ES15	125	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1249	31	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	HPB5174	45	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

ST: Sequence type, CC4: Part of clonal complex 4; which includes ST4,ST15 and ST109.

4.5.2.2 **Osmotolerance genes**

The survival of bacteria in PIF depends on its ability to resist osmotic stress and dry stress. The water activity (a_w) in dried infant formula is about 0.2 (Breeuwer *et al*. 2003). Bacteria usually protect themselves from high osmotic stress by rapid intracellular accumulation of K^+ ions followed by compatible solute accumulation, which includes the accumulation of proline, glycine betaine, and trehalose (Kempf and Bremer 1998; Breeuwer *et al*. 2003). Feeney and Sleator (2011) studied the osmotolerance response system in *C. sakazakii* strain 658 and found that bacterial osmotolerance was responsive in both hyper-osmotic and/or hypo-stress situations (Feeney and Sleator, 2011). Notably, hyper-osmosis in bacteria is controlled by primary and secondary responses (Feeney and Sleator, 2011).

Table 4.4 summarises the results of a BLAST search for 16 genes that encode proteins involved in the osmotolerance primary response. This response contributes to short-term protection against high osmotic stress via a K^+ uptake system (Feeney and Sleator, 2011). Of the 38 *C. sakazakii* CC4 and non-CC4 genome strains used in this study, all had the following genes: *trkA*, *trkE*, *kup (trkD)*, *trkH*, *kdpE*, *kdpA*, *kdpC*, *kdpB*, *kdpD*, *ompC*, *ompR*, *phoP*, *phoQ*, and *envZ*. However, the other genes were variably present in the different strains. Almost all strains (n=37; 97%) except strain 150 (ST16) had *ompF*, and *trkG* was found in 33 (87%) of the strains, but not in strains 6 (ST4), 680 (ST8), 1 (ST8), 520 (ST12), or strain ES15 (125). *trkG* is encoded by the pESA3 plasmid and has the Genbank number ESA_pESA3p05484.

Table 4. 4 The presence or absence of 16 primary response genes involved in K+ uptake in the osmotic stress response in *C. sakazakii.*

Gene			Genes involved in K+ uptake															
Species	Strains	ST	*trkH*	*trkG*	*trkA*	*trkE*	*kup (trkD)*	*kdpA*	*kdpB*	*kdpC*	*kdpD*	*kdpE*	*phoP*	*phoQ*	*ompC*	*ompF*	*ompR*	*envZ*
C. sakazakii ST4	SP291	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	6	4	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	20	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	553	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	557	4	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	558	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	721	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	767	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1219	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1220	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1221	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1225	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1231	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1240	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	701	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	694	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	730	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	377	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1533	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1537	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1542	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C. sakazakii CC4	1587	109	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	4	15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C. sakazakii non-CC4	658**	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1218	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1536	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	5	8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	680	8	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1	8	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	520	12	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	696	12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	140	40	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	150	16	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
	978	3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	984	3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	ES15	125	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1249	31	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	HPB5174	45	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

ST: Sequence type

CC4: Part of clonal complex 4; which includes ST4, ST15 and ST109.

The results of the BLAST searches of 38 bacterial strains for the presence of 28 osmotic stress-related genes are shown in **Tables 4.5** and **4.6**. These genes, which include the *proU*, *proP*, and *opuC* operon systems, encode proteins involved in the secondary response to osmotic stress that are responsible for compatible solute uptake. All of the *C. sakazakii* strains (n=38) had 22 of the 28 genes (**Tables 4.5, 4.6** and **4.7**). This included *proA*, *proB*, *proC*, $proP^{a}$, $proP^{b}$, $proP^{c}$, $proP^{f}$, $proP^{g}$, the *proU* operon *(proV*, *proW*, and *proX)*, $opuCA^{b}$, $opuCC^{b}$, $opuCD^{a}$, $opuCD^{b}$, *proQ*, *betA*, *betB*, *betI*, *betT*, *otsA*, and *otsB*. However, the other genes were variably present in the different strains. For example, 37/38 (97%) of the strains had $proP^{e}$ (ESA_01226) and *proW* (ESA_00588), the only exception being strain 680 (ST8). Similarly, 37/38 (97%) of the strains had the $opuCA^{b}$ (ESA_01738), $opuCB^{a}$ (ESA_01739), and $opuCC^{a}$ (ESA_01740) genes, with the exception being strain 557 (ST4). Interestingly, the $opuCB^{b}$ (ESA_01109) gene was absent from all of the *C. sakazakii* strains. The $opuCB^{b}$ (ESA_01109) gene was identified in 8/28 (21%) of the strains, including strain 1240 (ST4); ST1 strains 658 and 1218; ST8 strains 5, 680, and 1; and ST12 strains 520 and 696. In addition, 23/28 (89%) of the strains had the $proP^{d}$ (ESA_pESA3p05450) gene, with the exception of strains 6 (ST4), 680 (ST8), 1 (ST8), 520 (ST12), and ES15 (ST125). Additional research by our group found that these strains lacked the pESA3 plasmid and there was no clear patterns with regard to CC4 versus non-CC4 strain (unpublished data).

Table 4. 5 The presence or absence of genes reported to be involved in the secondary response to osmotic stress in *C. sakazakii*

Genes			proABC			proP(a, b, c ,d, e, f, g)							proU		
Species	Strains	ST	*proA*	*proB*	*proC*	*proP*^{a}	*proP*^{b}	*proP*^{c}	*proP*^{d}	*proP*^{e}	*proP*^{f}	*proP*^{g}	*proV*	*proW*	*proX*
***C. sakazakii* ST4**	SP291	4	+	+	+	+	+	+	+	+	+	+	+	+	+
	6	4	+	+	+	+	+	+	-	+	+	+	+	+	+
	20	4	+	+	+	+	+	+	+	+	+	+	+	+	+
	553	4	+	+	+	+	+	+	+	+	+	+	+	+	+
	557	4	+	+	+	+	+	+	+	+	+	+	+	+	+
	558	4	+	+	+	+	+	+	+	+	+	+	+	+	+
	721	4	+	+	+	+	+	+	+	+	+	+	+	+	+
	767	4	+	+	+	+	+	+	+	+	+	+	+	+	+
	1219	4	+	+	+	+	+	+	+	+	+	+	+	+	+
	1220	4	+	+	+	+	+	+	+	+	+	+	+	+	+
	1221	4	+	+	+	+	+	+	+	+	+	+	+	+	+
	1225	4	+	+	+	+	+	+	+	+	+	+	+	+	+
	1231	4	+	+	+	+	+	+	+	+	+	+	+	+	+
	1240	4	+	+	+	+	+	+	+	+	+	+	+	+	+
	701	4	+	+	+	+	+	+	+	+	+	+	+	+	+
	694	4	+	+	+	+	+	+	+	+	+	+	+	+	+
	730	4	+	+	+	+	+	+	+	+	+	+	+	+	+
	377	4	+	+	+	+	+	+	+	+	+	+	+	+	+
	1533	4	+	+	+	+	+	+	+	+	+	+	+	+	+
	1537	4	+	+	+	+	+	+	+	+	+	+	+	+	+
	1542	4	+	+	+	+	+	+	+	+	+	+	+	+	+
***C. sakazakii* CC4**	1587	109	+	+	+	+	+	+	+	+	+	+	+	+	+
	4	15	+	+	+	+	+	+	+	+	+	+	+	+	+
***C. sakazakii* Non-CC4**	658	1	+	+	+	+	+	+	+	+	+	+	+	+	+
	1218	1	+	+	+	+	+	+	+	+	+	+	+	+	+
	1536	1	+	+	+	+	+	+	+	+	+	+	+	+	+
	5	8	+	+	+	+	+	+	+	+	+	+	+	+	+
	680	8	+	+	+	+	+	+	-	-	+	+	+	-	+
	1	8	+	+	+	+	+	+	-	+	+	+	+	+	+
	520	12	+	+	+	+	+	+	-	+	+	+	+	+	+
	696	12	+	+	+	+	+	+	+	+	+	+	+	+	+
	140	40	+	+	+	+	+	+	+	+	+	+	+	+	+
	150	16	+	+	+	+	+	+	+	+	+	+	+	+	+
	978	3	+	+	+	+	+	+	+	+	+	+	+	+	+
	984	3	+	+	+	+	+	+	+	+	+	+	+	+	+
	ES15	125	+	+	+	+	+	+	-	+	+	+	+	+	+
	1249	31	+	+	+	+	+	+	+	+	+	+	+	+	+
	HPB5174	45	+	+	+	+	+	+	+	+	+	+	+	+	+

ST: Sequence type
CC4: Part of clonal complex 4; which includes ST4, ST15 and ST109.
proP(^{a,b,c,d,e,f,g}): (ESA_02131), (ESA_01706),(ESA_04214),(ESA_pESA3p05450),(ESA_01226) ,(ESA_00673) and (ESA_03328).

Table 4. 6 The presence or absence of genes reported to be involved in the secondary response to osmotic stress in *C. sakazakii* genome

Genes			Osmotic stress genes									Glycine betaine genes				Trehalose genes	
Species	**Strains**	**ST**	*opuCA*^{a}	*opuCA*^{b}	*opuCB*^{a}	*opuCB*^{b}	*opuCC*^{a}	*opuCC*^{b}	*opuCD*^{a}	*opuCD*^{b}	*proQ*	*betA*	*betB*	*betI*	*betT*	*otsA*	*otsB*
***C. sakazakii* ST4**	SP291	4	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
	6	4	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
	20	4	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
	553	4	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
	557	4	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+
	558	4	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
	721	4	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
	767	4	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
	1219	4	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
	1220	4	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
	1221	4	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
	1225	4	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
	1231	4	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
	1240	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	701	4	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
	694	4	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
	730	4	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
	377	4	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
	1533	4	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
	1537	4	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
	1542	4	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
***C. sakazakii* CC4**	1587	109	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
	4	15	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
C. sakazakii	658	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Non-CC4	1218	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1536	1	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
	5	8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	680	8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1	8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	520	12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	696	12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	140	40	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
	150	16	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
	978	3	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
	984	3	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
	ES15	125	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
	1249	31	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
	HPB5174	45	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+

ST: Sequence type
CC4: Part of clonal complex 4; which includes ST4,ST15 and ST109.
opuCA $^{a\&B}$: (ESA_01738) & (ESA_01110), *opuCB* $^{a\&B}$: (ESA_01739) and(ESA_01109), *opuCC* $^{a\&b}$: (ESA_01740) and (ESA_01108), *opuCD* $^{a\&b}$: (ESA_01741) and (ESA_01111)

Most of the 28 genes associated with the secondary response to osmotic stress, except for *proP*^{d}, *proP*^{e}, *opuCA*^{a}, *opuCB*^{a}, and *opuCB*^{b}, were present in most of the CC4 and non-CC4 *C. sakazakii* strains. The results of a BLAST search for 9 genes required for the hypo-osmotic stress response are shown in **Table 4.7.** This included the mechano-sensitive channel gene (*msc*), the K^{+} efflux (*kef*) gene, and the aquaporin gene (*aqpZ*). This system plays a role in maintaining the osmotic balance in bacterial cells; hence, when the medium changes from high to low osmolality, mechano-sensitive channels release unselective solutes from the cytoplasm to rapidly decrease the pressure in the cell (Feeney and Sleator, 2011).

Table 4. 7 The presence or absence of genes reported to be involved in the hypo-osmotic stress response

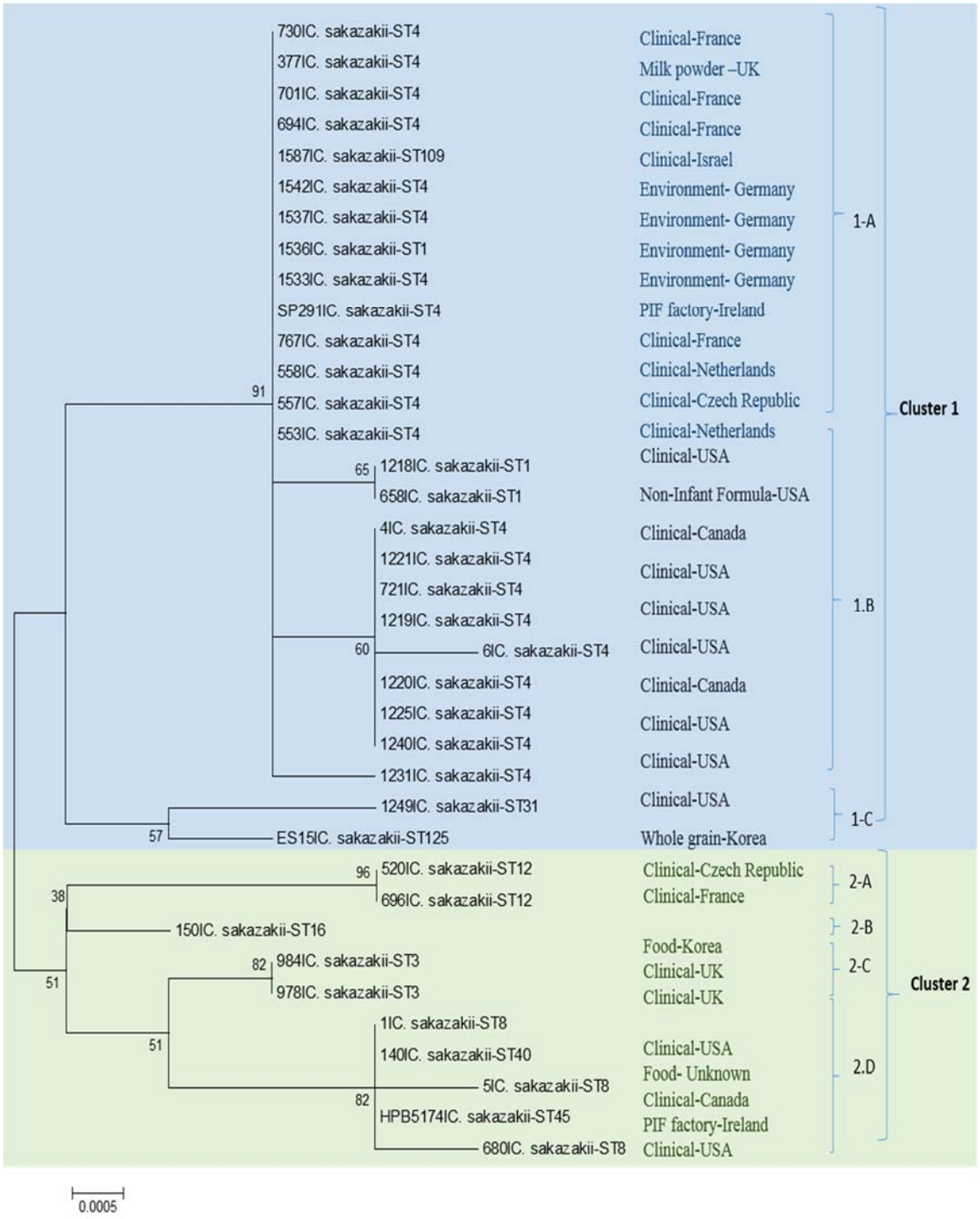
Gene			Mechano-sentitive channels			aquaporins	*kef* systems				
Species	Strains	ST	*mscL*	*mscS*	*mscK*	*aqpZ*	*kefA*	*kefB*	*kefC*	*kefF*	*kefG*
C. sakazakii ST4	SP291	4	+	+	+	+	+	+	+	+	+
	6	4	+	+	+	+	+	+	+	+	+
	20	4	+	+	+	+	+	+	+	+	+
	553	4	+	+	+	+	+	+	+	+	+
	557	4	+	+	+	-	+	+	+	+	+
	558	4	+	+	+	+	+	+	+	+	+
	721	4	+	+	+	+	+	+	+	+	+
	767	4	+	+	+	+	+	+	+	+	+
	1219	4	+	+	+	+	+	+	+	+	+
	1220	4	+	+	+	+	+	+	+	+	+
	1221	4	+	+	+	+	+	+	+	+	+
	1225	4	+	+	+	+	+	+	+	+	+
	1231	4	+	+	+	+	+	+	+	+	+
	1240	4	+	+	+	+	+	+	+	+	+
	701	4	+	+	+	+	+	+	+	+	+
	694	4	+	+	+	+	+	+	+	+	+
	730	4	+	+	+	+	+	+	+	+	+
	377	4	+	+	+	+	+	+	+	+	+
	1533	4	+	+	+	+	+	+	+	+	+
	1537	4	+	+	+	+	+	+	+	+	+
	1542	4	+	+	+	+	+	+	+	+	+
C. sakazakii CC4	1587	109	+	+	+	-	+	+	+	+	+
	4	15	+	+	+	+	+	+	+	+	+
C. sakazakii Non-CC4	658	1	+	+	+	+	+	+	+	+	+
	1218	1	+	+	+	+	+	+	+	+	+
	1536	1	+	+	+	+	+	+	+	+	+
	5	8	+	+	+	+	+	+	+	+	+
	680	8	+	+	+	+	+	+	+	+	+
	1	8	+	+	+	+	+	+	+	+	+
	520	2	+	+	+	+	+	+	+	+	+
	696	12	+	+	+	+	+	+	+	+	+
	140	40	+	+	+	+	+	+	+	+	+
	150	16	+	+	+	+	+	+	+	+	+
	978	3	+	+	+	+	+	+	+	+	+
	984	3	+	+	+	+	+	+	+	+	+
	ES15	125	+	+	+	+	+	+	+	+	+
	1249	31	+	+	+	+	+	+	+	+	+
	HPB5174	45	+	+	+	+	+	+	+	+	+

ST: Sequence type
CC4: Part of clonal complex 4; which includes ST4,ST15 and ST109

4.5.2.3 **Phylogenetic tree analysis of *rpoS***

Another sigma factor *rpoS* is associated with modulating the overall stress response, which was found in Gram-negative bacteria (Dodd and Aldsworth, 2002). Recently, Ordonez *et al*. (2012), showed that the *rpoS* gene, encoding for an alternative sigma factor σ^s provides *C. sakazakii* with its stress resistance capacity. According to their study, a positive relation, existing between the *rpoS* activity and the acid, alkaline, and osmotic stress resistance, had been equally observed (Ordonez *et al*. 2012). Their study concluded that the full length nucleotide sequence of the *rpoS* was essential and that the variations such as large insertions and deletions in this gene can affect the stress response of *C. sakazakii* isolates (Ordonez *et al.* 2012). In the present study, the *rpoS* gene sequence of *C. sakazakii* 658 (Genbank accession number: ESA_01532), nucleotide length (993bp) was extracted from the NCBI and aligned against all the strains in this study using BLAST. The full length *rpoS* gene was detected in all of the 38 sequenced isolates. A phylogenetic tree was constructed using MEGA5.2 software using the maximum likelihood method and analysed in order to check the variation in the *rpoS* gene sequence of 38 *C. sakazakii* CC4 (n=24) and non-CC4 (n=14) strains **(Figure 4.2)**. The bootstrap values of 1,000 replicates for the main lineages in the tree are presented as percentages. The scale bar (0.0005) indicates the relative phylogenetic distance.

The *rpoS* phylogenetic tree showed the genetic associations of the 38 isolates based on the alignment of the complete *rpoS* gene sequence (nucleotide length, 993 bp). The branch lengths are related to the number of nucleotide differences. Most of the isolate divisions and subdivisions indicate a single isolation source with regard to the country of isolation.



ST: Sequence type
USA: United States of America; UK: United Kingdom; PIF: powdered infant formula.

Figure 4. 2 The phylogenetic tree of *rpoS* gene of 38 *C. sakazakii* isolates.

The tree was constructed using the *rpoS* gene sequences (nucleotide sequences) of 38 *C. sakazakii* isolates. The sequences were obtained from the genomes in the *Cronobacter* MLST database. The NTU strain IDs are displayed at the top of each of the branches. The tree is drawn to scale using MEGA v5.2 with 1000 bootstrap replicates. *rpoS* tree analysis revealed that cluster 1 and cluster 2 are distinct based on the level of nucleotide sequence similarity. The number next to the branch point shows the bootstrap rate (1000 replicates). The scale bar shows the ratio of nucleotide substitution.

Overall, there was a high degree of sequence consensus amongst the CC4 and non-CC4 isolates. For comparison purposes, and to define the level of similarity amongst the isolates, CC4 strain SP291 was used as a reference strain. There was no clear subdivision between the CC4 and non-CC4 isolates. The phylogenetic tree (**Figure 4.2**) showed two larger clusters: cluster 1 had the CC4, ST1, ST31, and ST125 strains, while cluster 2 had isolates from different sequence types. The SNP analysis, which was based on variations in the nucleotide sequences, showed two SNPs for the Europe strains in sub-cluster 1.A. An interesting observation that there was a clear subdivision within cluster 1. Specifically, the strains within cluster 1 showed subclustering based on their geographic origins i.e. sub-cluster 1.B corresponded to isolates from Canada and the USA (**Figure 4.2**, blue shading), and sub-cluster 1.A corresponded to isolates from Europe (**Figure 4.2**). The sequence alignment analysis showed a high degree of sequence consensus within these subclusters. The isolates in Canadian and USA subcluster 1.B differed from the European isolates in cluster 1.A by two SNPs that arose due to microevolutionary events.

Additional subdivisions were noted within the American and Canadian subcluster 1.B. Strains 658 and 1218 in this subcluster showed a slightly longer branch length and had one SNP difference, C→T, at position 601 relative to the cluster 1.A strain (SP29). The longer branch length was due to an SNP in these strains. These two ST1 isolates were obtained from the USA in 2001 from non-infant formula and from CSF case, respectively.

In addition, CC4 isolate 6 showed a slightly longer branch length, which was also attributed to SNP differences. Specifically, there was a deletion in the start of the gene sequence of 84 nucleotides, possibly the result of sequence assembly or sequencing fault. Another CC4 strain, strain 1231, branched within cluster 1.B because of one SNP difference, G→A at position 192, relative to the cluster 1.A strains. Furthermore, isolates 1249 and ES15, which belong to ST31 and ST125, showed sub-branching with cluster 1 isolates due to multiple SNPs (5 and 4 SNP differences, respectively). Strains 1249 (ST31) and ES15 (ST125) were isolated from the UK and Korea, respectively.

In contrast, there was a lower degree of sequence consensus amongst the strains in cluster 2, possibly due to the presence of strains belonging to 8 different sequence types. There was a clear subdivision

within cluster 2 (subcluster 2.B) that corresponded to isolates from clinical cases in France and the Czech Republic (**Figure 4.2**, green shading). Moreover, ST3 strains 984 and 978, which were both isolated from the United Kingdom, clustered together. Variations were detected within the non-CC4 and CC8 isolates (e.g. strains 1, 5, and 680), since other ST strains, such as strain 140 (ST40) and HPB5174 (ST45), clustered within the CC8 cluster (**Figure 4.2**). These isolates were from Canada, the United States, and Ireland.

4.5.3 Heat tolerance assay

The heat tolerance experiment was performed using a desiccated series suspension of 13 *C. sakazakii* strains and one *C. koseri* strain 48 as a comparison control as described in Chapter 2 (**Section 2.8.6**). Briefly, the bacteria were incubated overnight at room temperature (20–25°C), and cells were counted after 18 hours of culture at 37°C on TSA media as described in Chapter 2 (**Section 2.8.6**). The initial viable cell counts prior to desiccation ranged from 8.89 to 10.43 $\log_{10}$ CFU/ml (**Figure 4.3**; **Figure 4.4**). The survival rates of the *C. sakazakii* strains were reduced after desiccation for 18 hours, showing decreases in viability of 1.38 to 4.18 $\log_{10}$ CFU/ml (**Figure 4.3**; **Figure 4.4**). However, one environmental strain, strain 1533 (CC4), showed higher resistance to desiccation than the other strains, with a reduction in viability of 1.38 $\log_{10}$ CFU/ml (**Figure 4.3**; **Figure 4.4**). In addition, strains 658, 1587, 701, 1542, and 1537 showed a large reduction in the viable cell count after desiccation, with reductions in viability of 3.19 to 4.18 $\log_{10}$ CFU/ml. *C. koseri* strain 48 showed a reduction in viability of 2.80 $\log_{10}$ CFU/ml, while strains 1536, 520, 696, 680, 1, 1221, and 721 showed relatively moderate resistance to desiccation, with reductions in viability of 2.11 to 2.93 $\log_{10}$ CFU/ml (**Figure 4.3**; **Figure 4.4**). The desiccated cultures were then exposed to dry heat for 30 minutes at three temperatures, 60°C, 80°C, or 100°C, and the effect of the dry heat on the dried cells was evaluated at two time points, 30 and 120 minutes.

4.5.3.1 **Viability of *C. sakazakii* after exposure to dry heat for 30 minutes**

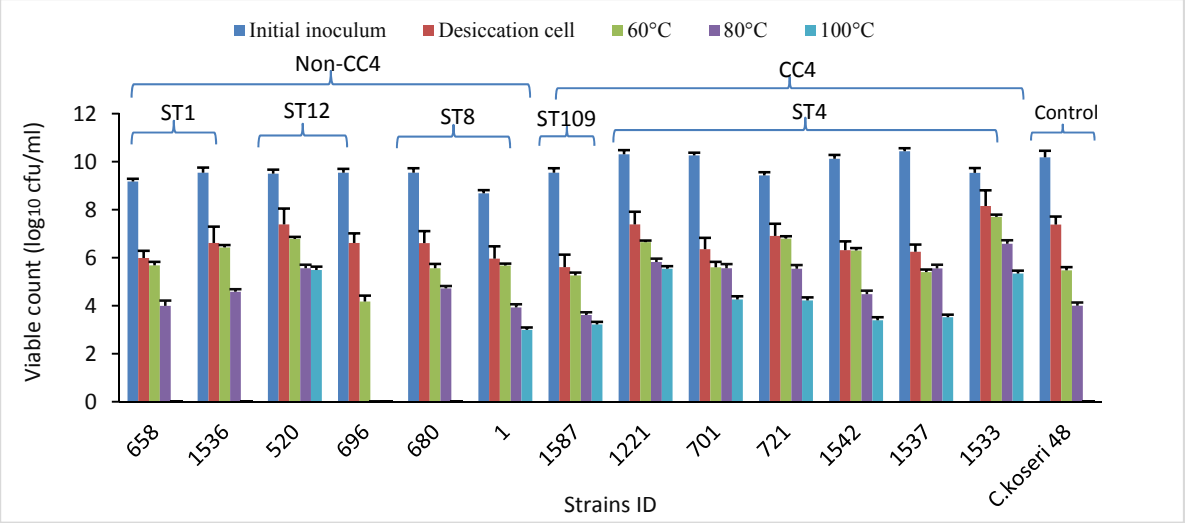
To define the heat resistance of the desiccated cells, the cell viability (log difference in CFU/ml) was determined post desiccation compared with post heat treatment of these cells. The number of cells that

recovered after dry heat treatment (60°C, 80°C, and 100°C) at each time point (30 minutes and 120 minutes) varied in the different strains (**Figure 4.3; Figure 4.4**).

After 60°C treatment for 30 minutes, strain 696 (ST12) showed highly reduced viability compared to the other strains, with a reduction of 2.44 $\log_{10}$ CFU/ml (**Figure 4.3**). Strains 520 (ST12), 680 (ST8), 1221(ST4), 701 (ST4), and 1537(ST4) showed moderate reductions in viability of 0.74 to 1.05 $\log_{10}$ CFU/ml (**Figure 4.3**). Finally, strains 658 (ST1), 1536 (ST1), 1 (ST8), 1587(ST109-CC4), 721 (ST4), 1542 (ST4) and 1533 (ST4) showed lower reductions in viability of 0.11 to 0.45 $\log_{10}$ CFU/ml. The viability of *C. koseri* strain 48 was greatly reduced by 1.91 $\log_{10}$ CFU/ml (**Figure 4.3**).

After 80°C treatment for 30 minutes, the viability of strains 658 (ST1), 1536 (ST1), 1 (ST8), 680 (ST8), 520 (ST12), 1542 (ST4), and 1587 (ST109-CC4) was greatly reduced by 1.89 to 2.03 $\log_{10}$ CFU/ml; this reduction in viability was greater than the reductions in viability of many the other strains. Moderate reductions in viability of 0.69 to 1.57 $\log_{10}$ CFU/ml were observed for CC4 strains 1221, 701, 721, 1537, and 1533. Notably, one clinical strain, strain 696 (ST12), were not viable after exposure to dry heat at 80°C. Furthermore, the viability of *C. koseri* strain 48 was highly reduced by 3.38 $\log_{10}$ CFU/ml (**Figure 4.3**).

After 100°C treatment for 30 minutes, most of the *C. sakazakii* strains, i.e. strains 520 (ST12), 1 (ST8), 1587(ST109-CC4), 1221 (ST4), 701 (ST4), 721 (ST4), 1542 (ST4), 1537 (ST4), and 1533 (ST4), recovered, but their viability was greatly reduced by 2.79 to 1.85 $\log_{10}$ CFU/ml (**Figure 4.3**). In contrast, four non-CC4 strains, i.e. strains 658 (ST1), 1536 (ST1), 696 (ST12), 680 (ST8), as well as *C. koseri* strain 48, were not viable after exposure to dry 100°C heat for 30 minutes. These strains were clinical isolates belonging to ST1, ST12, and ST8 (**Figure 4.3**).



ST: Sequence type
CC4: Part of clonal complex 4; which includes ST4 and ST109

Figure 4. 3 The viability of desiccated *C. sakazakii* CC4 and non-CC4 strains after exposure to dry heat at the indicated temperature for 30 minutes.

C. koseri strain 48 was used as a comparison control. Data are reported as the means ± standard deviation of the number of recovered cells in three independent experiments.

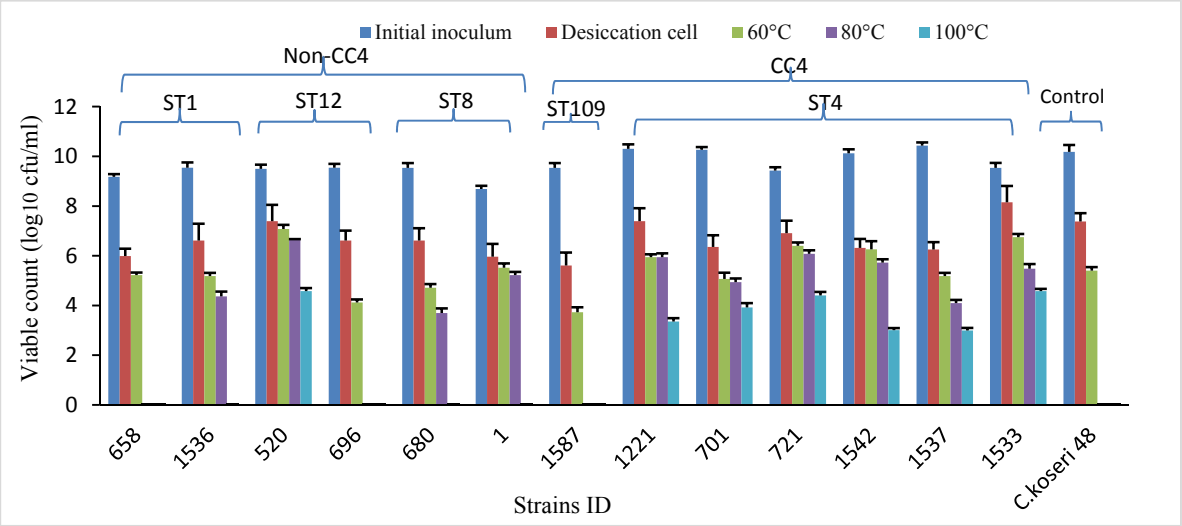
4.5.3.2 **Viability of *C. sakazakii* after exposure to dry heat for 1 hour**

Most of the desiccated cells of the *C. sakazakii* strains, both CC4 and non-CC4 strains, remained viable after being treated with heat at 60°C, 80°C, or 100°C for 1 hour (**Figure 4.4**). To define the heat resistance of the desiccated cells, the cell viability (log difference in CFU/ml) was determined post desiccation compared with post heat treatment of these cells.

After 60°C treatment for 60 minutes, all of the tested strains remained viable (**Figure 4.4**). After this treatment, strains 696 (ST12), 680 (ST8), and 1587 (ST109-CC4) showed high reductions in viability of 1.88 to 2.50 $\log_{10}$ CFU/ml. The viability of strains 520 (ST12), 1 (ST8), 721 (ST4) and 1542 (ST4) was reduced to a lesser extent, with reductions in viability of <0.44 $\log_{10}$ CFU/ml (**Figure 4.4**). Strains 658 (ST1), 1536 (ST1), 1221 (ST4), 701 (ST4), 1537 (ST4), and 1533 (ST4) showed moderate reductions in viability of 1.45 to 0.77 $\log_{10}$ CFU/ml (**Figure 4.4**). The viability of strains 696 (ST12), 680 (ST8), and 1587 (ST109-CC4) showed high reduction in the viability of 0.11 to 0.45 $\log_{10}$ CFU/ml. Finally, the viability of *C. koseri* strain 48 was greatly reduced after treatment by 1.98 $\log_{10}$ CFU/ml (**Figure 4.4**).

After 80°C treatment for 60 minutes, the viability of four strains, namely strains 1536 (ST1), 680 (ST8), 1537(ST4), and 1533 (ST4), was more reduced than that of the other strains, with reductions in viability of 2.15 to 2.91 $\log_{10}$ CFU/ml. Strains 520 (ST12), 1 (ST8), 1221 (ST4), 701 (ST4), 721 (ST4) and 1542 (ST4) showed moderate reductions in viability of 1.42 to 0.59 four $\log_{10}$ CFU/ml. Finally, four strains, strains 658 (ST1), 696 (ST12), 1587(ST109-CC4), and *C. koseri* strain 48, were not viable after exposure to 80°C dry heat for 60 minutes (**Figure 4.4**).

After 100°C treatment for 60 minutes, most of the CC4 strains, 1221, 701, 721, 1542, 1537, 1533, and one ST12 strain 520, remained viable but showed greatly reduced viability of 4.04 to 2.81 $\log_{10}$ CFU/ml (**Figure 4.3**). These strains were considered heat-resistant; other than ST12 strain 520, they were all CC4 strains. Notably, most of the non-CC4 strains 658, 1536, 696, 680, 1, and *C. koseri* strain 48, were not viable after exposure to dry heat at 100°C. These strains included clinical and environmental isolates that belonged to ST1, ST12, and ST8 (**Figure 4.3**) (**Table 4.17**).



ST: Sequence type
CC4: Part of clonal complex 4; which includes ST4 and ST109

Figure 4. 4 Viability of desiccated CC4 and non-CC4 *C. sakazakii* strains after exposure to dry heat at the indicated temperature for 1 hour.

The *C. koseri* strain 48 was used as a comparison control. Data are reported as the means ± standard deviation of the number of recovered cells in three independent experiments.

4.5.3.3 **Genes associated with thermal tolerance**

Gajdosova *et al.* (2011) identified an 18-kbp genomic region that contains 22 open reading frames in *C. sakazakii* ATCC 29544. BLAST was used to search for this region in the 38 *C. sakazakii* genomes, and this region was variably present (**Table 4.8**). Notably, genomic analysis showed substantial variation between CC4 and non-CC4 strains in terms of the presence of this region. This region was absent in most of the non-CC4 strains. The exception was strain 1(ST8), which had all of the genes in the 18-kbp region. Strain 680(ST8) and ES15 (ST125) had *orft5* and *orft4*, but lacked the rest of the genes, and strain 696(ST12) had the *orfABCDE* and *orfOP* genes, but lacked the rest of the genes.

Table 4. 8 Presence or absence of 22 thermo-tolerance genes in 38 *C. sakazakii* genomes (CC4 and non-CC4 strains)

Species	Strain	ST	*orfT5*	*orfT4*	*orfA*	*orfB*	*orfC*	*orfD*	*orfE*	*orfF*	*orfG*	*orfH*	*orfI (KT)*	*orfJ*	*orfK*	*orfL*	*orfM*	*orfN*	*orfO*	*orfP*	*orfQ*	*orfT3*	*orfT2*	*orfT1*
C. sakazakii CC4	20	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	557	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	558	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1219	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
	1220	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
	6	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
	1225	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
	1240	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
	4	15*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
	1587	109*	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
	1231	4	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
	1533	4	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
	1231	4	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
	SP291	4	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
	767	4	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
	701	4	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
	694	4	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
	730	4	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
	377	4	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
	1537	4	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
	1542	4	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
	553	4	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
	1221	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	721	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	658	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. sakazakii Non-CC4	1218	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1536	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	5	8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1	8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	680	8	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	ES15	125	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	520	12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	696	12	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
	140	40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	150	16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	978	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	984	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1249	31	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	HPB5174	45	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

ST: Sequence type, *CC4: Part of clonal complex 4; which includes ST4, ST15 and ST109.The grey shade indicate strains included in thermotolerance experiment **(Section 4.5.3)**

Genomic analysis showed substantial variation in the CC4 strains in terms of the presence of this region. The strains could be divided into 6 groups based on the presence or absence of the thermo-tolerance genes identified by Gajdosova *et al.* (2011) (**Table 4.8**). Group 1 included strains 557, 558, and 20, all of which had all 22 genes which was similar to strain 1(ST8). Group 2 included strains 6, 1219, 1220, 1225, 1240, and 4; these strains had all of the genes except the *orfQ* gene. Group 3 included strain 721 and strain 1221, which lacked the thermo-tolerance gene region. Group 4 included strains 553, 767, 701, 694, 730, 377, 1537 and 1542, which had 9/22 genes. Group 5 included strains 1231, 1533, and 1587, which had 7/22 thermo-tolerance genes. Group 6 comprised just one strain, SP291, which lacked 15/22 genes and which was similar to strain 696 (ST12) (**Table 4.9**).

Table 4. 9 Summary showing the *C. sakazakii* CC4 strains grouped according to the presence or absence of 22 thermo-tolerance genes.

Group	Strain number	Number of thermo-tolerance genes
1	557, 558, 20	22/22 genes are present; similar to strain 1 (ST8)
2	6, 1219, 1220, 1225, 1240, 4	21/22 genes are present; the *orfQ* gene is absent
3	721, 1221	22/22 genes absent; lacks the thermo-tolerance gene region (*orfHIJK*)
4	553, 767, 701, 694, 730, 377, 1537, 1542	9/22 genes are present
5	1231,1533, 1587	7/22 genes are present
6	SP291	7/22 genes are present; similar to strain 696 (ST12)

ST: Sequence type

4.5.4 Acid tolerance of *C. sakazakii* strains

4.5.4.1 Acid tolerance at pH 3.5

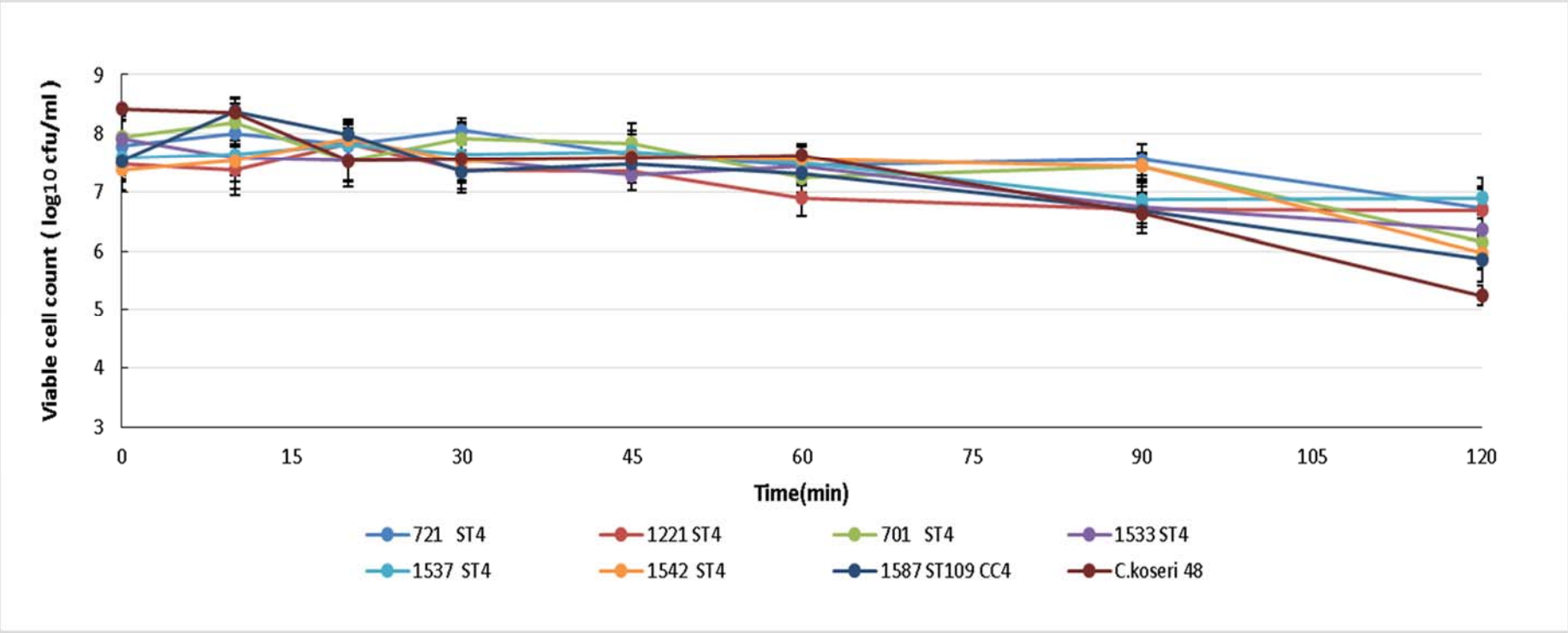
The acid tolerance of 13 *C. sakazakii* strains (both CC4 and non-CC4) plus *C. koseri* 48 was determined by treatment at pH 3.5. This experiment was performed in order to better understand the extent to which these cells could survive a pH resembling that of the neonatal stomach. *C. koseri* 48 was used as a comparison control. In the experiment, the strains were exposed to acidified infant formula (pH 3.5) for 120 minutes at 37°C. **Figures 4.5** and **4.6** show the results for the *C. sakazakii* CC4 and non-CC4 strains. The initial viability of the strains ranged from 7.95 to 8.4 $\log_{10}$ CFU/ml (**Figures 4.5** and **4.6**). The strains varied in their ability to tolerate the 2-hour challenge at pH 3.5, with reductions in viability ranging from 0.70 to 4.48 $\log_{10}$ CFU/ml (**Figures 4.5** and **4.6**). Treatment with acidified infant formula slightly reduced the viability of two *C. sakazakii* strains, 1221 (ST4) and 1537 (ST4), by 0.8 $\log_{10}$ CFU/ml (**Figure 4.5**). Two strains, 680 (ST8) and 696 (ST12), showed the greatest decreases, with reductions of about 4.48 $\log_{10}$ CFU/ml (**Figure 4.6**). *C. koseri* strain 48 showed a 3.18 $\log_{10}$ CFU/ml reduction.

Figure 4.5. shows the viability of the CC4 strains after 2 hours of incubation at pH 3.5. There was a reduction of 0.70 to 1.69 $\log_{10}$ CFU/ml after 2 hours of exposure to pH 3.5. The viable cell count was between 5.86 and 6.90 $\log_{10}$ CFU/ml for most of the ST4 strains. However, only two strains, 1221 (ST4) and 1537 (ST4), were stable at 60 and 90 minutes, with less reductions in viability of 0.70 and 0.81 $\log_{10}$ CFU/ml respectively (**Figure 4.5**). Strain 1542 (ST4) was stable until 60 minutes then showed a dramatic decline over the next hour, with reductions in viability of 1.42 $\log_{10}$ CFU/ml.

Figure 4.6 shows the variation in the viability of the non-ST4 strains after 2 hours of incubation at pH 3.5. In general, the strains could be grouped according to their adaptation to acid (**Figure 4.6** and **Figure 4.5**). The first group included the sensitive and highly sensitive strains 696 (ST12), 680 (ST1), 1536 (ST1), and 1 (ST8); the viability of these strains declined dramatically over the 2-hour experimental period and showed high reduction in the viability of 4.48 to 2.00 $\log_{10}$ CFU/ml. The second group included strains that are acid-resistant and showed only slight declines in viability after 60 minutes at pH 3.5, namely strains 721 (ST4), 1587 (ST109), 1542 (ST4), 658 (ST1), 701 (ST4), 520 (ST12), and

1533 (ST4); these strains showed reductions in viability of 1.07 to 1.79 log_{10} CFU/ml. The third group comprised two strains that were highly resistant to acid, strain 1537 (ST4) and 1221 (ST4), which remained viable throughout the 2-hour experimental period and which showed reductions in viability of just 0.70 and 0.81 log_{10} CFU/ml, respectively **(Table 4.17).**

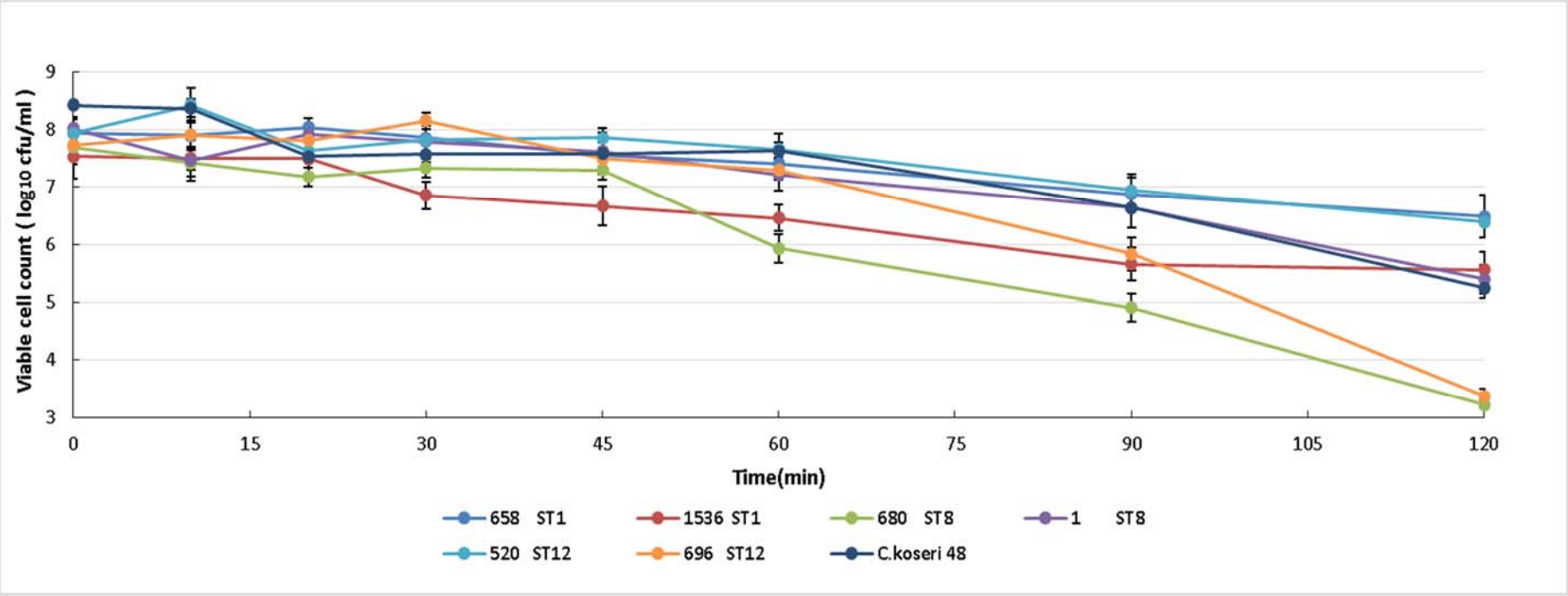
The acid tolerance experiments were repeated twice in triplicate, and the mean and standard deviation values were determined using Excel software. The *C. sakazakii* CFU/ml data were log-transformed and analysed. The student's unpaired t-test was performed to compare the CC4 and non-CC4 strains using the log differences of the T_0-T_{120min} values. There was a significant difference ($P<0.01$) between the reductions in strain viability over time for these two groups.



ST: Sequence type
CC4: Part of clonal complex 4; which includes ST4 and ST109

Figure 4. 5 The survival of *C. sakazakii* CC4 strains and a comparison strain at pH 3.5 at the indicated time points.

The survival curve shows the sensitivity of *C. sakazakii* CC4 strains to incubation in acidified (pH 3.5) infant formula for 2 hours at 37°C. Viability was measured at 0, 10, 20, 30, 45, 60, 90 and 120 minutes. Most of the CC4 strains showed acid tolerance, with little variation among the strains. Strain 1537 and strain 1221 showed the greatest acid resistance. The number of recovered cells was determined in triplicate in two independent experiments. The data are reported as means ± standard deviation.



ST: Sequence type
CC4: Part of clonal complex 4; which includes ST4, ST15 and ST109

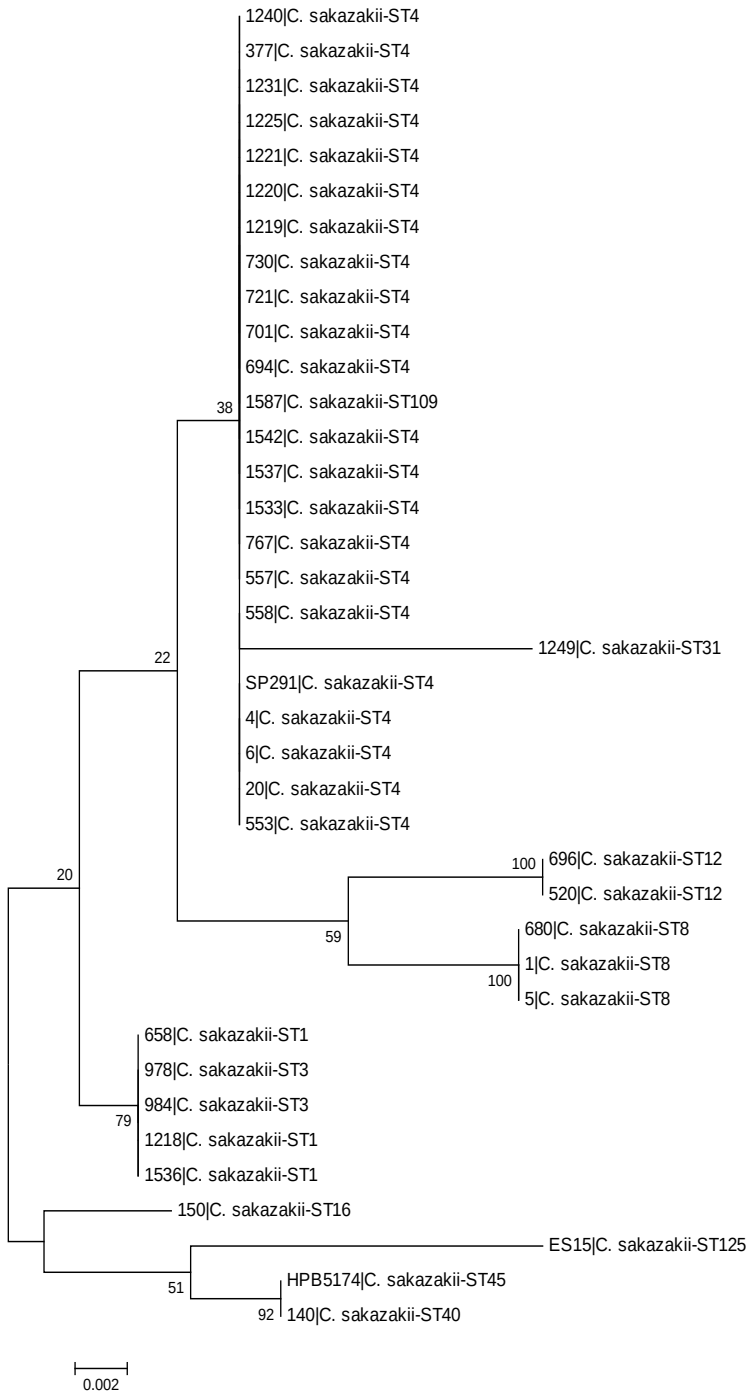
Figure 4. 6 The survival of *C. sakazakii* non-CC4 strains and a comparison strain at pH 3.5 at the indicated time points.

The survival curve shows the sensitivity of *C. sakazakii* non-CC4 strains (ST1, ST8, and ST12) to incubation in acidified (pH 3.5) infant formula for 2 hours at 37°C. Viability was measured at 0, 10, 20, 30, 45, 60, 90, and 120 minutes. Most of the non-CC4 strains were acid sensitive, although strain 1536 remained viable for the entire 2-hour period. The viability of strain 696 and strain 680 decreased dramatically starting at the 20-minute time point. The number of recovered cells was determined in triplicate in two independent experiments. The data are reported as means ± standard deviation.

4.5.4.2 **Phylogenetic analysis of the Acid tolerance associated gene *ompR***

Bang *et al.* (2000) showed that the expression of the envelope stress response gene *ompR* in *Salmonella enterica* is regulated by environmental stress and specifically by acidic pH. A recent study by Alvarez-Ordóñez *et al.* (2014) showed that *ompR* is the main gene that mediates *C. sakazakii* acid tolerance. A BLAST tool was used to search 38 *C. sakazakii* genomes and showed that this gene is present in all of the CC4 and non-CC4 strains.

The *ompR* gene homologue had been detected uniformly across analyses of CC4 and non-CC4 genomes in the present study. The *ompR* gene sequence of *C. sakazakii* 658 was extracted and aligned against the *ompR* gene of 38 *C. sakazakii* isolates using the *Cronobacter* pubMLST database. In order to check the variation in the *ompR* gene sequence of 38 *C. sakazakii* CC4 (n=24) and non-CC4 (n=14), a phylogenetic tree was constructed, using MEGA v.5.2 in **Figure 4.7.** As seen in the **Figure 4.7**, there were variations observed within CC4 and non-CC4 *C. sakazakii* isolates. The *ompR* phylogeny indicated close clustering of *C. sakazakii* CC4 isolates **(Figure 4.7).** However, variations were noted within the CC4 isolates, non-CC4 isolate 1249 (ST31), on the other hand, had been located on the same cluster of CC4 and showed longer branch length. Likewise, variations were noted within the non-CC4 cluster as ST1 isolates 1536, 1218, and 658 were located on the same cluster of ST3 isolates 984 and 978. Also, ST8 isolates 680, 1 and 5 and ST12 isolates, 520 and 696, were clustered on the different branches.



ST: Sequence type

Figure 4. 7 The phylogenetic tree of 38 *C. sakazakii* isolates, based on nucleotide sequences of *ompR* gene (720bp).

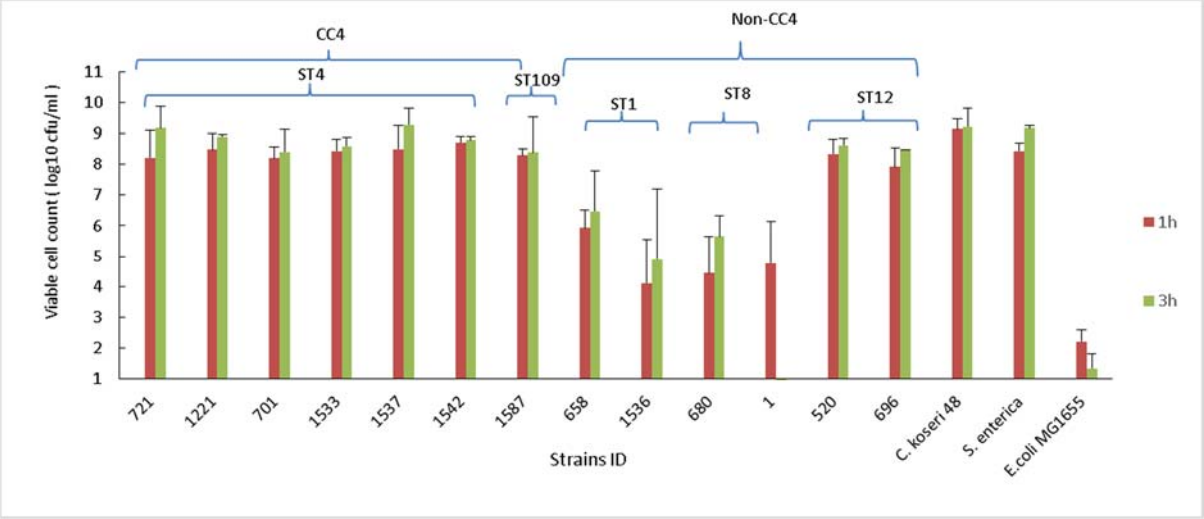
The NTU strain IDs are shown at the top of each branch. The tree is drawn to scale using MEGA v5.2 with 1000 bootstrap replicates. *ompR* tree analysis revealed four distinct clusters based on the level of nucleotide sequence similarity**.** The number next to the branch point shows the bootstrap rate (1000 replicates). The scale bar shows the ratio of nucleotide substitution.

4.5.5 Serum tolerance

4.5.5.1 **The sensitivity of *C. sakazakii* strains to human serum**

The *C. sakazakii* strains were examined for sensitivity to human serum by exposure for 3 hours at 37°C. The initial viable cell count was between 8.84 to 7.76 $\log_{10}$ CFU/ml for all of the strains (**Figure 4.8**). In the first hours after exposure to human serum, all of the CC4 and non-CC4 *C. sakazakii* strains exhibited significant reductions in the viable cell counts ($P<0.01$). Strain 658, strain 1536, strain 680, and strain 1 showed reductions in the viable cell count ranging from 2.79 to 4.39 $\log_{10}$ CFU/ml (**Figure 4.8**). After 3 hours of exposure to human serum, all of the *C. sakazakii* strains showed an increase in the viable cell count compared with their recovery after one hour. However, strain 1 displayed a notable reduction in cell viability (**Table 4.17).**

The unpaired student's t-test was used to compare the sensitivity of CC4 and non-CC4 strains to human serum. CC4 strains showed significant increases in the viable cell count after 3 hours ($P<0.01$), whereas no significant difference was observed for the non-CC4 strains. *C. koseri* 48 and *S. enterica* were used as positive controls, and both showed increases in viable cell count over 3 hours. This indicated that there is resistance to human serum. On the other hand, the negative control strain, *E. coil* MG1655, showed a reduction in the viable count over time, indicating sensitivity to human serum.



ST: Sequence type
CC4: Part of clonal complex 4; which includes ST4 and ST109

Figure 4. 8 Viability of CC4 and non-CC4 *C. sakazakii* strains after exposure to human serum.

Most of the *C. sakazakii* strains are serum resistant, although there is some variation in growth. However, strain 1 showed a sensitive phenotype after 2 hours. *S. enterica* strain 358 and *C. koseri* 48 were used as positive controls, and *E. coli* K12 (MG1655) was used as a negative control. The initial inoculum that used was 10^8 CFU/ml. The data are reported as means ± standard deviation of three independent experiments.

4.5.5.2 **Serum resistance genes**

A study by Franco *et al*. (2011b) found that the presence of *cpa*, a plasminogen activator and a *Cronobacter* outer membrane protease, was associated with resistance to human serum. In order to confirm the presence of the *cpa* locus in the genome, the *cpa* locus encoded in the pESA3 plasmid and has the Genbank number (ESA_pESA3p05434) was obtained from *C. sakazakii* strain ATCC BAA-894 and with a length of 816 bp. A BLAST search using the nucleotide sequence to determine the presence and absence of this *cpa* gene in *C. sakazakii* genomes (n=38) showed the presence of the 816-bp gene in all strains except ST8 strain 680 and strain 1, and ST4 strain 6 and ST12 strain 520 (**Table 4.10**).

A previous study showed that the murein lipoprotein *lpp* gene is strongly associated with serum resistance in *E. coli* (Phan *et al*. 2013). In order to confirm the presence of this gene in the genome, a protein BLAST search with the *E. coli* K-12 Lpp protein (78 amino acids) showed 76/78 (97%) identity with an amino acid sequence in the *C. sakazakii* ATCC BAA-894 genome encoded by the ESA_02087 gene. A second BLAST search showed that the ESA_02087 gene is present in all of the *C. sakazakii*

genomes (n=38). This might explain the resistance phenotype of strains 680 (ST8) and 520 (ST12), which are missing the *cpa* gene and thus showed resistance to human serum. However, strain 1 (ST8), which showed sensitivity to serum, had the *lpp* gene, indicating that there might be another factor involved in the resistance to serum, such as capsule polysaccharide and LPS.

Table 4. 10 The presence or absence of serum resistance genes in 38 *C. sakazakii* genomes.

Species	Strain number	ST	Genes associated with serum resistance		Serum resistance assay result
			***lpp* gene**	***cpa* gene**	
C. sakazakii CC4	721	4	+	+	+
	1221	4	+	+	+
	701	4	+	+	+
	1533	4	+	+	+
	1537	4	+	+	+
	1542	4	+	+	+
	1587	109	+	+	+
	SP291	4	+	+	NP
	6	4	+	-	NP
	20	4	+	+	NP
	553	4	+	+	NP
	557	4	+	+	NP
	558	4	+	+	NP
	767	4	+	+	NP
	1219	4	+	+	NP
	1220	4	+	+	NP
	1225	4	+	+	NP
	1231	4	+	+	NP
	1240	4	+	+	NP
	694	4	+	+	NP
	730	4	+	+	NP
	377	4	+	+	NP
	4	15	+	+	NP
C. sakazakii non-CC4	658	1	+	+	+
	1536	1	+	+	+
	680	8	+	-	+
	1	8	+	-	-
	520	12	+	-	+
	696	12	+	+	+
	ES15	125	+	-	NP
	1218	1	+	+	NP
	5	8	+	+	NP
	140	40	+	+	NP
	150	16	+	+	NP
	978	3	+	+	NP
	984	3	+	+	NP
	1249	31	+	+	NP
	HPB5174	45	+	+	NP

NP: Not performed; +: serum resistance, -: serum sensitive, ST: Sequence type, CC4: Part of clonal complex 4; which includes ST4, ST15 and ST109

4.5.6 Bacterial motility

4.5.6.1 **Motility assay**

A motility assay was used to characterize 13 *C. sakazakii* strains growing in tubes of LB broth plus 0.4% agar that were incubated at 37°C for 24 hours (**Section 2.8.3**). The strains showed variable motility (**Table 4.11**). Three ST4 *C. sakazakii* strains, 721, 1533, and 1542, showed high motility rates, while two strains, 680 (ST8) and 520 (ST12) showed no motility. The rest of the strains had low to medium motility rates, and most of the CC4 and non-CC4 strains were motile. To confirm these results, a bioinformatics analysis was performed to determine whether flagellar genes were present in these strains (**Table 4.11**).

Table 4. 11 Motility of 13 *C. sakazakii* strains.

Species	Strains	ST[a]	Motility rate
C. sakazakii	721	4	+++
C. sakazakii	1221	4	+
C. sakazakii	701	4	++
C. sakazakii	1533	4	+++
C. sakazakii	1537	4	++
C. sakazakii	1542	4	+++
C. sakazakii	1587	109[b]	++
C. sakazakii	658	1	++
C. sakazakii	1536	1	++
C. sakazakii	680	8	-
C. sakazakii	1	8	+
C. sakazakii	520	12	-
C. sakazakii	696	12	++

a: ST, sequence type.
b:CC4: Clonal complex 4.
The level of the motility production in the LB agar: +++: high motility, ++: medium motility, + Low motility and -: showed no motility.

4.5.6.2 **Flagellar genes**

Flagellar proteins associated with bacterial motility were identified previously in *Cronobacter* spp. genomes (Kucerova *et al*. 2010; Joseph *et al*. 2012a). The genomes of the 38 *C. sakazakii* strains used in this study were searched for the flagellar gene clusters *fli* (ESA_01248–61) and *flg* (ESA_02264–77) using BLAST. All of the *C. sakazakii* strains had the *flg* (ESA_02264–77) gene cluster. In contrast, the *fli* (ESA_01248–61) flagellar genes were variably present in the 38 *C. sakazakii* genomes (**Table 4.12**). All of the *C. sakazakii* strains showed the presence of *fliR*, *fliQ*, *fliP*, *fliO*, *fliF*, and *fliN* except for strain 680 (ST8) and strain 520 (ST12) (**Table 4.12**). Moreover, all had *fliM*, *fliL*, flagellar hook, *fliJ*, *fliH*, *fliG*, and *fliE* except strain 680 (ST8). The *fliI* gene was present in most of the *C. sakazakii* strains, with the exception of strain 577 (ST4) and strain 680 (ST8).

The motility test results (**Table 4.11**) showed that strain 680 (ST8) and strain 520 (ST12) were non-motile strains. In the BLAST genome analysis, strain 680 (ST8) was missing all of the flagellar genes from the *fli* cluster, whereas strain 520 (ST12) was missing only five *fli* genes (*fliR*, *fliQ*, *fliP*, *fliO*, and *fliN*) and had the rest of the *fli* genes.

Table 4. 12 The presence and absence of the *fli* (ESA_01248–61) genes reported by Joseph *et al.* (2012) in 38 *C. sakazakii* genomes.

Gene			*fliR*	*fliQ*	*fliP*	*fliO*	*fliN*	*fliM*	*fliL*	*fli-hook*	*fliJ*	*fliI*	*fliH*	*fliG*	*fliF*	*fliE*
Species	Strains	ST	ESA_01248	ESA_01249	ESA_01250	ESA_01251	ESA_01252	ESA_01253	ESA_01254	ESA_01255	ESA_01256	ESA_01257	ESA_01258	ESA_01259	ESA_01260	ESA_01261
C. sakazakii ST4	SP291	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	6	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	20	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	553	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	557	4	+	+	+	+	+	+	+	+	+	-	+	+	+	+
	558	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	721	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	767	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1219	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1220	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1221	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1225	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1231	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1240	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	701	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	694	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	730	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	377	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1533	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1537	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1542	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C. sakazakii CC4	1587	109	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		15	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C. sakazakii Non-cc4	658	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1218	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1536	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	5	8	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	680	8	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1	8	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	520	12	-	-	-	-	-	+	+	+	+	+	+	+	+	+
	696	12	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	140	40	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	150	16	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	978	3	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	984	3	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	ES15	125	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1249	31	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	HPB5174	45	+	+	+	+	+	+	+	+	+	+	+	+	+	+

ST: Sequence type
CC4: Part of clonal complex 4; which includes ST4, ST15 and ST109

4.5.7 Bacterial haemolysis

4.5.7.1 Haemolysis on blood agar

All of the *C. sakazakii* CC4 and non-CC4 strains (n=13) in this study had α-haemolysis activity on sheep blood agar and β-haemolysis activity on horse blood agar at 37°C (**Table 4.13**). *Staphylococcus aureus* was used as a control for α-haemolysis, and *Streptococcus pyogenes* was used as a control for β-haemolysis.

Table 4. 13 Haemolysis activity of *C. sakazakii* on blood agar.

Species	Strains	ST	Haemolysis	
			Sheep blood	Horse blood
C. sakazakii	721	4	α	β
C. sakazakii	1221	4	α	β
C. sakazakii	701	4	α	β
C. sakazakii	1533	4	α	β
C. sakazakii	1537	4	α	β
C. sakazakii	1542	4	α	β
C. sakazakii	1587	109*	α	β
C. sakazakii	658	1	α	β
C. sakazakii	1536	1	α	β
C. sakazakii	680	8	α	β
C. sakazakii	1	8	α	β
C. sakazakii	520	12	α	β
C. sakazakii	696	12	α	β

ST: Sequence type
CC4: Part of clonal complex 4; which includes ST4 and ST109

4.5.7.2 Haemolysis genes

The genomes of all of the *C. sakazakii* CC4 and non-CC4 strains (n=38) in this study contained the 6 haemolysis (*hly*) genes. The *hly* genes included: ESA_00102, ESA_00432, ESA_00643, ESA_02810, ESA_02937, and ESA_03540 (Joseph *et al.* 2012a). The genome analysis did not show a relationship between the presence of any of the haemolysis genes and sequence type.

4.5.8 Capsule formation

4.5.8.1 Capsule formation on milk agar

Capsule formation on whey-based formula milk agar at 37°C was investigated for all 14 strains, including 13 *C. sakazakii* and 1 *C. koseri* strain (**Table 4.14) (Section 2.8.2)**. Capsule formation varied among the strains (**Table 4.14**). Capsule production by the strains was categorized as follows: high capsule formation, ++; low capsule formation, +; or no capsule formation, -.

C. sakazakii strains 701, 1, 680 and 520 did not produce capsules (**Table 4.14**). *C. sakazakii* strain 696 and 1533 showed low capsule formation. Most of the *C. sakazakii* strains formed creamy beige mucoid colonies on milk agar, but strains 721 and 1587 produced leathery colonies that were hard to pick out on milk agar. No clear pattern emerged in terms of capsule formation on milk agar for CC4 strains versus non-CC4 strains.

Table 4. 14 Capsule formation by 13 *C. sakazakii* and 1 *C. koseri* bacterial strains on milk agar at 37°C.

Species	Strain number	ST	Milk agar at 37°C		
			Level of capsule production[a]	Colony morphology	Colony appearance[b]
C. sakazakii	721	4	++	Creamy beige, shiny, leathery	B
C. sakazakii	1221	4	++	Creamy beige, shiny, mucoid	A
C. sakazakii	701	4	-	Yellow, shiny	C
C. sakazakii	1533	4	+	Creamy beige, shiny, mucoid	A
C. sakazakii	1537	4	++	Creamy beige, shiny, mucoid	A
C. sakazakii	1542	4	++	Creamy beige, shiny, mucoid	A
C. sakazakii	1587	109*	+	Creamy beige, shiny, leathery	B
C. sakazakii	658	1	++	Creamy beige, mucoid	A
C. sakazakii	1536	1	++	Yellow, shiny, mucoid	A
C. sakazakii	680	8	-	Creamy beige	C
C. sakazakii	1	8	-	Yellow, shiny	C
C. sakazakii	520	12	-	Dark yellow	C
C. sakazakii	696	12	+	Creamy beige, shiny, mucoid	A
Citrobacter koseri	48		-	Yellow, shiny	C

*CC4: Clonal complex 4.,ST: Sequence type. ,a: Level of capsule production: high capsule formation, ++; low capsule formation, +; or no capsule formation, -. b: Colony appearance of capsule. A) Mucoid appearance; B) leathery appearance; C) no capsule formation.

4.5.8.2 **Capsule genes**

4.5.8.2.1 **Colanic acid capsule gene**

Bacterial capsule genes play critical roles in pathogenicity and in environmental interactions. Two capsular operons have been identified in *Cronobacter* spp. The first operon encodes colanic acid production genes (ESA_01155 to 01175; *wzABCKM*), while the second operon encodes capsular polysaccharide assembly and export (*kps*) genes (ESA_03349 to 03359) *(*Joseph *et al.* 2012a*).*

A BLAST search showed that all 38 *C. sakazakii* genomes had the first operon i.e. the colanic acid capsule genes region (ESA_01155–01175). This group of capsule genes is similar to the group 1 capsule genes in *Escherichia coli* (Rahn *et al.* 1999).

4.5.8.2.2 **Colanic acid regulator genes**

rcsA, *rcsB*, and *rcsF* are positive regulator genes that modulate the expression of colanic acid and the transcription of the *wca* operon in *E. coli* K-12, while the ATP-dependent Lon protease is the negative regulator of colanic acid expression (Gottesman *et al.* 1985; Gupte *et al*. 1997; Mao *et al*. 2001). The BLAST search results for *rcsA*, *rcsB*, and *rcsF* are shown in **Table 4.15**. All of the *C. sakazakii* genome strains (n=38) had the *rcsB* and *rcsF* genes. The *rcsA* gene was found in 36/38 (94%) of the *C. sakazakii* strains and was absent in two strains, strain 680 (ST8) and strain 520 (ST12).

4.5.8.2.3 **K-antigen capsule gene**

The second operon (K antigen genes), *kps* genes, was also found in the *C. sakazakii* genomes (Joseph *et al.* 2012a). The *kps* capsule cluster has three regions as defined by genomic analysis. Region 1 encodes the ESA_03349-53 genes, region 2 encodes three genes the ESA_03354-ESA_03356 and ESA_03357 genes, and region 3 encodes the ESA_03358-59 genes (**Table 4.15**) (Joseph *et al*. 2012a).

BLAST search results for the *kps* operon in all 38 *C. sakazakii* genomes are shown in **Table 4.15** All of the genomes had the region 1 *kpsEDCS* genes (ESA_03349-53). In addition, 32/38 (84%) of the genomes had the *kpsC* gene, while 6 strains did not have it: CC4 strains SP291, 20, 377, and 1587 plus strain 140 (ST40), and strain HPB5174 (ST45). Region 2, which comprises three genes (ESA_03354,

ESA_03356 and ESA_03357), was also used for a BLAST search. This region was missing in most of the strains (33/38; 86%), while it was present in 5/38 (13%): ST1 strains 658, 1218, and 1536 plus ST8 strains 5 and 680. Region 3 of the *kps* operon has two genes. One, *kpsT* (ESA_03358), was present in all 38 strains, and the second, *kpsM* (ESA_03359), was present in most of the strains (36/38; 94%); the exceptions were ST8 strain 680 and strain 1 (**Table 4.15**).

4.5.8.3 **Cellulose genes**

Cellulose genes, which include ESA_04204 (*bcsA*), ESA_04205 (*bcsB*), and ESA_04207 (*bcsC*), were found previously in *Cronobacter* species (Zogaj *et al*. 2003; Grimm *et al.* 2008; Hartmann *et al*. 2010). A BLAST search for *bcsA, bcsB*, and *bcsC* revealed that most of the 38 *C. sakazakii* strains had these genes. The exceptions were strain ES15 (ST125), which had none of the genes, and strain 20 (ST4), which lacked the *bcsC* (ESA_04207) gene (**Table 4.15**).

Table 4. 15 The presence or absence of the capsule biosynthesis (*kps*) genes (Joseph *et al*. 2012), the capsule positive regulator genes/colanic acid capsule regulator genes (*rcsABF*) and cellulose genes in 38 *C. sakazakii* genomes.

Gene description			Capsule Biosynthesis gene (*kps*)									Colanic Acid regulators Genes			Cellulose genes		
			Region 1				Region 2			Region 3							
			kpsE	*kpsD*	*kpsC*	*kpsS*	-	-	*capC*	*kpsT*	*kpsM*	*rcsA*	*rcsB*	*rcsF*	*bcsA*	*bcsB*	*bcsC*
Species	Strain	ST	ESA_03349	ESA_03350	ESA_03352	ESA_03353	ESA_03355	ESA_03356	ESA_03357	ESA_03358	ESA_03359	ESA_01247	ESA_00971	ESA_03142	ESA_04204	ESA_04205	ESA_04207
C. sakazakii ST4	SP291	4	+	+	-	+	-	-	-	+	+	+	+	+	+	+	+
	6	4	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+
	20	4	+	+	-	+	-	-	-	+	+	+	+	+	+	+	-
	553	4	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+
	557	4	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+
	558	4	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+
	721	4	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+
	767	4	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+
	1219	4	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+
	1220	4	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+
	1221	4	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+
	1225	4	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+
	1231	4	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+
	1240	4	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+
	701	4	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+
	694	4	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+
	730	4	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+
	377	4	+	+	-	+	-	-	-	+	+	+	+	+	+	+	+
	1533	4	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+
	1537	4	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+
	1542	4	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+
C. sakazakii CC4	1587	109	+	+	-	+	-	-	-	+	+	+	+	+	+	+	+
	4	15	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+
C. sakazakii Non-CC4	658	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1218	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1536	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	5	8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	680	8	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
	1	8	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
	520	12	+	+	+	+	-	-	-	+	+	-	+	+	+	+	+
	696	12	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+
	140	40	+	+	-	+	-	-	-	+	+	+	+	+	+	+	+
	150	16	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+
	978	3	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+
	984	3	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+
	ES15	125	+	+	+	+	-	-	-	+	+	+	+	+	-	-	-
	1249	31	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+
	HPB5174	45	+	+	-	+	-	-	-	+	+	+	+	+	+	+	+

+:the genes is presence with 80% of the nucleotide sequence,
-: the genes is missing less than 60% of the nucleotide sequence.
ST: Sequence type
CC4: Part of clonal complex 4; which includes ST4, ST15 and ST109

4.5.9 Genes associated with environmental fitness

The carbon starvation genes are one of the main types of stress response genes. These include the universal stress protein *uspA* (ESA_01955), the starvation response protein (ESA_03615), the carbon starvation sensing protein *rspA* (ESA_01752), and two carbon starvation proteins, ESA_00801 and ESA_00339 (Kucerova *et al*. 2010; Joseph *et al.* 2012). A BLAST search showed that the genomes of the *C. sakazakii* strains (n=38) in this study had most of the carbon starvation genes (**Table 4.16**). However, strain 1 was missing the ESA_01955 gene, strain 577 (ST4) was missing the ESA_01752 gene, and strain 680 (ST8) was missing the ESA_00801 gene (**Table 4.16**).

The carotenoid genes, which are involved in protecting bacteria from environmental stress, are also important in terms of environmental fitness. Lehner *et al*. (2006) identified an operon with 7 genes involved in carotenoid expression: *crtE*, *idi*, *crtX*, *crtY*, *crtI*, *crtB*, and *crtZ.* A BLAST search of these 7 genes revealed that most of the 38 *C. sakazakii* strains had the carotenoid genes. The exceptions were strain 680 (ST8), which had none of the genes, and strain 557 (ST4), which lacked the *crtX* (ESA_00345) gene (**Table 4.16**).

Genes associated with biofilm formation according to Hartmann *et al.* (2010) were analysed using BLAST search. Hartmann *et al.* (2010) introduced transposon mutations into two hypothetical proteins ESA_00281 and ESA_00282, found to have an effect on biofilm formation. The 38 genomes were screened for biofilm-associated genes, and these genes were present in all strains.

Table 4. 16 The presence or absence of genes associated with environmental fitness in 38 *C. sakazakii* genomes.

Genes description			Carbon starvation genes					Carotenoid gene								Biofilm associated genes	
			uspA	*sspA*	*rspA*	*cstA1*	*cstA2*	*crtZ*	*crtB*	*crtI*	*crtY*	*crtE*	*crtX*	*idi*	*crtZ*		
MLST	Strains	ST	ESA_01955	ESA_03615	ESA_01752	ESA_00801	ESA_00339	ESA_00341	ESA_00342	ESA_00343	ESA_00344	ESA_00347	ESA_00345	ESA_00346	ESA_00341	ESA_00281	ESA_00282
C. sakazakii ST4	SP291	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	6	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	20	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	553	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	557	4	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+
	558	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	721	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	767	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1219	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1220	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1221	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1225	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1231	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1240	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	701	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	694	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	730	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	377	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1533	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1537	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1542	4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C. sakazakii CC4	1587	109	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	4	15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C. sakazakii Non-CC4	658	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1218	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1536	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	5	8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	680	8	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+
	1	8	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	520	12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	696	12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	140	40	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	150	16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	978	3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	984	3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	ES15	125	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1249	31	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	HPB5174	45	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

-: Missing
+: presences
ST: Sequence type
CC4: Part of clonal complex 4; which includes ST4, ST15 and ST109

Table 4. 17 Summary of all of the experiments performed in this study and described in this chapter.

Species	Strains	ST[b]	Desiccation[c]	Heat resistance[f]	Acid resistance[g]	Serum resistance[H]	Capsule[d]	Motility[i]	Haemolysis[e]
C. sakazakii	721	4	S	R	R	R	Leathery	High	α
C. sakazakii	1221	4	MS	R	HR	R	Mucoid	Lower	α
C. sakazakii	701	4	R	R	R	R	NP	Medium	α
C. sakazakii	1533	4	HR	R	R	R	Mucoid	High	α
C. sakazakii	1537	4	HR	R	HR	R	Mucoid	Medium	α
C. sakazakii	1542	4	R	R	R	R	Mucoid	High	α
C. sakazakii	1587	109[a]	MS	S	R	R	Leathery	Medium	α
C. sakazakii	658	1	R	S	R	R	Mucoid	Medium	α
C. sakazakii	1536	1	R	S	S	R	Mucoid	Medium	α
C. sakazakii	680	8	MS	S	HS	S	NP	Non	α
C. sakazakii	1	8	MS	S	S	R	NP	Lower	α
C. sakazakii	520	12	R	R	R	R	NP	Non	α
C. sakazakii	696	12	MS	S	HS	R	Mucoid	Medium	α
C. koseri	48	6	MS	S	S	R	NP	NP	NP

HR: Highly resistant, MS: Medium sensitivity, S: Sensitive, R: Resistant

a: Clonal complex 4, which includes ST4 and ST109, b: Sequence type

c: Sub-lethally injured cells were classified as follows after desiccation in infant formula: **sensitive** (S) strains showed reductions in viability of 3.16 $\log_{10}$ CFU/ml; **moderate to sensitive** (MS) strains showed reductions in viability of 2.44 to 2.05 $\log_{10}$ CFU/ml; **resistant** (R) strains showed reductions in viability of 1.54 to 1.58 $\log_{10}$ CFU/ml; and **highly resistant** (HR) strains showed reductions in viability of 0.67 to 0.77 $\log_{10}$ CFU/ml (Section 4.5.1.3).

d: Capsule production on milk agar as in Table 4.14. Capsule appearance by the strains was categorized as follows: leathery or mucoid. NP: Not performed

e: Haemolysis in sheep blood (Section 4.5.7.1) i.e. α-haemolysis.

f: Heat resistance after 1 hour at 100°C (Section 4.5.3.2). Strains that remained viable were considered resistant (R), and strains that lost their viability were considered sensitive (S).

g: Acid resistance at pH 3.5 for 2 hours (Section 4.5.4.1). **Sensitive** (S) strains showed reductions in viability of 2.64 to 1.99 $\log_{10}$ CFU/ml; **highly sensitive** (HS) strains showed reductions in viability of 4.48 to 4.47 $\log_{10}$ CFU/ml; **resistant** (R) strains showed reductions in viability of 1.07 to 1.68 $\log_{10}$ CFU/ml; and **highly resistant** (HR) strains showed reductions in viability of 0.70 to 0.81 $\log_{10}$ CFU/ml.

h: Serum-resistance. Strains that showed increased viability of 0.09 to 1. 21 $\log_{10}$ CFU/ml were considered serum-resistant, while strains that showed reductions in viability were considered serum-sensitive.

i: The level of the motility production in the LB agar was scored as follows: high motility; medium motility; low motility or no motility as in Table 4.11 and Section 2.8.3.

4.6 Discussion

In recent years, there has been a rise in international concern about neonatal infections due to *Cronobacter* outbreaks (Holy and Forsythe, 2013). Many *C. sakazakii* outbreaks have been associated with reconstituted PIF (Himelright *et al*. 2002; Jarvis, 2005; Joseph and Forsythe, 2014), with CC4 strains being the most predominant lineages in clinical isolates and in samples from PIF and milk factories (Craven *et al*. 2010; Jacobs *et al*. 2011; Sonbol *et al*. 2013). *Cronobacter* spp. persist for a long time in food factory environments, such as in milk powder plants (Iversen *et al*. 2008; Jung *et al.* 2013; Kandhai *et al.* 2004). However, our understanding of the survival mechanisms underlying the persistence of *Cronobacter* spp. in these environments is still limited (Forsythe, 2014).

It is important to note that CC4 strains have not been studied previously with regard to their survival in situations involving various stresses. The aims of the study presented in this chapter were to investigate whether *C. sakazakii* CC4 strains were more resistant than non-CC4 strains to different stresses. Some of the specific *C. sakazakii* strains used for these experiments were selected after their sequence types were identified by MLST analysis. In addition, the collection of strains that was used was chosen so that diverse sequence types were represented. Accordingly, analyses were performed to characterise the physiological and virulence-related traits of the strains, which included 7 CC4 strains, 2 ST1 strains, 2 ST8 strains, and 2 ST12 strains.

4.6.1 Sub-lethal injury of cells during desiccation in infant formula

All of the *C. sakazakii* CC4 and non-CC4 strains were cultured on milk agar plates prior to desiccation resistance assay (**Section 2.8.5**). The experiments showed the effects of two microbiological media, TSA and VRBGA, on cell recovery after desiccation. Recovery was significantly different on TSA vs. VRBGA ($P<0.01$) (**Section 4.5.1.1 and 4.5.1.2**), and there was no correlation between sequence type (CC4 vs. non-CC4) and recovery. In particular, ST4 and ST1 strains isolated from milk factories and from milk powder showed higher recovery and fewer sub-lethally injured cells than the other STs **(Figure 4.1).** Notably, desiccation can damage the cell membrane during drying and thus reduce the viable cell count of the recovered bacteria (Gardiner *et al*. 2000; Ramos *et al.* 2001). Many factors

affect the recovery of bacteria after desiccation such as media composition, capsule formation that are discussed in the following paragraphs.

4.6.1.1 **The effect of media type on the recovery of injured cells**

PIF contains components that include lactose, milk fat, and proteins (Lian *et al*. 2002; Caubilla-Barron and Forsythe, 2007). These substances can protect the bacteria during milk drying and reconstitution of the milk and can affect their ability to survive desiccation. Milk may enhance the survival and recovery of desiccated bacteria, and this was evident in this experiment. For example, the milk component of infant formula includes factors such as lactose proteins and milk fat that may provide osmotic protection to bacteria during the formulation process, thus contributing to their ability to survive desiccation (Lian *et al*. 2002; Caubilla-Barron and Forsythe, 2007; Osaili and Forsythe, 2009). To investigate this, Lian *et al*. (2002) studied the survival of bifidobacteria isolated from spray-drying equipment and found that survival after desiccation varied according to the medium that was used. For example, recovery increased from 16.0% to 82.6% when the bacteria were dried in 10% skimmed milk. In contrast, bacterial recovery was greatly reduced from 63.74% to 8.20% when dried in 10% gelatin (Lian *et al*. 2002). Notably, the German strains 1533, 1537, 1542, and 1536 were isolated from spray-drying equipment in a factory that produced milk powder; this could explain the higher recovery of these strains compared with the clinical strains.

4.6.1.2 **Desiccation experiments and osmotolerance genes**

Previous studies showed the effects of storage temperature and storage conditions on the survival of desiccated *Cronobacter* cells in infant formula (Gurtler and Beuchat, 2005; Gurtler and Beuchat, 2007). In this study, all the 13 of the desiccated *C. sakazakii* CC4 and non-CC4 cells were recovered after desiccation at room temperature (**Section 4.5.1.1 and 4.5.1.2**). Previous studies demonstrated that at a low temperature (4°C), the survival rates of *Cronobacter* spp. strains stored in PIF were higher in low water activity conditions (a_w 0.25–0.30) than in high water activity conditions (a_w 0.43–0.50) (Gurtler and Beuchat 2005; Gurtler and Beuchat 2007).

Feeney and Sleator (2011) identified genes that are involved in osmotic stress adaptation in *C. sakazakii.* In this study, a BLAST search of 38 sequenced *C. sakazakii* strains showed that most of

the osmotolerance genes were present in the genomes of the CC4 and non-CC4 strains. Their presence might be explained as part of a resistance mechanism developed by these bacteria. The presence of *proP* genes increases the osmotolerance of many pathogenic bacteria (Feeney and Sleator, 2011). In this study, strain 680 was missing three *proP* genes, $proP^{e}$, *proW*, and $proP^{d}$. However, it still survived after desiccation. A previous study proposed that the reason that *C. sakazakii* can survive stress is because of the presence of 7 copies of the *proP* genes in the genome compared with one *proP* gene in *E. coli* (Feeney and Sleator 2011) **(Table 4.5)**. However, no variation was observed in the survival of desiccation cells between *C. sakazakii* CC4 and non-CC4 suggesting that these genes are important for the general stress response of *C. sakazakii* and not associated with a particular lineage. Further study is needed to identify the role of each bacterial gene in the response to diverse environmental stress conditions and in osmotolerance.

A previous study reported that stationary phase *Cronobacter* cells show greater resistance to dryness and to osmotic stress than other Enterobacteriaceae strains, including *Salmonella* and *E. coli* (Nazarowec-White and Farber, 1997a). This might be due to the presence of trehalose, which is a compatible solute that can withstand osmotic stress and drying and that promotes protein and membrane stability (Breeuwer *et al.* 2003). Breeuwer *et al*. (2003) showed that study showed that the accumulation of trehalose in stationary phase *Cronobacter* spp. increases more than 5-fold during desiccation. In this study, BLAST tools were used to search 38 *C. sakazakii* strains for the trehalose genes reported by Freeney and Slator (2011) **(Table 4.6).** These genes, *otsA* and *otsB*, were present in all of the strains **(Table 4.6),** indicating that these genes play critical roles in desiccation resistance and in osmotolerance. *E. coli* uses trehalose as the only carbon source in low and high osmolarity conditions, thereby preventing denaturation of the cellular membrane proteins (Horlacher and Boos, 1996). This suggests that further work is needed to investigate the link between trehalose and desiccation.

Additionally, stress response associated gene *rpoS* was also investigated (**Section 4.2.2.3**); the full length gene was noted in all the sequenced *C. sakazakii* isolates with very little sequence variation at the nucleotide level **(Figure 4.2).**

4.6.1.3 **The relationship between isolate source and desiccation resistance**

The result of this study showed that the isolate source was associated with the recovery of injured cells after desiccation **(Section 4.5.1).** For example, strains isolated from factory environments and exposed to stress had a lower level of injured cells, with reductions of only ~0.82 $\log_{10}$ CFU/ml for strain 1533 (ST4), strain 1537 (ST4), strain 658 (ST1), and strain 1536 (ST1). This contrasted with the greater reduction, about ~3.16 $\log_{10}$ CFU/ml, of the clinical isolates: 721 (ST4), 1221 (ST4), 701 (ST4), 1587 (ST4), 680 (ST8), 1 (ST8), 520 (ST12), and strain 696 (ST12) (**Section 4.5.1.3**). Strains exposed to environmental stress might develop adaptations that are adapted to the specific stressor. In this study strains 1533, 1536, 1542 and 1537 were isolated from the roller dryer and spray dryer. A study by Arku *et al.* (2008) found that a higher number of *Cronobacter* spp. strains that were exposed to spray drying survived desiccation for up to 12 weeks compared with desiccated cells that had not been exposed to spray drying. They also found that 35% of *Cronobacter* spp. were recovered in dry reconstituted skimmed milk after spray drying. Another researcher found a correlation between thermotolerance and the ability to withstand high osmotic and desiccation stress (Breeuwer *et al.* 2003; Dancer *et al*. 2009). The results of this study may support the hypothesis that strains exposed to environmental stress exhibit greater resistance than strains that are not exposed to stress. A study by Caubilla-Barron and Forsythe (2007) examined the recovery of desiccated *C. sakazakii* strains, including isolates from dried sources such as herbs, spices, and PIF, and found that there was no correlation between the source and the recovery. Most of these strains, which were recovered after 2.5 years, were isolated from clinical samples and infant formula.

4.6.1.4 **The effect of bile salt on the recovery of injured cells**

A previous study showed that the media type could influence the recovery of injured cells (Caubilla Barron and Forsythe, 2007). If the outer membrane is affected or damaged by desiccation stress, the injured cells will die rather than recover; such bacteria are considered to be sensitive to VRBGA medium (Merritt and Donaldson, 2009). In the desiccation study, VRBGA medium was used as a selective medium for investigating sub-lethally injured cells after desiccation. Medium containing bile salts has a toxic effect on Enterobacteriaceae, even on cells that have not been under stress. Some

Enterobacteriaceae are resistant to the high concentrations of bile found throughout the gastrointestinal tract. Bile functions as a bactericidal agent to help protect the host from pathogenic bacteria in the human digestive system. Bile salts can harm both bacterial DNA and the bacterial cell membrane (Merritt and Donaldson, 2009). Merritt and Donaldson (2009) demonstrated that bacterial strains with lower levels of sub-lethally injured cells in bile salt medium could be classified as bile-resistant strains. They further suggested that the bacteria that survive exposure to large quantities of bile salts might also have the ability to establish invasive infections.

This was observed in the present study by the recovery of the *C. sakazakii* strains on VRBGA medium (**Section 4.5.1.2**). The strains showed further reductions in viability on VRBGA, exhibiting greater sensitivity to desiccation stress; thus, the strains might not recover when exposed to bile salt in the stomach. The following strains showed high levels of sub-lethally injured cells: 721 (ST4), 1221 (ST4), 701 (ST4), 1587 (ST4), 680 (ST8), 1 (ST8), 520 (ST12), and 696 (ST12) **(Section 4.5.1.2; Figure 4.1).** This may be due to damage to the cell wall after desiccation that made the cells more sensitive and thus less able to recover on VRBGA.

4.6.2 Acid resistance

The gastric pH in the infant stomach is, on average, pH 3.5 after consumption of reconstituted PIF, pH 4.3 after consumption of ready-to-feed formula, and pH 2.5 after consumption of breast milk (Hurrell *et al.* 2009). Although many studies show that *C. sakazakii* can grow at pH ~4.5 to 4.1, acid tolerance varies according to the strain type and the acid type (Alvarez-Ordóñez *et al.* 2012a; Kim *et al.* 2012; Zhu *et al*. 2013). In addition, *C. sakazakii* can be resistant to low pH (pH 3.5) for a long time (Edelson-Mammel *et al*. 2006). Notably, the latter study was performed before the taxonomic revision of *Cronobacter* spp. in 2007 and before MLST was used to determine the sequence type.

Most CC4 strains were resistant to acid treatment and remained viable at pH 3.5 for up to 2 hours, with cell counts ranging between 5.86 and 6.90 $\log_{10}$ CFU/ml (a slight decrease compared to the starting cell count) (**Section 4.5.4.1**) (**Table 4.17**). Strain 1221 (ST4) and strain 1537 (ST4) were highly resistant to exposure to pH 3.5 for 2 hours. Strains 1221 and 1537 were obtained from a meningitis case and from a milk powdered processing plant, respectively (**Table 4.1**). In contrast,

most non-CC4 strains, 680 (ST8), 1 (ST8), 1536 (ST1), and 696 (ST12) showed dramatic declines in viability after exposure to pH 3.5 for 2 hours. However, unlike other non-CC4 strains, strain 520 (ST12) showed an acid-resistant phenotype against acid and tolerated pH 3. 5 treatment (**Table 4.17** and **Section 4.5.4.1**). A study by Kim and colleagues (2012) showed that pre-exposure of *C. sakazakii* to acidic conditions allowed the cells to adapt to harsher conditions, thereby improving their resistance to environmental stress such as heat, acidic pH, and organic acids (Kim *et al.* 2012). The observations in the present study may support that CC4 strains are predominant in clinical infections and that they can survive in stressful conditions, hence increased isolation from the environment, especially from the environment of milk powder processing factories (Joseph and Forsythe, 2011**).** The same observation is valid for ST1 strains e.g. strain 658, which show moderate to high resistance to acid stress and are predominantly isolated from clinical and infant formula (Joseph *et al*. 2012; Sonbol *et al.* 2013). Bacteria that can survive such low pH conditions may be more likely to infect neonates, and exposure after ingestion of contaminated infant formula may be one of the virulence factors.

The present study investigated the roles that the capsule plays in the survival of *C. sakazakii* CC4 and non-CC4 strains at pH 3.5. Capsule production is another factor that might contribute to the survival of bacteria in acid conditions. Most of the CC4 strains were acid resistant and formed capsular material e.g mucoid or leathery matrix on milk agar plate **(Table 4.14).** ST4 strains 1221 and 1537 that formed mucoid material showed high resistance to pH 3.5 over time **(Figure 4.5).** In addition, strain 1536, a non-CC4 strain, showed high resistance to acid, and this strain produced highly mucoid material **(Figure 4.6).** Strain 658 produced capsule material that formed a mucoid matrix. Many studies have shown the effects of bacterial biofilm and capsule formation in countering the acidity of the stomach (McNell and Hamilton, 2003; Iversen *et al*. 2004; Kim *et al.* 2006: Jung *et al*.2013). McNell and Hamilton (2003) studied the association between survival at low pH and increased biofilm formation. However, a study by Dancer *et al.* (2009) showed that there was no relationship between increased biofilm formation and survival at low pH. Iversen *et al*. (2004) reported that capsulated *Cronobacter* spp. strains form biofilms after growth in infant formula at 37°C for 24 hours, resulting in their adherence to various baby bottle materials, such as silicon, latex, and polycarbonate. This might be due to the colanic acid capsule. Another study by Kim *et al.* (2006) studied biofilm

formation by *Cronobacter* spp. in enteral feeding tubes in three different media: TSA, infant formula agar (IFA), and lettuce juice agar (LJA). They found that the strains formed more biofilms when they were grown in TSA and IFA than in LJA due to differences in media composition, such as protein levels. They suggested that the formation of biofilms in enteral feeding tubes caused cell clumps that might protect the bacteria from stomach acid due to capsule formation. Another study reported that growth methods and media formulation affect the attachment of *S. enterica* and *Listeria monocytogenes* to stainless steel surfaces (Hood and Zottola, 1997; Kim *et al.* 2006).

The variation in the viability of CC4 and non-CC4 isolates at low pH 3.5 was interesting. Most of the CC4 strains survived the low pH conditions with only 0.70 to 1.69 $\log_{10}$ CFU/ml reductions in viability while non-CC4 strains showed an average reduction of 5.86 to 6.90 $\log_{10}$ CFU/ml **(Figure 4.6).** A study by Dancer *et al.* (2009) found a correlation between the cross-resistance of strains to thermal and heat dehydration and to low a_w and low pH. Strain 1542 (CC4), which was isolated from a roller dryer, showed dramatic declines over time, indicating its sensitivity to low pH. When infants consume contaminated milk, the bacteria are subjected to acid stress in the baby's gastrointestinal tract. This may induce the expression of acid shock proteins that make the bacteria more tolerant of low pH in the stomach and intracellular environments and thus help them survive in the host (Alvarez-Ordóñez *et al.* 2014). The bacterial acid tolerance response (ATR) leads to greater resistance to lethally low pH levels after exposure to either low pH or after temporary exposure to slightly acidic environments (Alvarez-Ordóñez *et al.* 2012b). Researchers have studied the ATR in *C. sakazakii* in many different growth conditions (Edelson-Mammel *et al*. 2006; Kim *et al.* 2012; reviewed in Alvarez-Ordóñez *et al.* 2014). Alvarez-Ordóñez *et al*. (2014) confirmed the ability of *C. sakazakii* to develop an adaptive stationary-phase ATR, which may play a role in bacterial tolerance to low acid conditions and in the ability of the cells to multiply in the infant intestinal system after ingestion via contaminated milk formula. Recent study showed that OmpR is a response regulator of the acid stress response in *C. sakazakii* (Alvarez-Ordóñez *et al*. 2014). Alvarez-Ordóñez *et al*. (2014) introduced a transposon-mediated mutation into the *C. sakazakii ompR* gene and examined subsequent growth in two types of acidified media, i.e. acidified LB and acidified PIF broth. A study by Bang *et al*. (2000) showed an association between the *ompR* regulator gene and the *S. Typhimurium* stationary-phase

ATR during the adaptation of the bacteria after 2-hour exposure to pH 4.3 during culture in minimal media. Alvarez-Ordóñez *et al.* (2014) showed the induction of a stationary-phase ATR when *C. sakazakii* with a defective *ompR* gene was grown in LB broth with extra (1% w/v) glucose. This condition produces a stationary-phase ATR similar to that of the wild type strain (Alvarez-Ordóñez *et al.* 2014). In addition, when *C. sakazakii* DPC6529 is inoculated into pH 5 PIF, the growth rate increases by ~1.5 log after incubation at 37°C for 4 hours. As seen in the acid resistance experiment **(Figure 4.5; Figure 4.6)** that was performed in this study showed a variation in resistance to pH3.5, whereas most of the CC4 isolates were acid resistant. Additionally, the sequences of *ompR* were aligned to construct the phylogeny tree as shown in **Figure 4.7.** The analysis was interesting as all of the CC4 isolates clustered closely with each other (**Figure 4.7**). The non-CC4 isolates clustered separately according to the sequence type. The variation in *ompR* gene sequence between CC4 and non-CC4 could be important and may, to some extent explain the variation in the host's susceptibility.

Digestion and the emptying time of the stomach following ingestion of contaminated infant formula must also be considered when studying neonatal *C. sakazakii* infections. Indeed, work by Alvarez-Ordóñez *et al.* (2014) indicates that despite its low concentration in contaminated PIF, *C. sakazakii* in the neonate stomach may increase to critical levels prior to full completion of gastric digestion. Notably, limited data are available regarding neonate stomach emptying time, although Bodé *et al.* (2004) reports that the halftime for stomach emptying is 30–180 min (T1/2) for preterm infants.

4.6.3 Heat-tolerance

4.6.3.1 Heat tolerance of CC4 isolates after desiccation stress

Many researchers have reported that *C. sakazakii* can survive heat and dry conditions for long periods (Nazarowec-White and Farber 1997; Breeuwer *et al*. 2003; Edelson-Mammel *et al.* 2005; Dancer *et al.* 2009). In many outbreaks, neonatal infections due to *Cronobacter* spp. have been linked with contaminated PIF (Muytjens *et al.* 1983; Biering *et al*. 1989; Van Acker *et al*. 2001; Himelright *et al.* 2002). Several studies have isolated *Cronobacter* spp. from milk powder processing facilities (Kandhai *et al*. 2004; Mullane *et al*. 2007; Craven *et al.* 2010; Reich *et al*. 2010; Jacobs *et al*. 2011; Hochel *et al*. 2012). Also, several MLST studies demonstrated that *C. sakazakii* CC4 is more often

associated with neonatal meningitis cases than any other sequence type and that CC4 is the most predominant sequence type isolated from PIF factories (Joseph *et al*. 2012b; Sonbol *et al.* 2013).

Heat-tolerance experiments were carried out in this part of the study to investigate whether the survival of CC4 versus non-CC4 strains differed at different temperatures after drying, which could provide insights into the survival mechanism used by the more heat-resistant CC4 strains **(Section 2.8.6).** In this study, *C. sakazakii* CC4 and non-CC4 strains were investigated for their ability to tolerate a range of temperatures 60°C, 80°C, and 100°C, for 30 and 60 minutes in the dry (desiccated) state **(Sections 4.5.3.1 and 4.5.3.2)**. Generally, after 30 minutes of heat exposure, there was a trend of reduced viable cell count with increasing temperature **(Figure 4.3).** All of the CC4 strains showed survival after 30 minutes of exposure to the different temperatures. The CC4 strains 1533, 1537 and 1542 were isolated from a roller dryer and spray dryer at a milk powder processing factory in Germany that has a temperature range between 150°C to 160°C (Jacobs *et al*. 2011). On the other hand, the non-CC4 strains showed variation **(Figure 4.3).** For example, strains 680 (ST8), 696 (ST12), 658 (ST1), and strain 1536 (ST1) showed reduction in viability when they were rehydrated after being exposed in their desiccated state to 100°C heat for 30 minutes **(Figure 4.3).**

In contrast, after 60 minutes of heat exposure, there was little variation between strains within the same sequence type **(Figure 4.4).** After exposure to 100°C for 60 minutes, all of the CC4 strains remained resistant to heat except for strain 1587. On the other hand, all of the non-CC4 strains, i.e. ST12 strain 696, ST1 strains 658 and 1536, and ST8 strains 680 and 1, remained viable after rehydration only after heat treatment at 60°C, with very few viable cells detected after exposure to 100°C, with the exception of strain 520 (ST12) **(Figure 4.4).** Hence at 100^{o}C, CC4 strains appear to be more heat tolerant than non-CC4 strains **(Figure 4.4).** The cross-tolerance conditions to which bacteria are exposed, such as desiccation following high temperature, may influence their survival after rehydration. Particularly, the PIF manufacturing environment presents several types of stress to bacteria, which could induce cross-tolerance to these stressors (Gruzdev *et al.* 2011). For example, when *Salmonella* is desiccated, it develops cross-tolerance to other types of stress compared to non-desiccated cells (Gruzdev *et al*. 2011). Another factor that may contribute to survival during heat-tolerance is the ability of bacteria to persist in low water activity environments of a_w 0.25–0.30, such

as the PIF, which might be associated with bacterial osmotolerance (Gurtler and Beuchat, 2005; Gurtler and Beuchat, 2007; Feeney and Sleator, 2011). Other researchers suggested that the observation of heat resistance in desiccated *Salmonella* and *E. coli* strains is a physical phenomenon and not a genetic criteria that is linked to a reduction in the denaturation of bacterial intracellular proteins as a result of low water content (Hiramatsu *et al.* 2005).

4.6.3.2 **Heat tolerance and capsule**

The composition of the capsule polysaccharides is another factor that might affect heat-tolerance. *Cronobacter* spp. produce exopolysaccharide (EPS) capsular materials such as colanic acid that provides a protective physical barrier to environmental stresses and help the bacteria avoid the host immune system (Harris *et al*. 1989; Lehner *et al*. 2005). In addition, colanic acid contributes to bacterial attachment to the host cell and to biofilm formation, and it also helps protect *E. coli* cells against different types of environment stress (Mao *et al*. 2001;Whitfield, 2006). Mao *et al*. (2001) inserted a kanamycin resistant gene cassette into two genes namely *wcaD* and *wcaE*, in *E. coli* O157:H7. These 2 genes are part of the 21 *wca* operon genes necessary for the colanic acid synthesis. The resultant mutant strains showed less tolerance to heat (60°C) than the parent strain (Mao *et al*. 2001). BLAST search revealed that *wcaE* and *wcaF* were present in all (n=38) of the *C. sakazakii* sequenced strains**.** Previous studies reported that some *C. sakazakii* strains such as strain ATCC 12868 and strain ATCC 29004 secreted EPS colanic acid (Harris *et al*. 1989; Lehner *et al.* 2005).

At 37^{o}C, this study found that most of the *C. sakazakii* CC4 strains 1221, 1533, 1537 and 1542 produced creamy beige mucoid colonies (**Figure 4.4; Table 4.14**). However, with regards to heat tolerance there is some variation among the leathery strains e.g 721 was heat resistant whereas 1587 was heat sensitive at 100^{o}C for 1 hour **(Figure 4.4; Table 4.14).** Furthermore, it must be noted ST1 strains 658, 1536 and ST12 strain 696 produced mucoid colonies on milk agar but were all sensitive to heat at 100^{o}C for 1 hour **(Figure 4.4).** The genome analyses revealed that all of the 13 *C. sakazakii* strains used in heat tolerance assay in this study were positive for colanic acid synthesis genes **(Section 4.5.8.2.1)**. The heat tolerance assays indicated that all of the tested strains were able to survive at 60^{o}C for up to 1 hour. On the other hand, at 100^{o}C only CC4 strains were able to survive

for one hour whereas all except one non-CC4 strains (520-ST12) were not viable. It is suggested that CC4 might have additional factors responsible for their heat tolerance at 100^{O}C and therefore warrant further investigation.

4.6.3.3 **Thermotolerance genes**

C. sakazakii in the milk powder factory environment and in PIF is proposed to be a route of transmission in many neonatal outbreaks (Nazarowec-White and Farber 1997; van Acker *et al*. 2001; Caubilla-Barron *et al.* 2007; Craven *et al*. 2010). *Cronobacter* thermotolerance genes have been investigated by many researchers (Nazarowec-White and Farber 1997; Breeuwer *et al*. 2003; Iversen *et al*. 2004; Dancer *et al*. 2009). A study by Edelson-Mammel and Buchanan (2004) investigated the survival of *Cronobacter* strains in rehydrated infant formula at 58°C and found two heat-tolerant phenotypes, i.e. thermosensitive and thermotolerant phenotypes, that had 20-fold differences in their D-values (0.5–9.9 minutes).

An 18kb region that includes 22 open reading frames (ORFs) has been associated with thermotolerance in *C. sakazakii* (Gajdosova *et al.* 2011). Additionally the study suggested that 4 of these 22 ORFs i.e. *orfHIJK* were linked experimentally with thermotolerance in *E. coli* at 58^{o}C as cloning of these ORFs caused a two-fold increase in thermotolerance (Gajdosova *et al*. 2011). Interestingly, Gajdosova *et al*. (2011) found that this region is missing from *C. sakazakii* strain BAA-894, which was associated with a PIF recall in response to the Tennessee outbreak (Himelright *et al.* 2002). In the present study, genomic analysis of the above mentioned region containing these thermotolerance genes showed variation between CC4 and non-CC4 strains **(Table 4.8).** With the exception of strain 1 (ST8), the remaining non-CC4 strains in this study lacked these genes **(Table 4.8).** On the other hand, the genes were variably present in CC4 strains. In addition, the current study revealed that the CC4 strain *C. sakazakii* SP291 which has been regarded as a persistent thermotolerant isolate (Power *et al*. 2013; Yan *et al*. 2013) lacked this region **(Table 4.8).** Another CC4 strain 1533 isolated from roller dryer isolate lacked this region. Similarly, the non-CC4 isolate, 520 (ST12) lacking this region showed high resistant to heat at 100^{o}C for 1 hour **(Figure 4.4; Table 4.8)**. Furthermore, in this study BLAST search showed that *C. sakazakii* ST1 strain BAA-894 (NTU

ID 658) which was associated with a PIF recall in response to the Tennessee outbreak (Himelright *et al.* 2002) also lacked this region. Hence, the present analysis suggested that the thermotoerance genes proposed by Gajdosova *et al*. (2011) may not be the only genetic factors linked with thermotolerance in *C. sakazakii.*

4.6.4 Serum resistance

The ability of *C. sakazakii* to invade a host and cause infections is related to a strategy that protects it from the bactericidal effects of human serum. The cell surface of each bacterium has outer membrane proteins, a capsule, and a lipopolysaccharide layer that all play roles in mediating resistance to serum (Rautemaa and Meri, 1999; Schwizer *et al*. 2013). A study by Franco *et al*. (2011) found that the plasmid borne plasminogen activator gene (*cpa*) which encodes an outer membrane protease, is essential for serum resistance in *C. sakazakii*. A number of *C. sakazakii* strains in this study were assayed for their ability to survive in human serum. Most of the strains persisted and replicated in serum, similar pattern to the positive controls, *C. koseri* and *S. enterica*. Only strain 1 (ST8) was sensitive to human serum after 3 hours, with the viable count declining dramatically to under the detection limit **(Figure 4.8; Table 4.10).**

With regard to the presence of the *cpa* gene in *C. sakazakii* genomes, most of the strains that had this gene were serum resistant **(Figure 4.8; Table 4.10).** However, the present analysis could not determine correlation between serum resistance and presence of *cpa* as the strain 520 and 680 which were found to be serum resistant **(Figure 4.8)** lacked *cpa* (these were plasmid less strains, Sumyya Hariri, Unpublished data). These observations suggest that another factor might contribute to serum resistance.

It is possible that proteolytic activity could differ among strains and influence serum resistance. A plasminogen activator *(cpa*) gene has been regarded as essential for serum resistance in *C. sakazakii* by Franco *et al*. (2011). Their study showed that the *cpa* expression level affects the proteolytic activity of the strains in serum. Specifically, they found that the overexpression of *cpa* in the recombinant *C. sakazakii* BAA-894 strain enhanced plasminogen activation, α2-AP inactivation, and the cleavage of complement factors compared to normal *cpa* expression levels in the wild-type strain

BAA-894 (Franco *et al.* 2011). They suggested that the low proteolytic activity of the BAA-894 strain could be due to repression by the O-antigen (Franco *et al*. 2011). Notably, the long O-antigen factors in smooth lipopolysaccharide (LPS) inhibit the proteolytic activity of omptin proteins (Kukkonen *et al*. 2004; Franco *et al*. 2011). The serum resistance assay conducted in the present study indicated that *C. sakazakii* BAA-894 (658) was serum resistant **(Figure 4.8; Table 4.10)**

Although the *cpa* gene is considered to be a key factor in mediating serum resistance in *C. sakazakii*, many other genes may also contribute to serum survival. A transposon mutagenesis study conducted by Schwizer *et al.* (2013) showed that deletion of certain structural and regulatory genes in *C. sakazakii* strain ES5 improved its survival in serum as compared to the parent strain. Notably, the YbaJ protein a part of the antitoxin YbaJ-Hha complex; deletion of ybaJ results in lower expression of the main component of type 1 fimbriae, leading to improved survival in human serum. It is worth mentioning that the absence of the main element of the type 1 fimbriae probably is the reason for the enhanced survival of this mutant in human serum. Although this mutant showed increased serum resistance, type 1 fimbriae are important for bacterial adhesion and pathogenicity. Furthermore, there is a link between the invasion of human brain microvascular cells by *E. coli* K1 and the presence of type 1 fimbriae (Adegbola and Old, 1983; Teng *et al.* 2005). In a parallel PhD study, the type 1 fimbriae genes (*fimABCDEFGHI*) of *E. coli* K-12 were aligned with *C. sakazakii* genomes, however none of the homologue was identified in any of the isolate (Naqash Masood; unpublished data).

Phan *et al.* (2013) used the transposon mutagenesis method to study the association between serum resistance and LPS in *E. coli* and found that 46 genes were involved in serum resistance. They reported that mutation of the *wcaF* gene, which encodes a protein needed for colanic acid biosynthesis, resulted in an altered LPS profile and in sensitivity to high osmolarity. This mutant strain also altered the synthesis of colanic acid, which led to a decrease in membrane integrity (Phan *et al.* 2013). There is high homology (82%) between the proteins encoded by the *E. coli* K-12 *wcaF* gene and by the *C. sakazakii* (Genbank number: ESA_01164) **(Table 4.15)**; the latter was present in all of the strains in the present study **(Section 4.5.8.2).** The homology in WcaF protein sequences of *E. coli* and *C. sakazakii* suggests that its role in *C. sakazakii* is similar to that of *wcaF* in *E. coli*. Phan *et al*. (2013) also identified the murein lipoprotein *lpp* gene, which is strongly associated with serum resistance in

E. coli (Phan *et al*. 2013). This gene was found in the genomes of all of the *C. sakazakii* strains in this study **(Table 4.10).**

Furthermore, EPS plays a significant role in protecting and repairing the cell wall after damage by serum components (Miajlovic *et al*. 2014). The colanic acid capsule is another factor that may contribute to bacterial survival in serum (Gupte *et al*. 1997). The Rcs pathway regulates colanic acid biosynthesis triggered by stress that affects the envelope structure or murein integrity (Miajlovic *et al*. 2014). Previous studies showed that *rcsA* is the main regulator of the colanic acid capsule genes (Gottesman and Stout, 1991; Gupte *et al*. 1997). The present study analysed the *rcsA* gene in *C. sakazakii* genomes, however the presence of this gene was not correlated with the serum resistance assays. The strains 520 and 680, which lacked *rcsA*, were serum resistant, but strain 1 (ST8), which had *rcsA*, was serum sensitive **(Table 4.10).**

4.6.5 Motility

Joseph *et al.* (2012) identified the flagellar genes in *Cronobacte*r spp. In this study, a motility assay was performed using selected *C. sakazakii* CC4 and non-CC4 strains to investigate a possible correlation between motility and CC4 and non-CC4 strains **(Section 4.5.6 and Table 4.11).** The present study indicated that *C. sakazakii* strains belonging to specific sequence types showed variation in their motility rates **(Table 4.11).** For example, ST4 strains 721, 1533, and 1542 had high motility rates, whereas strain 1221 (ST4) had a low motility rate **(Table 4.11).** For the non-CC4 strains, ST12 strain 520 and ST8 strain 680 were non-motile, whereas strain 696 (ST12) showed lower motility **(Table 4.11).**. The genomes of the strains were searched for flagellar gene sequences using BLAST, and the non-motile strain 520 (ST12) was missing the *fliR*, *fliQ*, *fliP*, *fliO*, and *fliN* genes. In addition, strain 680 (ST8) was missing all of the flagellar genes in the *fli* cluster **(Table 4.12).** The only strains that did not show any kind of motility were strains 520 (ST12) and 680 (ST8). This analysis determined that motility is associated with the presence of five flagellar genes *fliRQPON*. Therefore, the absence of these genes led to a reduced motility rate in *C. sakazakii.* A recent study showed that the flagella of *C. sakazakii* contribute to bacterial survival in the human macrophages (Cruz-Córdova *et al*. 2012). A study by Cruz-Córdova *et al*. (2012) showed that flagella and the flagellin protein in

C. sakazakii ST1 and ST4 strains elicit a high inflammatory response in human macrophages by stimulating the production of IL-8 and TNF-α, which are neutrophil chemoattractants. They further demonstrated that this inflammatory response was due to TLR5 signalling produced by flagella.

Hartmann and co-workers (2010) have observed a relationship between flagella and environmental persistence in terms of biofilm formation. Their study showed that the flagella of *C. sakazakii* contribute to biofilm production on abiotic surfaces and to their attachment to Caco-2 intestinal epithelial cells. Hartmann *et al*. (2010) introduced transposon mutations into two (hypothetical) proteins i.e. ESA_00281 and ESA_00282 and reduced the biofilm formation. In the present analysis biofilm associated genes ESA_00281-ESA_00282 were screened in 38 *C. sakazakii* isolates. The analysis suggested that both of these genes were present uniformly in all *C. sakazakii* strains indicating their presence is not specific to any particular lineage and hence important for *C. sakazakii* in general.

4.6.6 Haemolysis

Virulence factors contribute to the pathogenicity of bacteria, and bacterial toxins such as haemolysin are some of the most important virulence factors. Some bacteria like *E. coli* O:157 are capable of haemolysing red blood cells which is important to begins infection such as Haemolytic Uremic Syndrome (Wong *et al.* 2012). There are three types of haemolysin: α-haemolysin, γ-haemolysin, and β-haemolysin. In this study, different *C. sakazakii* sequence types (ST1, ST4, ST12, and ST8) were assayed for their ability to lyse sheep and horse erythrocytes **(Section 4.5.7.1)**.

Assaying haemolysis activity on blood agar in the laboratory did not show any variations among CC4 and non-CC4 strains **(Table 4.13).** All of the *C. sakazakii* strains showed α-haemolytic activity on sheep blood and ß-haemolytic activity on horse blood **(Table 4.13).** Previous genomic analysis on *C. sakazakii* had identified six haemolysin-related genes, which involved ESA_00102, ESA_02810, ESA_00432, ESA_00643, ESA_02937, and ESA_03540 (Joseph *et al.* 2012b). All 38 *C. sakazakii* CC4 and non-CC4 genomes in this study, had all of the haemolysis genes **(Section 4.5.7.2)**.

Furthermore, a study, which is related to haemolysis genes in *E. coli*, clarified that the *hly* deletion reduced toxicity in mice significantly (Hacker *et al.* 1983; Scheffer *et al.* 1995). In a parallel PhD

study, haemolysin associated genes *hlyABCD* of *E. coli* O157:H7 were screened for their presence in 30 *C. sakazakii* genomes, however none of the homologue was detected in any of the isolate (Naqash Masood; unpublished data). To summarize, it is still unclear whether these are the only genes responsible for haemolytic activity or if other virulence factors are also involved. Additional studies are needed to understand the function of these genes and virulence potential in pathogenicity.

4.6.7 Capsule production

Cell surface structures are among the many factors that contribute to bacterial resistance to environmental stress, and they represent the first line of protection for a bacterium. Capsule polysaccharides play significant roles in mediating resistance to desiccation (Reckseidler-Zenteno 2012). The polysaccharide capsule is a common characteristic of bacteria, and it causes infections in the host by involvement in serum survival (Reckseidler-Zenteno *et al*. 2005). It also protects the bacteria from the harmful effects of desiccation by forming a hydrated gel that surrounds the bacterial cell surface (Reckseidler-Zenteno 2012). In addition, mucoid strains of *E. coli*, *Acinetobacter calcoaceticus*, and *Erwinia stewartii* are more desiccation-resistant than non-mucoid strains (Reckseidler-Zenteno, 2012). In *E. coli* K-12, exopolysaccharides capsule (EPS) is linked to desiccation resistance and to the final stages of biofilm formation (Danese *et al.* 2000). Studies indicated that *Cronobacter* spp. can produce many types of extracellular material; as a result, mucoid colonies and biofilms can form in devices such as nasogastric feeding tubes and infant feeding equipment (Caubilla-Barron *et al.* 2007; Hurrell *et al.* 2009a; Hurrell *et al.* 2009b; Joseph *et al.* 2012).

In this study, it was found that most of the CC4 strains produced capsule materials, mucoid or leathery, more than the non-CC4 strains except strain 701 **(Table 4.14).** The non-CC4 strains 1 (ST8), 520 (ST12) and 680 (ST8) did not produce mucoid colonies despite their clinical association (**Table 4.14**). Most of the environmental *C. sakazakii* strains 1533 (ST4), 1537 (ST4), 1542 (ST4), 1536 (ST1) and 658 (ST1) produced creamy beige mucoid colonies while the CC4 strains 721 and 1587 which caused meningitis produced hard to pick leathery colonies when grown on the milk agar medium. (**Table 4.14**). The study indicated that there were different levels of mucoid production in

different sequence types. The mucoid colony production in *C. sakazakii* is likely to be associated with capsule production however it requires verification using the Indian Ink test.

4.6.8 Capsule genes

4.6.8.1 Colanic acid capsule genes

Desiccation increases the expression of the colanic acid polysaccharide capsule biosynthetic genes (*cps* genes) in *E. coli* (Ophir and Gutnick, 1994; Reckseidler-Zenteno, 2012). Joseph *et al.* (2012) identified the *cps* genes in *C. sakazakii* genomes. In bacteria, the capsule is a highly hydrated surface layer that provides protection against host defences, especially phagocytosis. As with many Enterobacteriaceae, *E. coli* produces many EPSs, including colanic acid (Ophir and Gutnick, 1994; Mao *et al*. 2001; Reckseidler-Zenteno, 2012). Colanic acid contains L-fucose, pyruvate, D-galactose, D-glucuronic acid, and D-glucose and creates a thick mucoid material on cell surfaces (Mao *et al*. 2001). In *E. coli* K-12, colanic acid biosynthesis is linked to the *wca* gene cluster, which contains 21 open reading frames (ORFs) (Stevenson *et al*.1996; Mao *et al*. 2001). In these ORFs, two genes, *wcaC* and *wcaE*, encode colanic acid glycosyl transferases, and *wcaD* encodes a colanic acid polymerase. These three genes are responsible for the serial transfer of the colanic acid sugar elements and for construction of the colanic acid polymer (Stevenson *et al*.1996; Mao *et al*. 2001). Colanic acid contributes to bacterial attachment to the host cell and to biofilm formation, and it also helps protect *E. coli* cells against different environment stresses (Whitfield, 2006).

The present study suggested that 7 of 13 tested *C. sakazakii* isolates were able to produce creamy beige mucoid colonies on milk agar (**Table 4.14**); this was similar to the *E. coli* colanic acid capsule described by Mao *et al*. (2001), which contributes to bacterial resistance to heat and acid.

In this study, capsule production was shown to contribute to acid survival (**Figures 4.5** and **4.6**). Two ST4 strains, strain 1221 and 1537, as well as ST1 strain 1536, showed high resistance to pH 3.5 over time. Notably, these strains produced highly mucoid material (**Table 4.14**). However, due to the presence of these genes in all the *C. sakazakii* genomes **(Section 4.5.8.2),** it may not correlate with the mucoid production as it varied amongst all the tested strains **(Table 4.14)**

4.6.8.2 **Colanic acid capsule regulator genes**

This study investigated the genes involved in colanic acid capsule production and in the mucoid phenotype in the *C. sakazakii* genome. Two strains 520 and 680, did not show mucoid appearance when grown on milk agar **(Table 4.14).** This might be because they were missing the *rcsA* gene, which encodes RcsA, the main regulator of the colanic acid capsule genes (Gottesman and Stout, 1991; Gupte *et al*. 1997). The Rcs pathway regulates colanic acid biosynthesis triggered by stress that affects the envelope structure or murein integrity (Miajlovic *et al*. 2014). This includes cell interactions with solid surfaces, and acid stress can trigger Rcs activation (Miajlovic *et al*. 2014).

4.6.8.3 **The polysaccharide capsule genes (K-antigen)**

The *kps* capsule cluster has three regions as defined by the genomic analysis. Region 1 encodes the ESA_03349-53 genes, region 2 encodes three genes the ESA_03354, ESA_03356 and ESA_03357 genes, and region 3 encodes the ESA_03358-59 genes (**Table 4.15**) (Joseph *et al*. 2012a). As in *E. coli*, this capsular polysaccharide (K- antigen genes) has three regions, two regions (regions 1 and regions 3) have homologies with region 1 and 3 in *C. sakazakii* genome. Regions 1 and 3 of the capsule operon of *E. coli* K1 include gene clusters implicated in the biosynthesis of enzymes and transport proteins (Jiménez *et al*. 2013).

Three clusters of K-antigen genes were analysed using *Cronobacter* BLAST **(Section 4.5.8.2).** The cluster 2 was missing uniformly from all the CC4 and non-CC4 isolates apart from ST1 and ST8 strains **(Table 4.15).** The remaining two clusters showed no specific pattern for the presence/absence between CC4 and non-CC4. The presence of these genes did not correlate with the mucoid production as it varied amongst all the tested strains **(Table 4.15).** All of the CC4 strains apart from strain 701, showed the production of creamy, beige and shiny colonies while the appearance of the mucoid production varied in non-CC4 isolates. However, the colony morphology did not correlate with STs in non-CC4 isolates as differences in the appearance were observed between the isolates of same sequence type **(Table 4.15).** Region 2 of *E. coli* K1 encodes glycosyltransferases and other enzymes needed for the biosynthesis of the K-antigen-specific gene (Jiménez *et al*. 2013). Region 2 of *E. coli* K1 contains the *neu* genes, which direct the biosynthesis, activation, and polymerization of N-

acetylneuraminic acid (NeuNAc), a polysialic acid (Leying *et al*. 1989; Vann *et al*. 2004). The protein encoded by region 2 of *E. coli* K1 generates a polysialic layer that provides resistance to the alternative complement pathway and mediates resistance to serum killing (Leying *et al*. 1989; Vann *et al*. 2004).

Also, in the desiccation study, there was a high level of sub-lethally injured cells in strains 721, 701, 1221, 1587, 680, 520, 696 and 1 **(Section 4.5.1.3).** However, strain 1533, 1542, and 1537 showed lower levels of sub-lethally injured cells after desiccation **(Section 4.5.1.3).** A study by Lehner *et al.* (2005) assessed the production of extracellular materials and the persistence of *Cronobacter* (unspeciated) strains on different media. They found that 24 out of 56 isolates produced milky viscous colonies that they assumed to be extracellular polysaccharide capsules. Caubilla-Barron and Forsythe (2007) studied the correlation between capsulation of *Cronobacter* spp. strains and the levels of sub-lethally injured cells. They found that capsulated *Cronobacter* spp. strains had significantly ($P<0.05$) lower levels of sub-lethally injured cells than non-capsulated strains. In contrast, our study found no difference between capsulated and non-capsulated strains.

Lehner *et al.* (2005) used high-performance liquid chromatography to analyse these materials and found glucose, galactose, fucose, and glucuronic acid. Another study by Riedel and Lehner (2007) used proteomics to study the surface structures of *C. sakazakii* strain z236 and found many proteins related to desiccation survival and osmotic tolerance. In their study they found that four genes (ESA_03349, ESA_03350, ESA_03352, ESA_03353) encoded for capsule polysaccharide transport *kpsE, kpsD, kpsC* and *kpsS* genes respectively, these proteins were up regulated during desiccation. The genome analysis showed that *kpsE, kpsD* and *kpsC* genes were presence in all the 38 *C. sakazakii* geneome except *kpsC* gene which was presence in 32/38 (84%) of the genomes and missing in 6 strains SP291(ST4), 20(ST4), 377(ST4), 1587(ST4), 140 (ST40), and strain HPB5174 (ST45).

4.6.8.4 **Cellulose genes**

Studies have shown that cellulose is involved in the interactions of bacteria with host tissues and in the interactions of cells with surfaces that are resistant to bacterial colonization. Many Gram-negative bacteria, including *Salmonella* spp. and *E. coli*, have genes that are essential to cellulose biosynthesis

and regulation (Ross *et al*. 1991; Zogaj *et al*. 2001). These genes are encoded by the *bcsABZC* and *bcsEFG* operons, and the *bcs* genes are known to be important for bacterial cellulose synthesis (Ross *et al*. 1991; Zogaj *et al*. 2001). Studies have also identified genes that are homologues of genes in the cellulose synthase operon in *Cronobacter* species (Frank and Chmielewski 2001; Zogaj *et al*. 2003; Grimm *et al.* 2008; Hartmann *et al*. 2010). A study performed by Hartmann and co-workers showed that the generation of *C. sakazakii* ES5 mutants by random transposon mutagenesis of *bcsA*, *bcsC*, and *bcsG* results in reduced biofilm formation in a microtiter plate assay. Recently, an isogenic mutagenesis study of the *bcsA* and *bcsB* genes in *C. sakazakii* BAA894 strains by Hu *et al.* (2015) showed that the *bcsA* and *bcsB* genes are required to produce cellulose and are involved in biofilm production and in cell-cell aggregation. Cellulose synthase genes are also involved in the persistence of *Cronobacter* in the environment and in foods (Hu *et al.* 2015). Our study analysed 38 sequenced *Cronobacter* genomes and found variations in the cellulose genes in different STs. Most of the 38 *C. sakazakii* strains had all three cellulose genes, i.e. *bcsA, bcsB*, and *bcsC*. The exceptions were strain ES15 (ST125), which had none of the genes, and strain 20 (ST4), which lacked the *bcsC* (ESA_04207) gene (**Table 4.15**).

4.6.9 Other genes associated with environmental fitness

4.6.10 Carotenoid genes

It has been suggested that carotenoids might mediate resistance to drying, and a study by Johler *et al.* (2010) showed the impact of pigmentation on cell growth on osmotic stress media and persistence under desiccation conditions. Specifically, the researchers evaluated the persistence of *C. sakazakii* strain ES5 in environmental stress conditions using the genes responsible for pigment expression. Their hypothesis was that carotenoids had structural roles in the bacterial cell membrane and that they might help protect the cells against desiccation (Wiesyaw *et al.* 2005; Johler *et al.* 2010). Notably, desiccation leads to higher levels of reactive oxygen species that damage the bacterial cells, and carotenoids decrease the level of oxidative destruction by decreasing oxygen radicals (Johler *et al.* 2010). They found that the colourless mutants were more sensitive than the wild type strain to long-term desiccation. In this study, a BLAST search showed that the carotenoid genes were present

in most of the strains. However, strain 680 (ST8) lacked all of the genes, strain 557 (ST4) lacked the *crtX* (ESA_00345) gene, and strain 4 (ST15) had a partial match with the same gene (**Table 4.16**). The desiccation resistance assays conducted in the present study showed that all of the studied *C. sakazakii* isolates were able to resist desiccation including 680; hence it is difficult to correlate the presence of carotenoid genes with desiccation resistance in *C. sakazakii*.

4.6.11 Carbon starvation genes

The Csr system plays an important role as a universal regulator for many bacterial physiological and virulence mechanisms (Dubey *et al.* 2003). The Csr system consists of three main factors: the RNA binding protein CsrA plus two small untranslated RNA molecules, CsrB and CsrC. CsrA controls the inhibition of gluconeogenesis, glycogen production and catabolism, and biofilm production, and it also initiates glycolysis, acetate uptake, and flagellum production (Dubey *et al.* 2003).

A previous study showed that the expression of the *cstA* gene in *E. coli* was induced by carbon starvation (Dubey *et al.* 2003). Indeed, Dubey *et al.* (2003) studied the expression of a cstA′-′lacZ translational-fusion in wild type and *csrA* mutant strains and found that the expression levels in a *csrA* mutant strain grown in Luria broth (LB) were two-fold greater than in the wild type. In addition, the *csrA* mutant showed greater expression (about a 5–10-fold increase) compared to the wild type when grown in LB with additional glucose (Dubey *et al.* 2003). In this study, a BLAST search showed that most of the 38 *C. sakazakii* strains had the carbon starvation genes (**Table 4.16**). However, strain 1 was missing the ESA_01955 gene, strain 577 (ST4) was missing the ESA_01752 gene, and strain 680 (ST8) lacked the ESA_00339 gene (**Table 4.16**). These genes are important regulator proteins, and *C. sakazakii* is known to persist in many environmentally stressful conditions. Accordingly, these genes may play important roles in persistence under starvation conditions, and further study is important.

The experiments presented in this chapter investigated the associations of *C. sakazakii* persistence with a number of key genes involved in osmotolerance and desiccation, capsule production, thermotolerance, and carbon starvation. The results of the study of the survival of bacteria confirmed the survival of the *C. sakazakii* in different stress conditions and this may support the presence of

bacteria in PIF as well as its ability to survive spray drying. Hence, *C. sakazakii* may replicate when infant formula is reconstituted, and exposure to heat may cause it to acquire adaptations to high temperature during the preparation of infant formula. The analysis showed that these genes were not unique to specific STs. Moreover, the findings indicated that there were no correlations between sequence type and the presence or absence of these genes. Hence, the attention of the present study shifted to focus on the outer membrane proteins profile.

CHAPTER 5: Primary characterisation of the outer membrane proteins of the neonatal meningitic pathovar *C. sakazakii* ST4

5.1 Introduction

The Gram-negative bacterial cell wall has three principal layers: the outer membrane (OM), the peptidoglycan cell wall, and the inner membrane (IM). The two membrane layers delineate two aqueous compartments, the periplasm and the cytoplasm (Nikaido, 2003; Ruiz *et al.* 2006; Galdiero *et al.* 2012). Outer membrane proteins (OMPs) are considered potential cell surface virulence factors for many Gram-negative bacteria. They not only play important roles in adaptation to changes in environmental conditions, they also contribute to bacterial adhesion and invasion and in bacterial resistance to toxic agents (Carlsohn *et al.* 2006; Galdiero *et al.* 2012). Indeed, although the general structure of the OM is highly preserved in Gram-negative bacteria, there are differences in the OMs of different species (Nikaido, 2003; Ruiz *et al.* 2006) (**Figure 5.1**). There are two main types of integral OMPs: **lipoproteins** that are attached to the periplasmic side of the OM via N-terminal lipid modifications and **β-barrel**-containing proteins that extend from the OM and facilitate molecular exchanges and cell interactions with the environment (Ricci and Silhavy, 2012). The β-barrel structure serves two major functions: it allows the transport/insertion of the protein into the OM, and it helps facilitate solute transport through the OM. In addition, small bacterial β-barrel proteins can act as membrane protein anchors, helping the bacteria adhere to host cells and bind to membrane-tethered enzymes, such as phospholipases and proteases (Misra, 2012). Porins are proteins with 3 channels (a trimer of β-barrel proteins) that span the cellular membranes and act as pores that allow the diffusion of some types of molecules (Misra, 2012) (**Figure 5.1**). Notably, porins act as non-specific transporters that allow hydrophilic molecules smaller than ~700 Da, such as sugars and ions, to diffuse across the OM (Ricci and Silhavy, 2012). OmpF, PhoE, and OmpC are well-characterized porins in *E. coli* (Ricci and Silhavy, 2012). Other types of OMPs, including the maltodextrin transporter LamB and the sucrose channel ScrY, also aid in the diffusion of specific substrates (Van Gelder *et al.* 2001). OMPs are involved in many cellular activities, like energy-dependent efflux (TolC), active transport (FhuA and BtuB), adhesion (Ag43), secretion (autotransporter proteins), pilus synthesis (FimD), OM syntheses (BamA and LptD), proteolysis (OmpT), peptidoglycan binding (OmpA), and the stress response (OmpL) (Tamm *et al.* 2004; Ricci and Silhavy, 2012).

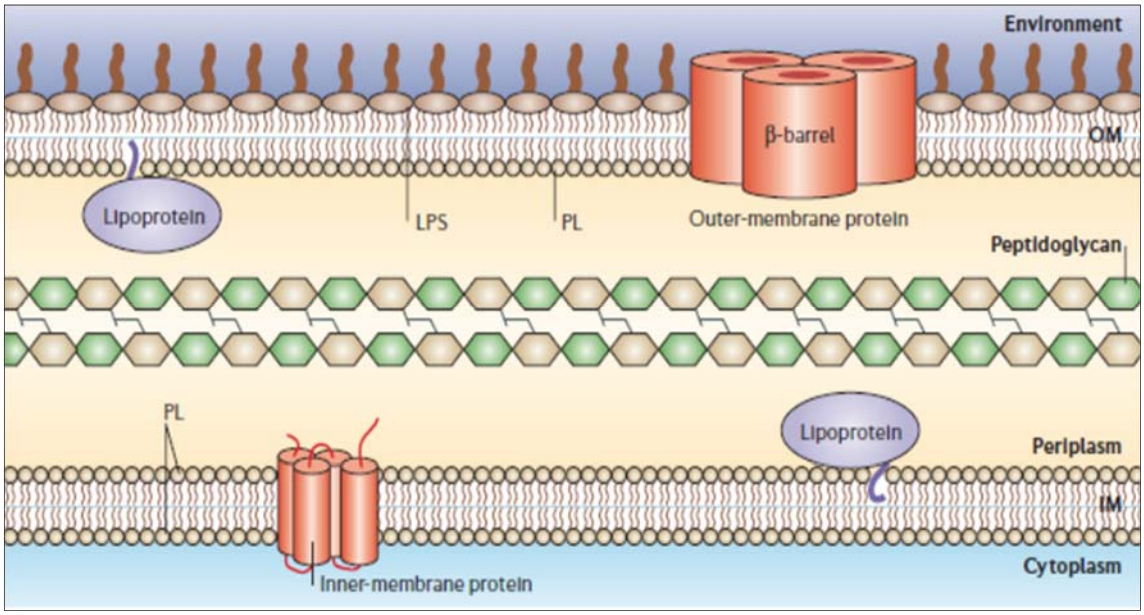


Figure 5. 1The general structure of the cell envelope in Gram-negative bacteria (From Lagha *et al.* 2012).

A bacterial cell undergoes physiological changes in response to changing environmental conditions. Previous studies showed that OMP expression depends on the growth conditions and on the surrounding environment (Puente *et al.* 1991; Wibbenmeyer *et al.* 2002; Lagha *et al.* 2012). In addition, many studies have investigated the effects of different types of stress, such as heat, acid, and serum stress, on OMP expression profiles in many Gram negative bacteria (Smith, 2003; Wu *et al.* 2009; Gu *et al.* 2012; Walczak *et al.* 2012). For example, changes in pH stimulate the expression of many OMPs, including OstA and TolC (Wu *et al.* 2009; Walczak *et al.* 2012). Further, in most bacteria, low pH affects the function of the cell wall by inducing changes in lipid composition that reduce membrane fluidity (Brown *et al.* 1997; Jain and Sinha, 2008). Notably, membrane fluidity is essential for membrane functionality and can affect biochemical synthesis, protein transport, and protein secretion (Brown *et al.* 1997; Jain and Sinha, 2008). *Cronobacter* can tolerate acidic or alkaline pH environments, both short- and long-term, but growth at low or high pH can affect its virulence, its survival in food products, and its development of control strategies (Smith, 2003). The acidic environment of the stomach acts as the host's first line of defence and as a natural barrier against pathogens (Smith, 2003). A recent study by Alvarez-Ordóñez *et al.* (2014) showed that the envelope stress response gene *ompR* is the main gene that mediates *C. sakazakii* acid tolerance. Riedel and Lehner (2007) used MALDI-TOF mass spectrometry (MS) to show that OmpC is expressed at

high levels in high osmolarity conditions and during desiccation stress in *C. sakazakii* strains. In addition to MALDI-TOF MS, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) has been used widely to characterise OMP profiles (Helmuth *et al.* 1985; Kim *et al.* 2010b).

C. sakazakii can cause systemic infections, such as meningitis and bacteraemia (Muytjens *et al*. 1983; Van Acker *et al*. 2001; Block *et al.* 2002; Caubilla-Barron *et al*. 2007). Until now, few studies have investigated the virulence factors and the pathogenic mechanisms of *Cronobacter* spp. and their associations with neonatal infections (Mohan Nair and Venkitanarayanan, 2007; Kim and Loessner, 2008; Singamsetty *et al.* 2008; Kim *et al.* 2010b). However, infection begins with the colonisation of mucosal surfaces, followed first by translocation of the bacteria into the intestinal lumen and second by bacteria crossing the blood-brain barrier, thus evading the host immune system (Kim and Loessner, 2008). Townsend *et al.* (2007) reported that *Cronobacter* spp. can penetrate rat brain endothelial cells and persist inside human macrophages, and other studies showed that *Cronobacter* can invade many human and animal tissues, especially epithelial and endothelial cells (Mohan Nair and Venkitanarayanan, 2007; Kim and Loessner, 2008; Singamsetty *et al.* 2008). A study by Mohan Nair *et al.* (2009) reported that *C. sakazakii* OmpA, a virulence factor that is required for HBMEC invasion, also plays a role in the pathogenesis of neonatal *C. sakazakii* meningitis. Another study by Kim *et al.* (2010a) reported that in *C. sakazakii,* both OmpX and OmpA are essential for the basolateral invasion of host cells such as spleen and liver cells.

5.2 Aims

C. sakazakii OMPs play significant roles in bacterial survival by allowing the cells to adapt to various environmental conditions as well as to the host defence system (Riedel and Lehner,2007; Kim *et al.* 2010a). These proteins have a huge effect on the bacterial cell wall, and they mediate cell protection and enhance bacterial persistence in different environments. The primary aim of the analysis presented in this chapter was to construct the outer membrane proteins (OMP) profile of the selected *C. sakazakii* isolates in order to observe if there is any variation between *C. sakazakii* CC4 and non-CC4 strains. The study had the following objectives:

- *In silico* analysis of the selected OMPs that were identified previously in *Cronobacter* spp. and *E. coli*-mediated bacterial adaptation response to the host environment using the *Cronobacter* BLAST
- Extraction of outer membrane proteins (OMPs) of selected *C. sakazakii* strains after growing them at pH 6 and 3.5 using SDS-PAGE analysis
- Correlation of the OMP profiles and serotypes

5.3 Materials and Methods

The detailed methods, including the buffers, media, and incubation conditions used in the OMP studies, are described in **Sections 2.5, 2.6**, and **2.9**. **Tables 5.1** and **5.2** list the strains used in OMP profile construction and genomic analysis.

Table 5. 1 Details of the *C. sakazakii* strains used in the outer membrane protein extraction and profile analysis

Bacterial species	NTU ID	ST[a]	Clonal complex[b]	Source	Year	Country	Details
C. sakazakii	721	4	4	Clinical	2003	USA	Neonate aged 2 weeks; Meningitis
C. sakazakii	1221	4	4	Clinical	2003	USA	Neonate > 1 month; Meningitis
C. sakazakii	701[c]	4	4	Clinical	1994	France	Peritoneal fluid isolate, PFGE 2, fatal NECIII infant case.
C. sakazakii	1533[d]	4	4	Environment	2006	Germany	Drying milk powder tower
C. sakazakii	1537[d]	4	4	Environment	2009	Germany	Roller dryer powder
C. sakazakii	1542[d]	4	4	Environment	2009	Germany	Roller dryer
C. sakazakii	1587	109	4	Clinical	2000	Israel	baby girl, 36 week gestation, Fed infant formula. CSF isolate
C. sakazakii	658	1	1	Environment	2001	USA	Non-infant formula
C. sakazakii	1536[d]	1	1	Environment	2009	Germany	Roller dryer
C. sakazakii	680	8	8	Clinical	1977	USA	Spinal fluid isolate
C. sakazakii	1	8	8	Clinical	1980	USA	Throat isolate from child (ATCC 29544)
C. sakazakii	520	12	12	Clinical	1983	Czech Republic	Details unknown
C. sakazakii	696	12	12	Clinical	1994	France	Faecal isolate. NECII infant
Citrobacter koseri	48	6	-	-	-	US	Comparative control organism

a: Sequence types (ST).
b: clonal complex (CC).
c: French outbreak isolates (Caubilla-Barron *et al.* 2007)
d: Strains isolated from milk powdered factory in Germany (Jacobs *et al.* 2011)
NECII: necrotizing enterocolitis.
CSF: cerebrospinal fluid.
-: not available.

Table 5. 2 Details of the *C. sakazakii* strains used in the genomic analysis.

C. sakazakii strain NTU ID (International culture collection code)	ST[a]	Country of origin	Source	Year of isolation
1	8	USA	Clinical (throat)	1980
4	15(CC4)	Canada	Clinical	1990
5	8	Canada	Clinical	1990
6	4	Canada	Clinical	2003
20	4	CR	Clinical (faeces)	2004
140	40	Unknown	Non-clinical (spice)	2005
150	16	Korea	Non-clinical (spice)	2005
377	4	UK	Milk powder	1950
520	12	Czech Republic	Clinical	1983
553	4	Netherlands	Clinical	1977
557	4	Czech Republic	Clinical	1979
558	4	Netherlands	Clinical	1983
658 (ATCC BAA-894)	1	USA	Non-infant formula	2001
680	8	USA	Clinical	1977
696	12	France	Clinical	1994
694	4	France	Clinical	1994
701	4	France	Clinical	1994
721	4	USA	Clinical (CSF)	2003
767	4	France	Clinical (trachea)	1994
730	4	France	Clinical	1994
978	3	UK	Clinical (internal feeding tube)	2007
984	3	UK	Clinical (internal feeding tube)	2007
1218	1	USA	Clinical (CSF)	2001
1219	4	USA	Clinical (CSF)	2002
1220	4	USA	Clinical (CSF)	2003
1221	4	USA	Clinical (CSF)	2003
1225	4	USA	Clinical (blood)	2007
1231	4	NZ	Clinical (faeces)	2004
1240	4	USA	Clinical (CSF)	2008
1249	31	UK	Clinical	2009
HPB5174	45	Ireland	Powdered infant formula factory	Unknown
SP291	4	Ireland	Powdered infant formula factory	2012
ES15	125	Korea	Whole grain	Unknown
1533	4	Germany	Environment(drying milk powder tower)	2006
1536	1	Germany	Environment(roller dryer)	2009
1537	4	Germany	Environment(Roller dryer)	2009
1542	4	Germany	Environment(roller dryer)	2009
1587	109(CC4)	Israel	Clinical	2000

[a]:Sequence types (ST).
[b]:Clonale complex 4 (CC4), this included ST4, ST15 and ST109.
CR: Czech Republic; NZ: New Zealand; UK: United Kingdom; USA: United States of America

5.4 Results

5.4.1 *In silico* analysis of selected OMPs mediated bacterial adaptation response to environmental stress

In Gram-negative bacteria, the production of OMPs is often regulated by environmental conditions (Lin *et al.* 2002). Moreover, OMPs play essential roles in the pathogenicity of bacteria by improving environmental adaptations (Lin *et al.* 2002). The final part of the current PhD study aimed to improve our understanding of the *C. sakazakii* strains used in this study and to confirm the importance of specific OMPs associated with virulence or environmental persistence, such as the iron transport system and desiccation associated genes (http://PubMLST.org/perl/bigsdb/). Specifically, to predict similarities in OMP function, the amino acid sequences of selected well characterised OMP proteins of *C. sakazakii* and *E.coli* that are known to be associated with virulence and physiologic adaptation **(Table 5.3)** were used in a BLASTp search to predicted amino acid sequences encoded by *C. sakazakii*. This was followed by BLAST searches to determine the presence or absence of the genes (nucleotide sequences) in the *Cronobacter* PubMLST database (n=38 *C. sakazakii* sequenced genomes).

Table 5. 3 List of the selected OMPs protiens search for homologys in *C. sakazakii*

***E. coli* K12 from Kim *et al.* (2014)**		***C. sakazakii***			
Gene	**Protein**	**BLASTp**	**AA**	**Strain**	**Reference**
fimD	P30130	ESA_01974	878	ATCC BAA-894	Kucerova *et al.*2010
fepA	P05825	BN126_1669	746	680	Joseph *et al.*2012
fecA	P13036	BN126_RS04970	774	680	Joseph *et al.*2012
fhuA	P06971	BN129_1161	747	701	Joseph *et al.*2012
fhuE	P16869	ESA_02242	729	ATCC BAA-894	Kucerova *et al.*2010
ompA	P0A910	CSSP291_11140	346	SP291	Power *et al.*2013
ompX	P0A917	ESA_02526	171	ATCC BAA-894	Kucerova *et al.*2010
ompC	P06996	CSSP291_04915	367	SP291	Power *et al.*2013
ompF	P02931	CSSP291_RS11130	362	SP291	Power *et al.*2013
ompR	EDV67786	ESA_04334	239	ATCC BAA-894	Kucerova *et al.*2010
ompT	P09169	CSSP291_20538	317	SP291	Power *et al.*2013
pcoB	Q47453	BN128_3022	296	696	Power *et al.*2013
phoE	P02932	CSSP291_RS14270	351	SP291	Power *et al.*2013
lamB	P02943	CSSP291_RS00385	446	SP291	Power *et al.*2013
ostA	P31554	CSSP291_RS15090	784	SP291	Power *et al.*2013

- **Iron receptors**

Kim *et al*. (2001) reported that in *E. coli*, the most well characterised iron receptors are FepA (81 kDa), the ferric dicitrate transport protein FecA (80.5 kDa), FhuA (78 kDa), and FhuE (76 kDa). BLAST was used to search the genomes of 38 *C. sakazakii* strains for the nucleotide sequences of the genes encoding the FepA, FecA, FhuA, and FhuE proteins. *fepA* and *fhuE* were present in all of the *C. sakazakii* CC4 and non-CC4 strains. *fecA* was present in 8 CC4 strains 6, 721, 1220, 1221, 1240, 1225, 1533, and strain 4, 2 ST8 strains 680 and 1, and was absent from the other strains. *fhuA* was present in all of the CC4 strains and most of the non-CC4 strains except (n=5) including ST12 strains 520 and 696; ST125 strain ES15; ST40 strain 140; ST45 strain HPB5174 (**Table 5.4**).

- **Desiccation resistance, acid tolerance and osmotolerance related proteins**

BLAST was used to search *C. sakazakii* genomes for the genes encoding *ompA, ompF, ompX, ompR* and *ompC* (**Table 5.3).** The *ompA*, *ompC, ompR* and *ompX* genes were present in all 38 genomes, while *ompF* was present in all of the CC4 and most of the non-CC4 strains. It was absent from 6 strains, this included ST1 strains 658, 1536 and 1218; from ST3 strains 978 and 984; and from ST31 strain 1249 (**Table 5.4).**

- **Serum resistance**

OmpT was characterised previously as an OM plasminogen activator protease that is involved in serum resistance in *E. coli* strain (Hui *et al.* 2010). A BLAST search of the 38 *C. sakazakii* genomes showed that *ompT* was present in most of the CC4 strains except strain 6 (ST4) and in most of the non-CC4 strains, except for ST8 strains 680 and 1, ST12 strain 520, and ST125 strain ES15 (**Table 5.4**)

- **Solvent resistance**

E. coli shows sensitivity to n-hexane, and the 87-kDa OMP OstA protein is involved in bacterial resistance to solvents (Abe *et al.* 2003). BLAST search revealed that the *ostA* (*lptD*) gene was in all the *C. sakazakii* CC4 and non-CC4 strains (**Table 5.4**).

- **Antibiotic resistance**

PhoE is an OM porin that is induced in *E. coli* K-12 when it is grown under phosphate-limited conditions (Bauer *et al.* 1989). A BLAST search showed that *phoE* was present in all of the *C. sakazakii* CC4 and in most of the non-CC4 strains except ST16 strain 150 (**Table 5.4**).

- **Maltose uptake and transport**

The LamB protein (47 kDa) is involved in the uptake and transport of maltose through the OM in *E. coli* and is required for bacterial growth under limited maltose condition (Braun *et al.* 2001; Vazquez-Juarez *et al.* 2004; Baldwin *et al.* 2011). Previous studies using genomic hybridization and genomic sequence analysis revealed the presence of LamB in *Cronobacter* spp. (Kucerova *et al.* 2010; Joseph *et al.* 2012). A BLAST search showed that the *lamB* gene was present in all 38 *C. sakazakii* genomes (**Table 5.4**).

- **Copper transport**

The element copper is important for bacterial growth and is essential as a cofactor for many enzymes. *pcoB* is involved in transferring copper into the periplasm, where it is oxidised by PcoA, a multicopper oxidase (Djoko *et al.* 2008). Genomic analysis by Joseph *et al.* (2012) revealed the presence of the copper resistance gene cluster in *C. sakazakii* BAA-894 (ESA_04238–45). A BLAST search showed that the *pcoB* gene was present in most of the CC4 strains except strains 20,721, 767, 1221, 701 and 694 while detected in 8/15 non-CC4 strains. The non-CC4 isolates 680 (ST8), 520 (ST12), 140 (ST14), 150 (ST16), 978 (ST3), 984 (ST3), ES15 (ST125) and 1249 (ST31) lacked *pcoB* (**Table 5.4**).

- **Adhesion-associated OMPs**

In *E. coli*, the OM fimbrial usher protein FimD (MW 91.4 kDa) is essential for the assembly of adhesive type 1 pili and for their translocation through the OM (Nishiyama *et al.* 2003). BLAST searches found that this gene was only present in *C. sakazakii* ST1, ST3, and ST125 strains (6 out of 38 strains). These strains were 658 (ST1), 1218 (ST1), 1536 (ST1), 978 (ST3), 984 (ST3), and strain ES15 (ST125) (**Table 5.4**).

Table 5. 4 BLAST search for the presence or absence of genes encoding selected OMPs in the genomes of 38 *C. sakazakii* strains plus the role(s) of the proteins in bacterial interactions with the host or environment.

Protein function			OM usher protein fimD	Ferrienterobactin receptor	Fe(3+) dicitrate transport protein FecA	Ferrichrome-iron receptor	Iron Ion Binding/Receptor Activity	OM protein A	OM protein X Adhesin	OM protein C	OM protein F	ompR transcriptional regulator	Protease 7	Copper resistance protein B	Stress Response OM pore protein E	Maltoporin	LPS-assembly protein LptD
CC	Strains	ST	*fimD*	*fepA*	*fecA*	*fhuA*	*fhuE*	*ompA*	*ompX*	*ompC*	*ompF*	*ompR*	*ompT*	*pcoB*	*phoE*	*lamB*	*ostA*
CC4 strains	SP291	4	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+
	6	4	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+
	20	4	-	+	-	+	+	+	+	+	+	+	+	-	+	+	+
	553	4	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+
	557	4	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+
	558	4	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+
	721	4	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+
	767	4	-	+	-	+	+	+	+	+	+	+	+	-	+	+	+
	1219	4	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+
	1220	4	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1221	4	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+
	1225	4	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1231	4	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+
	1240	4	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	701	4	-	+	-	+	+	+	+	+	+	+	+	-	+	+	+
	694	4	-	+	-	+	+	+	+	+	+	+	+	-	+	+	+
	730	4	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+
	377	4	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+
	1533	4	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1537	4	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+
	1542	4	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+
	1587	109	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+
	4	15	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
non-CC4	658	1	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+
	1218	1	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+
	1536	1	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+
	5	8	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+
	680	8	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+
	1	8	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+
	520	12	-	+	-	-	+	+	+	+	+	+	-	-	+	+	+
	696	12	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+
	140	40	-	+	-	-	+	+	+	+	+	+	+	-	+	+	+
	150	16	-	+	-	+	+	+	+	+	+	+	+	-	-	+	+
	978	3	+	+	-	+	+	+	+	+	-	+	+	-	+	+	+
	984	3	+	+	-	+	+	+	+	+	-	+	+	-	+	+	+
	ES15	125	+	+	-	-	+	+	+	+	+	+	-	-	+	+	+
	1249	31	-	+	-	+	+	+	+	+	-	+	+	-	+	+	+
	HPB5174	45	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+

+: Gene present.-: Gene absent .ST: Sequence type. CC: clonal complex

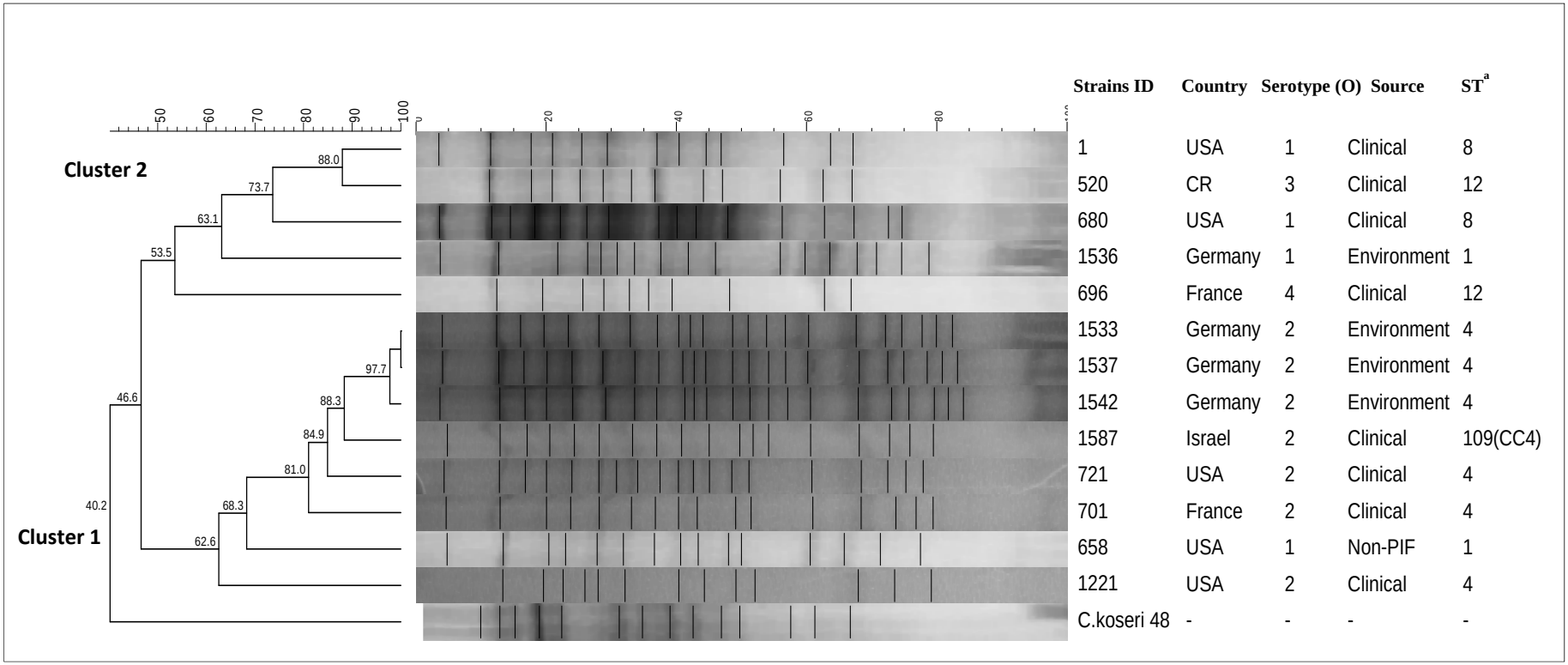
5.4.2 Investigation of the OMP profiles at pH 6 versus pH 3.5

5.4.2.1 OMP profiling at pH 6

5.4.2.1.1 Analysis of the *C. sakazakii* OMP profiles at pH 6

C. sakazakii strains were cultured at pH 6 in BHI medium to an optical density of 0.9–1.3 at 600 nm, and then the OMPs were extracted as described in **Chapter 2**, **Section (2.9.2).** A total of 10 µg of protein was loaded per lane for each sample for SDS-PAGE separation. The stained gels were analysed using BioNumerics software version 6.5, as described in **Chapter 2**, **Section (2.9.2, 2.9.3**, **and 2.9.4**). The analysis, which clusters proteins with similar profiles, was performed after band assignment and generation of a dendrogram.

In this study, 13 *C. sakazakii* ST4 and non-ST4 strains that were isolated from clinical and environmental sources were selected for OMP profile analysis. The *C. sakazakii* strains included 7 CC4 strains and 6 non-CC4 strains: 2 ST8 strains, two ST1 strains, and two ST12 strains (**Table 5.1**). One *C. koseri* strain was included for comparative purposes. There were 6–22 bands per OMP sample, with an average of 13 bands per sample. The BLUeye Prestained Protein Ladder (10 bands; 10–245 kDa; S6-0024; Geneflow, UK) was used as the MW ladder (**Figures 5.2 and 5.3**). The OMP dendrogram showed 13 OMP patterns and grouped the strains into two main clusters (**Figure 5.2**).



Strains ID	Country	Serotype (O)	Source	ST[a]
1	USA	1	Clinical	8
520	CR	3	Clinical	12
680	USA	1	Clinical	8
1536	Germany	1	Environment	1
696	France	4	Clinical	12
1533	Germany	2	Environment	4
1537	Germany	2	Environment	4
1542	Germany	2	Environment	4
1587	Israel	2	Clinical	109(CC4)
721	USA	2	Clinical	4
701	France	2	Clinical	4
658	USA	1	Non-PIF	1
1221	USA	2	Clinical	4
C.koseri 48	-	-	-	-

Figure 5. 2 Cluster analysis of the outer membrane protein (OMP) SDS-PAGE profiles of 13 *C. sakazakii* strains and one *C. koseri* strain (strain 48) from cells cultured at pH 6.

The analysis was performed using BioNumerics software, version 6.5. Dice's coefficient and the unweighted pair group method with arithmetic mean (UPGMA) were used for cluster analysis. ST, sequence type. Dendrogram clusters were defined at a 47% similarity level, while subclusters were defined at a 70% similarity level. Cluster 1 had one subcluster that included six strains, strains 1533, 1537, 1542,1587, 721, and 701, plus two outlier strains, strains 658 and 1221. Cluster 2 had one subcluster that included strains 1, 520, and 680, plus two outlier strains, strains 1536 and 696.The isolates were grouped based on the Dice coefficients (relative band intensity) with optimisation at 0.5% and band match tolerances of 1%.

The OMP dendrogram showed 13 OMP patterns and clustered the strains into two main groups and the level of similarity for these strains was 47%.(**Figure 5.2**).

Cluster 1 had mostly CC4 strains and just one non-CC4 strain, strain 658 (ST1) (**Figure 5.2**). This OMP cluster C.sakC6–C.sakC12, had one sub-cluster , that included six isolates: 1533 (ST4), 1537 (ST4), 1542 (ST4), 1587 (ST109), 721 (ST4) and 701 (ST4), plus two outlier strains, 1221 (ST4), and 658 (ST1) (**Table 5.5**). Interestingly, the level of similarity for strains 1533, 1537, and 1542 was 98%. These strains showed similar OMP profiles, and about 22 bands were identical in all three strains. In addition, strains 1537 and 1533 had identical protein profiles and had a level of similarity of 100%. Strain 1587, which is ST109 and belongs to CC4, was clustered in the middle of cluster 1 with the CC4 strains. This strain showed 18 OMP bands. Within the CC4 strains, strains 1587 (ST109), 721 (ST4), and 701 (ST4) had very similar OMP profiles and had a level of similarity of 80%.

Notably, strain 1221, which is a CC4 strain, was distant from the other CC4 strains in the OMP dendrogram (**Figure 5.2**) and showed a level of similarity of 62.6%. Strain 658 (ST1) shared some OMP bands with the CC4 strains, as seen in **Figure 5.2**. Strain 658 had 15 OMP bands and a level of similarity of 68% with the other strains.

Cluster 2 in the OMP dendrogram contained non-CC4 strains (**Figure 5.2**). Cluster 2 had one sub-cluster, C.sakC1–C.sakC5 that included three strains 1 (ST8), 520 (ST12), and 680 (ST8), plus two outlier strains, strains 1536 (ST1) and 696 (ST12). These isolates had a level of similarity of 46.6% with the other strains. Strains 1 and 520 had very similar OMP bands: 12 of 13 bands were identical, and the level of similarity was 88%. Strains 680, 1536, and 696 showed considerable variations in their OMP profile patterns and had a level of similarity of 53%. Overall, the non-CC4 strains showed much more diversity than the CC4 strains.

Table 5. 5 The OMP profiles at pH 6 (corresponding to Figure 5.2).

Cluster	Species	Strain	ST	Serotype (O)	OMP profile type
Cluster 2	*C. sakazakii*	1	8	O:1	C. sakC1
	C. sakazakii	520	12	O:3	C. sakC2
	C. sakazakii	680	8	O:1	C. sakC3
	C. sakazakii	1536	1	O:1	C. sakC4
	C. sakazakii	696	12	O:4	C. sakC5
Cluster 1	*C. sakazakii*	1533, 1537	4	O:2	C. sakC6
	C. sakazakii	1542	4	O:2	C. sakC7
	C. sakazakii	1587	109(CC4)	O:2	C. sakC8
	C. sakazakii	721	4	O:2	C. sakC9
	C. sakazakii	701	4	O:2	C. sakC10
	C. sakazakii	658	1	O:1	C. sakC11
	C. sakazakii	1221	4	O:2	C. sakC12

Cluster 2: OMP-type C.sakC1–C.sakC5.
Cluster 1: OMP-type C.sakC6–C.sakC12.
ST:Seqace type
CC4: Clonal complex

5.4.2.1.2 Correlation between the OMP profile at pH 6 and serotype

In Gram-negative bacteria, the O-antigen is a highly dissimilar component of lipopolysaccharide (LPS). The sugar residues within the O-antigen region vary in number and order, creating a characteristic serotype (Mullane *et al*. 2008b; Jarvis *et al*. 2011; Sun *et al*. 2012b). To date, 18 variant serotypes have been described in *Cronobacter,* corresponding to 7 O-antigen types (O:1–O:7) in *C. sakazakii* (Mullane *et al.* 2008; Jarvis *et al*. 2011; Sun *et al*. 2012b; Jarvis *et al*. 2013; Ogrodzki and Forsythe, 2015). In order to identify the O-antigen regions of *C. sakazakii* strains used in this study, the genomic regions, which correspond to 7 defined *C. sakazakii* serotypes, were retrieved from GenBank **(Table 5.6)** and compared against all 13 *C. sakazakii* genomes using BLAST to search the *Cronobacter* database. The *C. sakazakii* serotypes were defined based on information from the studies in **Table 5.6.** The results of the genomic comparison indicated that 13 *C. sakazakii* isolates belonged to 4 serotypes, O:1–O:4.

Table 5. 6 List of the O-antigen in *C. sakazakii*

C. sakazakii Strains	Serotype	GenBank number	Reference
NCTC 8155	O:1	EU076545	Mullane *et al.* (2008)
NCTC 11468	O:2	EU076546	Mullane *et al.* (2008)
2156	O:3	HQ646168	Jarvis *et al.* (2011)
G2594	O:4	JQ674747	Sun *et al.* (2012)
G2706	O:5	JQ674748	Sun *et al.* (2012)
G2704	O:6	JQ674749	Sun *et al.* (2012)
G2592	O:7	JQ674750	Sun *et al.* (2012)

In this study, there were some correlations between the OMP patterns and the serotypes of the strains. There were 12 OMP profiles for the 13 *C. sakazakii* strains, and these strains had 4 serotypes: O:1, O:2, O:3, and O:4 (**Tables 5.5)**. The majority of the strains belonged to serotype O:2. Most of the strains in cluster 1 belonged to serotype O:2, including strains 721, 1221, 701, 1533, 1537, 1542, and 1587. The exception in cluster 1 was strain 658, which belonged to serotype O:1. This serotyping result corresponded to the OMP analysis with regard to the MLST sequence type divisions (ST4 versus non-ST4). Cluster 2 included strains belonging to different serotypes: strains 1536 (O:1), 680 (O:1), 1 (O:1), 520 (O:3), and strain 696 (O:4) (**Table 5.5**).

5.4.2.1.3 The MWs of OMP profile at pH 6

The GeneTools analysis of the SDS-PAGE OMP patterns assigned a MW value to each band for all of the strains grown at pH 6 using a protein ladder for the MW calibration (**Table 5.7**). The majority of the MW values were in the 13–267 kDa range. The 13 *C. sakazakii* strains that were used in the GeneTools analysis included 7 *C. sakazakii* CC4 strains, 6 *C. sakazakii* non-CC4 strains, and one *C. koseri* strain for comparative purposes. Some proteins were common to both CC4 and non-CC4 isolates (**Table 5.7**). This included protein bands with apparent MW ranges of 267-183**,** 80–91,79–70, 59–53, 35–30, and 28–29 kDa (**Table 5.7**). These proteins could be major *C. sakazakii* OMPs. A few protein bands were identified only in the CC4 strains; these had apparent MW ranges of 160–150, 140–149, 138–136, 123–128, 24-27 and 14-13 kDa (**Table 5.7**). **Table 5.8** summarises the

predicted MWs of the protein bands identified by the GeneTools software in the *C. sakazakii* OMP profiles of cells cultured at pH 6 as well as the predicted OMPs as identified in *E.coli* **Table 5.12**.

Table 5. 7 Molecular weights of the outer membrane proteins of the indicated *C. sakazakii* strains cultured at pH 6.

Strain ID	Clonal complex 4							Non-clonal complex 4						No.[a]	No.[b] sample %
	1587 ST109	**1542** ST4	**1537** ST4	**1533** ST4	**701** ST4	**1221** ST4	**721** ST4	**696** ST12	**520** ST12	**1** ST8	**680** ST8	**1536** ST1	**658** ST1		
MW, **kDa**															
267-183		183.23	183.23	192.88					183.11	253.54	266.91			6	**46**
160-150					154.90		160.04							2	**15**
140-149	142.42			149.22		149.22	145.10							4	**31**
138-136		138.49	136.56	137.20										3	**23**
130-131	130.34										131.01			2	**15**
123-128		127.33		123.82	128.53									3	**23**
111-119	118.72	117.07	117.07				116.53	117.95		112.75				6	**46**
101-109	101.78	101.31	101.31	108.65	108.65		103.70					104.60		7	**54**
99-93				95.79	94.02				95.44		99.23	97.69	97.69	6	**46**
80-91	89.31	88.49	89.31	85.64		86.44	89.73				86.27		88.99	11	**85**
					84.45		80.60	81.06		82.98					
79-70	78.01	77.29	78.01	74.80	73.42		70.07		75.00		72.73		75.00	9	**69**
69-66		69.42	69.75	68.78		68.78						66.31		5	**38**
61-65	64.13	63.83	64.13		63.53		62.94			61.12	61.31		61.87	8	**62**
60-60.9		60.35	60.92	60.92					60.21	60.39				5	**38**
59-53		54.46	54.72	54.46	57.07	56.80	53.46		56.51	54.99	56.85	54.82	53.52	11	**85**
50-53					52.22			52.87		52.87	55.16	52.24	51.14	8	**62**
			50.31						50.53		50.68		50.07		
49-46		49.61		49.61	48.47						49.17			4	**31**
46-44		45.62	45.62						47.32	46.66	46.00	48.15	46.66	7	**54**
44-41	44.36							44.08		43.31	44.08		43.61	5	**38**
40-36		39.84	40.78	36.80	36.80			40.34			41.65	40.34	38.79	8	**62**
												38.79	37.71		
												37.18	36.78		
35-30	34.64	35.96	34.16				35.96		34.42	34.03	35.37	34.61	34.71	12	**92**
				30.25		32.90		31.29	31.11	31.20	30.42	30.34	32.18		
28-29	29.42	27.43	27.95	27.30	29.15	27.43	29.42			29.75	26.89		29.33	10	**77**
24-27		24.52	25.82	25.10	25.70									4	**31**
23-20	23.08	22.87	22.97	23.63								23.31		5	**38**
	20.83	20.44	21.22		21.12							21.32			
15-19		18.97		19.33					19.62					7	**54**
		17.12	17.61						15.01		15.49	15.17	16.47		
14-13		12.47	13.18	14.34	13.50									4	**31**

MW, kDa: the molecular weight in kilodaltons; No.[a]: the number of bands in each size range in **Figure 5.3**; No.[b]: %: the percentage of strains with proteins in the indicated MW range; ST: sequence type

Table 5. 8 Summary of the outer membrane protein (OMP) bands from *C. sakazakii* cells cultured at pH 6 that have predicted molecular weights in the indicated ranges. GeneTools software was used to determine the predicted molecular weights.

Molecular weight, kDa	No. of samples, %	No. of strains	*C. sakazakii* strains	
			CC4	Non-CC4
267–183	46	6	1542, 1537, 1533	520, 1, 680
160 –150	15	2	701, 721	-
140–149	31	4	1587, 1533, 1221, 721	-
138–136	23	3	1542, 1537, 1533	-
130–131	15	2	1587	680
123–128	23	3	1542, 1533, 701	-
111–119	46	6	1587, 1542, 1537, 721	696, 1
101–109	54	7	1587, 1542, 1537, 1533, 701, 721	1536
99–93	46	7	1533, 701,	520, 680, 1536, 658
80–91	85	11	1587, 1542, 1537, 1533, 1221, 701, 721	696, 1, 680, 658
79–70	69	9	1587, 1542, 1537, 1533, 701, 721	520, 680, 658
69–66	38	5	1542, 1537, 1533, 1221	1536
61–65	62	8	1587, 1542, 1537, 701, 721	1, 680, 658
60–60.9	38	5	1542, 1537, 1533	520, 1
59–53	85	11	1542, 1537, 1533, 701, 1221, 721	520, 1, 680, 1536, 658
50–53	62	7	701, 1537	696, 1, 680, 1536, 658, 520
49–46	31	4	1542, 1533, 701	680
46–44	54	7	1542, 1537	520, 1, 680, 1536, 658
44–41	38	5	1587	696, 1, 680, 658
40–36	64	8	1542, 1537, 1533, 701	696, 680, 1536, 658
35–30	92	12	1587, 1542, 1537, 1533, 1221, 721	696, 520, 1, 680, 1536, 658
28–29	77	10	1587, 1542, 1537, 1533, 701, 1221, 721	1, 680, 658
24–27	31	4	1542, 1537, 1533, 701	-
23–20	38	5	1587, 1542, 1537, 1533, 701	1536
15–19	54	7	1542, 1537, 1533	520, 680, 1536, 658
14–13	31	4	1542, 1537, 1533, 701	-

No. of samples: the percentage of strains with proteins in the indicated molecular weight ranges; **No. of strains**: the number of strains in the indicated molecular weight range in Figure 5.2; **ST**: sequence type

5.4.2.2 **OMP profiling at pH 3.5**

5.4.2.2.1 **Analysis of the *C. sakazakii* OMP profiles of cells grown at pH 3.5**

BioNumerics software version 6.5 was used to analyse the OMP profiles of 13 *C. sakazakii* CC4 and non-CC4 strains and 1 *C. koseri* strain (strain 48) grown at pH 3.5. After separation of the proteins by SDS-PAGE, a protein ladder was used to assign a MW value to each band as described previously in Chapter 2, **Section (2.9.4** and **2.9.4.6).** The OMP patterns of cells grown at pH 3.5 (**Figure 5.3**)

differed from the patterns of bacteria cultured at pH 6 (**Figure 5.2**). Thirteen OMP patterns were identified and grouped into two main clusters with 40% similarity (**Figure 5.3**; **Table 5.7**). **Table 5.9** summarizes the OMP profile data in **Figure 5.3.**

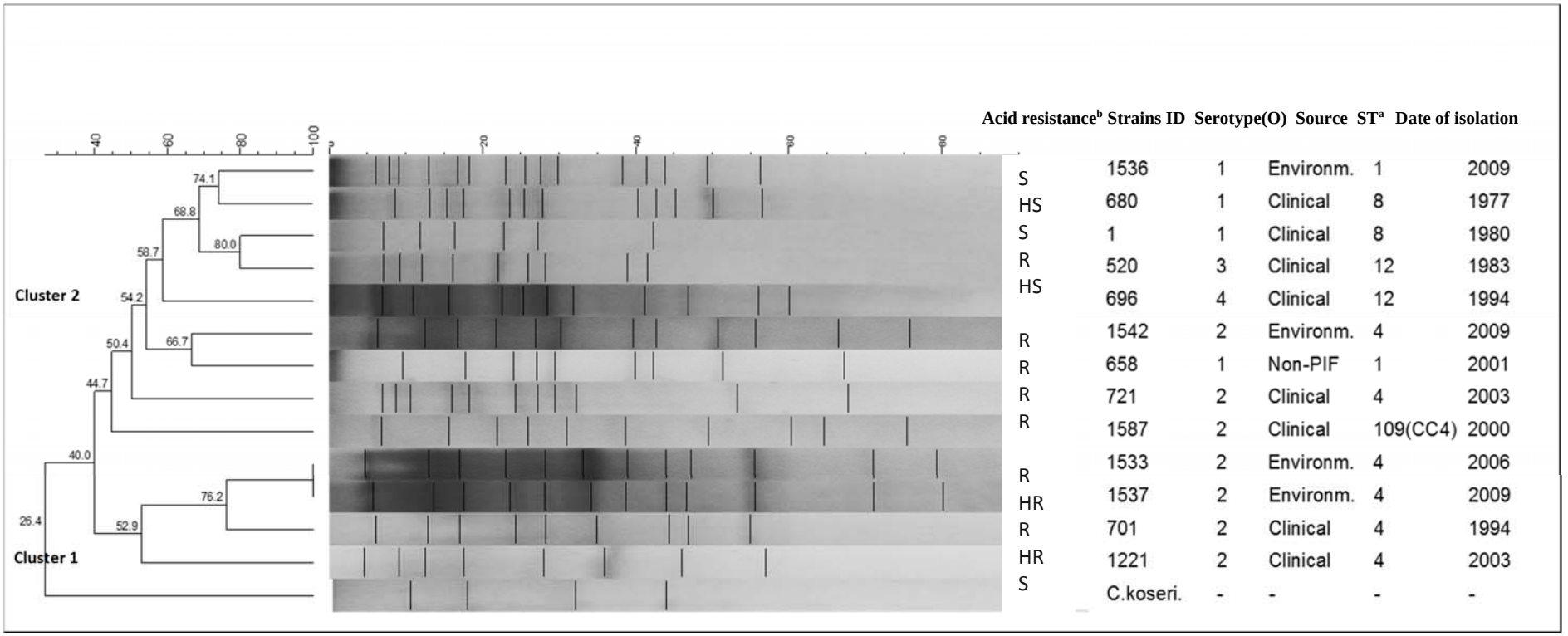
The relationship between acid resistance and the OMP clusters and sub-clusters at pH 3.5

OMP profiling at pH 3.5 divided the strains into two large clusters. The strains in clusters 1 and 2 ended up grouped mainly according to sequence type, except for ST1 strains 1536 and 658. An attempt was made to determine whether the OMP profiling findings correlated with the results of the acid resistance assays (**Figure 5.3** and **Figure 4.5**). The strains in clusters 1 showed resistant or highly resistant strains and the strains in cluster 2 indicated sensitive, highly sensitive and resistant to acid treatment at pH 3.5, with reductions in viability of an average of 0.70 to 1.79 $\log_{10}$ CFU/ml for cluster 1 and 1.07 to 1.68 for cluster 2 (**Figure 4.5**).

There were two sub-clusters within cluster 2. Sub-cluster 2.1 included strains 1536(ST1), 680(ST8), 1(ST8) and 520 (ST12), which clustered in one group with 58.7% level of similarity plus one outlier strain, strain 696 (ST12). The sub-cluster 2.1 mainly included strains, which were sensitive and highly sensitive to acid i.e. , strains 1536 (ST1), 680 (ST8), 1 (ST8), and 696 (ST12), with exception of strain 520 (ST12) which showed a resistant phenotype to acid pH 3.5 (**Figure 4. 5**). This cluster showed reductions in viability of 2.00 and 4.48 $\log_{10}$ CFU/ml after acid treatment for strains 1536 (ST1), 680 (ST8), 1 (ST8), and 696 (ST12), and showed reductions in viability of 1.55 $\log_{10}$ CFU/ml after acid treatment for strain 520 (ST12).

Subcluster 2.2 included strains 1542 (ST4) and 658(ST4), plus two outlier strains, 721(ST4) and 1587 (ST4). Strains 1542 (ST4) and 658 (ST1) that were resistant to pH 3.5 treatment showed reductions in viability of 1.42 and 1.45 $\log_{10}$ CFU/ml after acid treatment.These strains showed a level of similarity of 66.7% and were obtained from a powered milk processing plant and a powered formula, respectively. The cluster 2 also had two singlet clinical strains, strain 721 (ST4) and strain 1587 (ST109), which branched closely and had similarity levels of 54% and 50.4%, respectively with other strains in cluster 2. Strains 721 (ST4) and 1587 (ST109) showed reductions in viability of 1.07 and 1.68 $\log_{10}$ CFU/ml after acid treatment, respectively (**Figure 4.5**).

Cluster 1 included four strains that were resistant or highly resistant to acid, and showed reductions in viability of 0.70 to 1.79 $\log_{10}$ CFU/ml after acid treatment. There was one subcluster within cluster 1, which included three strains, strains 1533(ST4), 1537(ST4), and 701(ST4), plus one outlier strain, strain 1221(ST4) (**Figure 5.3**). Sub-cluster 1.1 included two environmental strains, 1533 (ST4) and 1537 (ST4) that, in the acid assay, showed reductions in viability of 0.70 and 1.56 $\log_{10}$ CFU/ml, respectively (**Figure 4.5**). Strains 1533 and 1537 (both ST4) showed 100% similarity in their OMP profiles (**Figure 5.3**). Strain 701 showed similarity levels of 76.2% with the other strains in subcluster 1.1. Cluster 1 indicated one singlet strain 1221 (ST4) on the dendrogram. This strain showed similarity levels of 52.9% with the other strains in subcluster 1.1. Strains 701 (ST4) and 1221 (ST4) showed reduced viabilities of 0.81 and 1.79 $\log_{10}$ CFU/ml, respectively, after acid treatment (**Figure 4.5**). The analysis indicated that the strains in OMP profile cluster 1 were either resistant or highly resistant to acidic stress (**Section 4.5.4.1**)(**Figure 5.3** and **Figure 4.5**). Of the strains in the cluster 1, isolates 1533 and 1537 were obtained from the same milk powder processing factory in Germany, 701 was isolated from the CSF of a neonate with meningitis in 1994 in France, and 1221 was a meningitis isolate from the USA in 2003. It was interesting that the isolates in OMP profile cluster 1, despite their geographic, temporal, and source diversity, were closely clustered. The analysis indicated that these isolates might all have OMPs that are responsible for their acid tolerance. Further experimental analysis using 2D gel electrophoresis is warranted in order to identify the specific OMPs that are responsible for the enhanced acid resistance of these isolates.



Acid resistance[b]	Strains ID	Serotype(O)	Source	ST[a]	Date of isolation
S	1536	1	Environm.	1	2009
HS	680	1	Clinical	8	1977
S	1	1	Clinical	8	1980
R	520	3	Clinical	12	1983
HS	696	4	Clinical	12	1994
R	1542	2	Environm.	4	2009
R	658	1	Non-PIF	1	2001
R	721	2	Clinical	4	2003
R	1587	2	Clinical	109(CC4)	2000
R	1533	2	Environm.	4	2006
HR	1537	2	Environm.	4	2009
R	701	2	Clinical	4	1994
HR	1221	2	Clinical	4	2003
S	C.koseri.	-	-	-	-

a: Sequence type
b: Acid-resistance experiment

Figure 5. 3 Cluster analysis of the outer membrane protein SDS-PAGE profiles of 13 *C. sakazakii* strains and *C. koseri* (strain 48) from cells cultured at pH 3.5.

The analysis was performed using BioNumerics software, version 6.5. Dice's coefficient and the unweighted pair group method with arithmetic mean (UPGMA) were used for cluster analysis. Dendrogram clusters were defined at a 40% similarity level, while subclusters were defined at a 66% similarity level. Cluster 1 had one subcluster that included three strains, strains 1533, 1537, and 701, plus one outlier strain, strain 1221. Cluster 2 had two subclusters. Subcluster 2.1 included strains 1536, 680, 1 and 520, plus one outlier strain, strain 696. Subcluster 2.2 included strains1542 and 658, plus two outlier strains, strains 721 and 1587.The isolates were grouped based on the Dice coefficients relative band intensity) with optimisation at 0.5% and band match tolerances of 1%. A 40% similarity cut-off was used to differentiate the OMP profiles (clusters and subclusters).

SDS-PAGE analysis of the OMPs from bacteria cultured at pH 3.5 revealed two main clusters (**Figure 5.3; Table 5.7)**.

Cluster 1 included the following CC4 strains: 1533, 1537, 701, and 1221. The strains had a 52.9% level of similarity (**Figure 5.3**). Interestingly, **Figure 5.3** shows that strains 1533 and 1537 were clustered together in the SDS-PAGE dendrogram and had a level of similarity of 100%. This indicates that the OMP profiles of these two strains are identical when the cells are cultured at pH 3.5. Cluster 2 included 9 isolates, some of which were CC4 and some of which were non-CC4 isolates (**Figure 5.3**). The non-CC4 isolates were strain 1536 (ST1), 680 (ST8), 1 (ST8), 520 (ST12), 696 (ST12), and 658 (ST1). The CC4 isolates in this cluster were strains 721 (ST4), 1542 (4), and 1587 (ST4). This cluster had a 50.4% level of similarity. Analysis of the OMP patterns in cells cultured at pH 3.5 showed no relationships between clonal complexes and OMP patterns

Table 5. 9 The OMP profiles at pH 3.5 (corresponding to Figure 5.3)

Cluster	Species	Strain ID	ST[a]	OMP profile type*
Cluster 2	*C. sakazakii*	1536	1	C.sakC1
	C. sakazakii	680	8	C.sakC2
	C. sakazakii	1	8	C.sakC3
	C. sakazakii	520	12	C.sakC4
	C. sakazakii	696	12	C.sakC5
	C. sakazakii	658	1	C.sakC6
	C. sakazakii	721	4	C.sakC7
	C. sakazakii	1587	109[b]	C.sakC8
	C. sakazakii	1542	4	C.sakC9
Cluster 1	*C. sakazakii*	1533,1537	4	C.sakC10
	C. sakazakii	701	4	C.sakC11
	C. sakazakii	1221	4	C.sakC12

*Cluster 2: OMP-type C.sakC1–C.sakC9
Cluster 1: OMP-type C.sakC10–C.sakC12
a: Sequence type (ST)
b: Clonal complex 4

5.4.2.2.2 The MWs of OMP profile at pH 3.5 using GeneTools software

The GeneTools analysis of the SDS-PAGE OMP patterns showed that most of the OMPs from cells cultured at pH 3.5 had a MW range of 13–126 kDa. Most of the CC4 strains had more OMP bands than the non-CC4 strains (**Tables 5.10** and **5.11**), and some proteins in the 44–41 and 24–27 kDa MW ranges were found in both CC4 and non-CC4 strains. These proteins could be major OMPs in *C. sakazakii*. There were no bands in the 101–109 kDa molecular weight range. **Table 5.10** lists the protein bands shown in **Figure 5.3**. Certain protein bands were predominant in the CC4 strains; these were found in the following apparent MW ranges: 123–93, 99–93, 69–66, 61–65, 50–55, and 35–30 kDa (**Table 5.10**). **Table 5.11** summarises the predicted MWs of the protein bands identified by the GeneTools software in the OMP profiles of *C. sakazakii* cells grown at pH 3.5 as well as the predicted OMPs of *E. coli* as showed in **Table 5.12**.

Table 5. 10 Molecular weights of the outer membrane proteins of the indicated *C. sakazakii* strains cultured at pH 3.5 identified using GeneTools software.

MLST	CC4							Non-CC4						No. of bands	No. of samples (%)
Strain	1587	1542	1537	1533	701	1221	721	696	520	1	680	1536	658		
MW, kDa	ST 109	ST 4	ST 4	ST 4	ST 4	ST 4	ST 4	ST 12	ST 12	ST 8	ST 8	ST 1	ST 1		
267–183														0	0
160 –150														0	0
140–149														0	0
138–136														0	0
130-131														0	0
123–128			126.10											1	7
111–119	114.62						113.06							2	15
101–109														0	0
99–93		98.57	95.78	93.06								93.06		4	31
80–91						90.42	84.15	91.58	85.23		83.21			5	38
79–70								76.82	74.16			79.95	76.82	4	31
69–66	67.49	69.05	67.49											3	23
61–65		63.58	62.36	64.46	64.76	65.96	64.46	65.16						7	53
60–60.9					60.16	60.16	60.47							3	23
59–53	54.57						58.33		58.95		59.31	58.24	55.16	6	46
50–53		53.46	52.37	51.84	52.64	51.57								5	38
49–46			47.79				48.50	49.17	49.17	47.54	46.42			6	46
46–44	46.17							46.42						2	15
44–41		44.02	42.89	43.64	44.21	44.21	41.97	41.78			40.99	44.25	43.41	10	77
40–36							40.55		40.40	40.79	40.02	37.07	36.72	6	46
35–30	34.26	33.89	32.70	30.99	34.13	34.13		32.94						7	46
28–29								28.93				28.69		2	15
24–27	26.57	25.82	24.79	25.45	26.76			25.00		26.89	26.78	27.67	27.00	10	77
23–20		22.36					22.94				21.01	20.82		4	31
15–19	18.44	18.24	18.64	18.40				19.46					19.68	7	53
		14.87	17.55	15.53			16.78	18.50							
14–13			13.76	13.24										2	15

MW: molecular weight; No. of samples: the percentage of strains with proteins in the indicated molecular weight range; No. of bands: the number of bands in the indicated molecular weight range in **Figure 5.3**; ST: sequence type

Table 5. 11 Summary of the OMP bands from *C. sakazakii* cells grown at pH 3.5 that have predicted molecular weights (MW) in the indicated ranges. GeneTools software was used to determine the predicted molecular weights.

Molecular weight kDa	No. of samples %	No. of strains	CC4	Non-CC4
267–183	0	0	-	-
160 –150	0	0	-	-
140–149	0	0	-	-
138–136	0	0	-	-
130-131	0	0	-	-
123–128	7	1	1537	-
111–119	15	2	1587, 721	-
101–109	0	0	-	-
99–93	31	4	1542, 1537, 1533	1536
80–91	38	5	1221, 721	696, 520, 680
79–70	31	4	-	696, 520, 1536, 658
69–66	23	3	1587, 1542, 1537,	-
61–65	53	7	1542, 1537, 1533, 701,1221, 721	696
60–60.9	23	3	701, 1221, 721	-
59–53	46	6	721, 1587	658, 520, 680, 1536
50–53	38	5	1542, 1537, 1533, 701,1221	-
49–46	46	6	1537, 721	696, 520, 1, 680,
46–44	15	2	1587	696
44–41	77	10	1542, 1537, 1533, 701, 1221, 721	696, 680, 1536, 658
40–36	46	6	721	520, 1, 680, 1536, 658
35–30	53	7	1587, 1542, 1537, 1533, 701, 122	696
28–29	15	2	–	696, 1536
24–27	77	10	1587, 1542, 1537, 1533, 701	696, 1, 680, 1536, 658
23–20	31	4	1542, 721	680, 1536
15–19	53	7	1587, 1542, 1537, 1533, 721	696, 658
14–13	15	2	1537, 1533	-

No. of samples: the percentage of strains with proteins in the indicated molecular weight range; No. of bands: the number of bands in the indicated molecular weight range in **Figure 5.3**; ST: sequence type.

Table 5. 12 Presumed identification of *C. sakazakii* outer membrane protein (OMP) bands. The presumed identification obtained by comparison with previously reported *E. coli* OMPs by Kim *et al.* (2014).

Molecular weight (kDa)	No. of samples (%) found in this study		OMP[a]
	pH 6	pH 3.5	
267–183	46	0	Uncharacterized protein YuaO
160–150	15	0	OM protein YpjA, YuaQ
140–149	31	0	Autotransporter • Serine protease (Sat) • Serine protease EspC, EspP, • EatA • Temperature-sensitive haemagglutinin Tsh • Haemoglobin-binding protease Hbp
139–136	23	0	Autotransporter Serine protease (Pet)
130–132	15	0	Autotransporter (AIDA-I)
123–128	23	7	Unknown
111–119	46	15	Bacteriophage N4 adsorption protein A (NfrA)
101–109	54	0	Adhesin/invasin TibA autotransporter, intimin (Eae), OM usher protein CS3-2, antigen 43 (Ag43)
99–93	46	31	OM usher proteins (AggC, YraJ, AfaC, ElfC, HtrE, SfmD, FocD, FimD, YfcU)
80–91	85	38	Iron transport proteins including: • Ferric aerobactin receptor (IutA) • FhuE receptor • Catecholate siderophore receptor Fiu • Ferrienterobactin receptor (FepA) • Ferrichrome-iron receptor (FhuA) • Fe(3+) dicitrate transport protein FecA • LPS-assembly protein OstA, YabG • OM protein assembly factor BamA (Omp85) • Poly-beta-1,6-N-acetyl-D-glucosamine export protein (PgaA) • OM usher proteins (FaeD,YehB, FasD, cssD, PapC, YqiG, YbgQ, YejO, FanD, YhcD)
79–70	69	31	Colicin I receptor (CirA) Probable TonB-dependent receptor (YncD)
69–66	38	23	Vitamin B12 transporter BtuB
61–65	62	53	Protein TraN
60–60.9	38	23	Cryptic OM porin BglH
59–53	85	46	Unknown
50–53	62	38	OM protein TolC (Multidrug efflux pump subunit TolC)
49–46	31	46	Long–chain fatty acid transport protein, FadL Maltoporin (LamB)
46–44	59	15	Membrane-associated protein UidC
44–41	38	77	OmpN Putative OM protein YedS

40–36	64	46	OmpA, phoE (OmpE), OmpF, Putative OM porin NmpC, OmpC
35–30	92	53	• Copper resistance protein B (PcoB). • Phospholipase A1 (OmpLA (PldA) • Nucleoside-specific channel-forming protein Tsx • OmpG • OM protease OmpP • Protease 7 (OmpT)
28–29	77	15	MltA-interacting protein, MipA (YeaF) Probable N-acetylneuraminic acid OM channel protein NanC (YjhA)
24–27	31	77	Porin OmpL Putative OM protein YiaT
23–20	38	31	PagP, OmpW (YciD) Putative OM protein YiaT
15–19	54	53	OmpX (YbiG)
14-13	31	15	Unknown

No: Number of samples
a: OMPs from Kim *et al.* (2014)

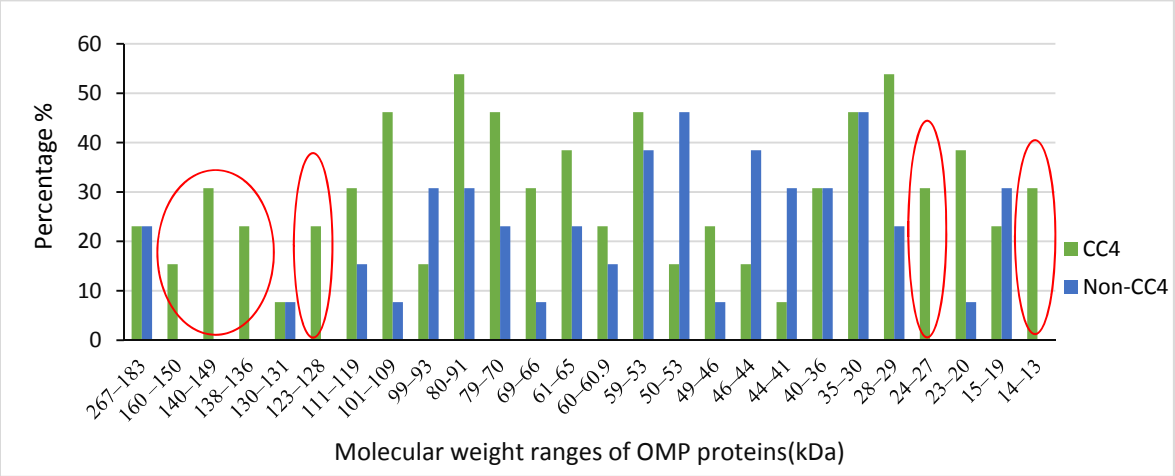


Figure 5. 4 The expression of OMPs by *C. sakazakii* strains cultured at pH 6.

Figure 5.4 shows the percentage of samples at pH 6 with outer membrane protein (OMP) bands in the indicated molecular weight ranges (data from **Table 5.8**). The OMPs analysis demonstrated that the CC4 strains had specific OMPs bands that missing in non-CC4 strains at pH 6. This included bands with molecular weight ranges between 160–150, 140–149, 138–136, 123–128, 24-27 and 14-13 kDa(indicated by ovals) **(Table 5.8; Figure 5.4)**. Certain proteins were expressed by all CC4 and non-CC4 strains when they were grown at pH 6, especially in the following MW ranges 267-183, 80–91, 79–70, 59–53, 35–30, 40-36 and 28–29 kDa **(Table 5.8; Figure 5.2)**.

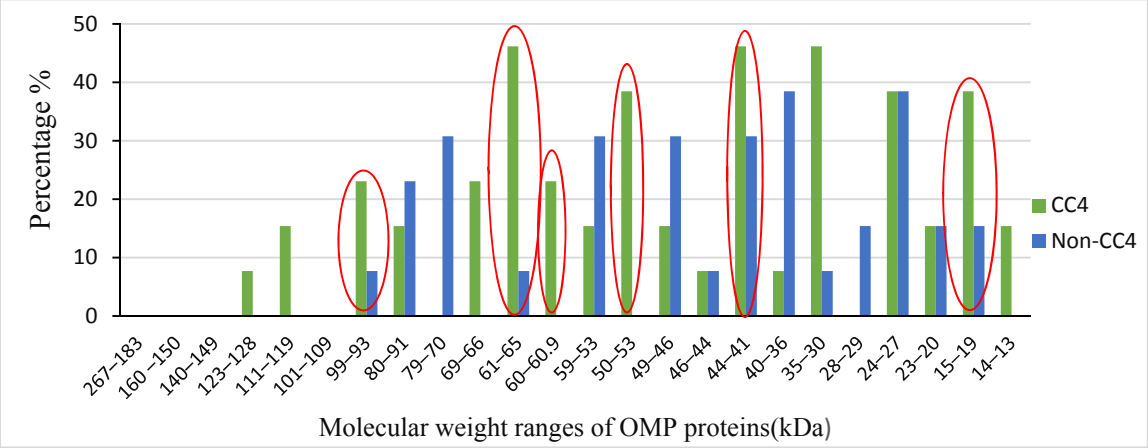


Figure 5. 5 The expression of OMPs by *C. sakazakii* strains cultured at pH 3.5.

Figure 5.5 shows the percentages of samples of cells cultured at pH 3.5 with OMP bands in the indicated molecular weight ranges (data from **Table 5.11**). The OMP analysis demonstrated that the CC4 strains had more OMPs bands than the non-CC4 strains when cultured at pH 3.5. This included bands in the MW ranges of 99-93, 61–65, 60-60.5, 50–53, 35–30 and 15-19 kDa (indicated by ovals) **(Table 5.11; Figure 5.3)**. Also, the OMPs analysis demonstrated that the CC4 strains had specific OMPs bands that were missing in non-CC4 strains at pH 3.5. This included bands with molecular weight ranges 123-128, 111-119, 69–66, 60-60.9, 50-55 and 14-13 kDa. Some proteins were expressed by all CC4 and non-CC4 strains when they were grown at low pH (pH 3.5), especially in the MW ranges of 44–41 and 24–27 kDa **(Table 5.11; Figure 5.3)**.

5.5 Discussion

The association of *C. sakazakii* CC4 strains with neonatal meningitis was demonstrated by Joseph and Forsythe (2011) and by Hariri *et al.* (2013). *C. sakazakii* CC4 is also the predominate clone found in powder milk factories (Sonbol *et al.* 2013). However, the genetic factors underlying the predominance of *C. sakazakii* CC4 in clinical cases and its frequent isolation from PIF are unknown. It is now known that the outer membrane proteins (OMPs) play important roles in bacterial pathogenicity, in antimicrobial resistance, and in immunogenicity (Nikaido, 1999; Lin *et al.* 2002). In a hostile environment, bacterial OMPs represent the first line of bacterial defence (Nikaido, 1999; Lin *et al.* 2002). OMPs are also involved in cellular survival by binding to many molecules and substances, by mediating adhesion to other cells and to bactericidal agents, and by helping cells adapt to changes in environmental conditions (Nikaido, 1999; Lin *et al.* 2002). SDS-PAGE analysis of OMPs is widely used in epidemiological studies of bacterial pathogens, including studies of *Neisseria meningitidis* and *Haemophilus influenzae* type B (Helmuth *et al.* 1985; Kim *et al.* 2010b). The importance of OMPs in bacterial pathogenicity prompted this analysis of the OMP profiles of *C. sakazakii* isolates and a comparison of the OMP profiles of CC4 and non-CC4 isolates.

Different types of environmental stress can influence bacterial OMPs. More specifically, low pH, heat, and osmotic stress alter the expression of some OMPs in pathogenic bacteria. For example, exposure of bacteria to host stressors and to stressful environmental conditions can induce the expression of proteins that make the bacteria more resistant to these or other stressors. Many studies in *E. coli* show that OMP expression changes in response to osmotic and acid stress (Nikaido and Vaara, 1987; Lagha *et al.* 2012). Although *C. sakazakii* is the most acid tolerant of the Enterobacteriaceae, most Enterobacteriaceae have high resistance to low pH conditions (Dancer *et al.* 2009). Accordingly, the OMP profiles of *C. sakazakii* isolates were determined at acidic conditions i.e. pH 6 and 3.5. Due to the limited number of strains, the diversity of all *C. sakazakii* OMPs in CC4 and non-CC4 isolates was not represented in this study. The primary aim of the study was to compare the OMP profiles of *C. sakazakii* CC4 and non-CC4 isolates. In addition, the study also attempted to determine the relationship of OMP profiles to MLST and serotype.

5.5.1 BLAST analysis of selected OMPs

Bacterial OMPs play major roles in both pathogenicity and environmental fitness. A number of OMPs and the genes that encode them have already been described in *C. sakazakii* and *E. coli*. The present study analysed these genes in *Cronobacter* using BLAST searches of 38 sequenced *C. sakazakii* isolates representing both CC4 and non-CC4 strains.

Iron plays a critical role in the survival and multiplication of Gram-negative bacteria, and several iron adaptation mechanisms have been demonstrated in different species of *Cronobacter* (Kucerova *et al.* 2010; Grim *et al*. 2012). There are several Gram-negative bacterial OMPs that function as iron-associated protein receptors and as siderophore complexes and in ferric dicitrate transport through the OM into the environment (Rogers *et al.* 2000; Rabsch *et al.* 2003; Grim *et al.* 2013). Joseph *et al.* (2012a) found iron utilisation genes in all *Cronobacter* spp.; however, the specific functions of these genes have not been studied yet. Grim *et al.* (2013) recently reported that all *Cronobacter* have 5 common OMP iron receptors: the hydroxamate type siderophore ferrichrome FhuA; the siderophore receptor YncD; the TonB receptor FoxA; the ferric rhodotorulic acid/coprogen receptor FhuE; and the ferric enterobactin receptor PfeA (FepA). These iron receptors are essential for bacterial virulence (Rabsch *et al.* 2003; Rogers *et al.* 2000; Grim *et al.* 2013).

Kim *et al*. (2014) reported that in *E. coli*, the most well characterised iron receptors are FepA (81 kDa), the ferric dicitrate transport protein FecA (80.5 kDa), FhuA (78 kDa), and FhuE (76 kDa). Grim and colleagues (2012) characterised the iron receptor genes *fepA, fecA, fhuA, fhuE* in *Cronobacter*. The finding revealed that *fepA* was present in all *C. sakazakii* CC4 and non-CC4 strains. *FecA* was present in 8 CC4 strains 6, 721, 1220, 1221, 1240, 1225, 1533 and 4, 2 ST8 strains 680 and 1, and were absences in the other genome strains. In the absence of another iron uptake system, bacterial proteins that are part of the ferric dicitrate transport system, such as FecA, help the organism grow in iron-limited conditions (Veitinger *et al.* 1992).

fhuA was present in all of the CC4 strains and in most of the non-CC4 strains except 5 strains: ST12 strains 520 and 696, ST125 strain ES15, ST40 strain 140, and ST45 strain HPB5174. *fhuE* was present in all of the CC4 and non-CC4 strains (**Table 5.4).**

The presence of the iron outer membrane receptors in *C. sakazakii* strains, as found by this study, suggested that this organism might have many heterologous siderophores of both fungal and bacterial origin (Grim *et al*. 2012). This also supports the hypothesis that *Cronobacter* arose from a shared ancestor that had a plant-associated lifestyle before it had been species–level bi-directionally divided.

Riedel and Lehner (2007) identified three differentially expressed OMPs associated with desiccation and osmotolerance OmpA, OmpC, and OmpF. The presence of these genes in *C. sakazakii* strains was confirmed by the present study. The OmpA, OmpC and OmpF proteins play critical roles in desiccation and in the osmotic stress response (Riedel and Lehner, 2007). Notably, in such conditions, these proteins are up- and/or down regulated. The genes encoding these proteins were found in most of the strains in this study, which supports the notion that these proteins are critical for the survival of *C. sakazakii* in environmentally stressful conditions.

In terms of the effects of environmental stress on *ompA*, Riedel and Lehner (2007) reported that the OmpA protein is considerably upregulated in desiccation conditions. However, the OmpA protein is downregulated or absent when the bacteria are cultured in high-salt medium containing 1 M NaCl (Riedel and Lehner, 2007). A study by Mittal *et al.* (2009) showed that *ompA* is essential for serum resistance in *Cronobacter* spp. In a recent study using 2-DE (two-dimensional gel electrophoresis) and RT-PCR it was suggested that OmpX expression was significantly high (two-fold) in the virulence of *C. sakazakii* isolate G362 compared to an attenuated *C. sakazakii* isolate L3101 (Ye *et al.* 2015). A recent study by Alvarez-Ordóñez *et al.* (2014) demonstrated that the *C. sakazakii* envelope stress response protein OmpR is the main regulator of OMP expression in acidic conditions. *ompR* also plays a role in preserving the integrity of the cell envelope as the main defence against acid stress (Alvarez-Ordóñez *et al.* 2014).

BLAST analysis showed that the *ompA, ompX*, *ompC*, and *ompR* genes were present in the genomes of all of the sequenced *C. sakazakii* strains (**Table 5.4**).

The presence of the *ompC* gene in all of the *C. sakazakii* strains might indicate that this protein is important for surviving desiccation (**Table 5.4**). Importantly, the OmpC protein is upregulated 2.6-

fold in response to desiccation and 2.8-fold in response to culture with 1 M NaCl (Riedel and Lehner, 2007).

In this study, BLAST searches found *ompF* (ESA_02413) in 32/38 of the sequenced *C. sakazakii* strains, with the exception of the following strains: ST1 strains 658, 1536, and 1218; ST3 strains 978 and 984; and ST31 strain 1249 (**Table 5.4**). OmpF is important for membrane transport in *E. coli* in response to changes in the pH of the medium (Nikaido and Vaara, 1985; Ozkanca and Flint, 2002), and thus it might play an important role in the adaptation of *C. sakazakii* strains to the low pH of the stomach. The OmpC and OmpF proteins are also involved in membrane transport, specifically allowing the transport of hydrophilic molecules of MW less than 600 Da to be transported through the OM (Nikaido and Vaara, 1985; Ozkanca and Flint, 2002).

OmpT cleaves plasminogen to plasmin, which is rapidly inhibited by α2-AP (Hui *et al.* 2010; Franco *et al*. 2011). The OM protease encoded by this gene, OmpT, appears to play a role in pathogenicity by mediating resistance to killing by serum (McCarter *et al.* 2004; Franco *et al.* 2011). OmpT mediates *E. coli* resistance to serum (Hui *et al.* 2010), but until now, no studies have investigated its role in *C. sakazakii*. The present study found that the *ompT* gene was present uniformly in all CC4 strains with exception of isolate 6 whereas it was also noted in most (12/15) non-CC4 strains, with the exception of strains 680 (ST8), 1 (ST8), and 520 (ST12) (**Table 5.4**). Hence, the analysis suggested that the *ompT* might be important for the serum resistance of *C. sakazakii* in general.

The OstA is another OMP linked with solvent resistance in *E. coli* (Abe *et al.* 2003). Similarly the LamB protein (47 kDa) is associated with the uptake and transport of maltose in *E. coli* helping the bacteria to survive under maltose limiting conditions (Braun *et al*. 2001; Vazquez-Juarez *et al*. 2004; Baldwin *et al*. 2011). The homologue of the *lamB* gene has been reported in *Cronobacter* spp. (Kucerova *et al.* 2010; Joseph *et al.* 2012). The BLAST analysis in the present study indicated the presence of *ostA* and *lamB* uniformly in all of the CC4 and non-CC4 strains indicating no specific association with CC4 (**Table 5.4**).

Copper is essential for the growth and survival of bacteria. The PcoB is an OMP linked with the transport of copper from the environment into the periplasm in *E. coli* (Djoko *et al.* 2008). The

BLAST search showed a variable pattern of presence across CC4 and non-CC4 strains and did not show specificity to any of the lineage. The *pcoB* gene was present in all CC4 strains except strains 20, 721, 767, 1221, 701 and 694, while it was noted in almost half (8/15) non-CC4 strains. The non-CC4 strains that lacked *pcoB* included 680 (ST8), 520 (ST12), 140 (ST14), 150 (ST16), 978 (ST3), 984 (ST3), ES15 (ST125) and 1249 (ST31) (**Table 5.4**). Furthermore, another important OMP known fimbrial usher protein FimD is linked with the assembly and translocation of adhesive type 1 pili (Nishiyama *et al.* 2003). The BLAST analysis indicated the gene encoding *fimD* was absent largely from the studied *C. sakazakii* isolates and was detected only in 6/38 strains representing ST1, ST3 and ST125, suggesting a recent acquisition of this gene in *C. sakazakii* (**Table 5.4**). An antibiotic resistance associated OMP called PhoE encoding for porin protein is expressed in *E. coli* K-12 under phosphate limiting conditions (Bauer *et al.* 1989). The BLAST analysis indicated that *phoE* was present in all of the *C. sakazakii* CC4 and in most (17/18) non-CC4 strains except 150 (ST16) (**Table 5.4**). The analysis indicates that porin protein PhoE is important for the stress response of *C. sakazakii* in general.

5.5.2 Analysis of *C. sakazakii* OMPs

5.5.2.1 OMP profiles and molecular weight analysis of bacteria cultured at pH 6

The OMP profiling was undertaken at pH 3.5 and 6. The OMP profiling at pH 6 showed distant clustering of *C. sakazakii* CC4 and non-CC4 isolates. All the CC4 isolates clustered closely at pH 6 for instance for example strains 1533, 1536, and 1537, had closely related OMP patterns with a 98% level of similarity. Two strains, 1533 and 1537, had identical OMP profiles with a 100% level of similarity (**Figure 5.2)**. After reviewing the MLST database and examining the isolate sources, this analysis showed that CC4 strains were grouped together in one cluster with a 60% level of similarity. This might be explained by the clustering of strains from the same source with the same ST. This is supported by the observation that strains isolated from one factory in Germany, i.e. strains 1533, 1536, and 1537, had closely related OMP patterns with a 98% level of similarity. Two strains, 1533 and 1537, had identical OMP profiles with a 100% level of similarity. These two strains were isolated from a spray-drying tower in 2006 and from a roller dryer in 2009, respectively (Jacobs *et al.* 2011).

These strains had identical serotypes (O2) and sequence types (ST4). Kline *et al.* (1989) found that epidemiologically related *Citrobacter diversus* strains had similar OMP profiles, whereas epidemiologically unrelated strains had profiles that were more dissimilar. The result of this study indicates that epidemiologically related isolates have similar OMP patterns. This finding indicated that strains that had indistinguishable OMP profiles that were isolated from the same place (such as equipment from the same factory) might be the same strains that show the possible contamination routes in milk powder factories.

Three CC4 strains, 1587, 721, and 701, had a level of similarity of 80%. The CC4 strains 1587, 721, and 701 were very important as they caused very severe illnesses in neonates; the isolate 1587 was a CSF isolate while 721 and 701 were isolates from meningitic and fatal NEC cases respectively. The non-CC4 isolate 658 clustered with the CC4 strains, and its OMP pattern shared some characteristics with the CC4 strains (**Figure 5.2**). Strain 658 has a 64% level of similarity with the other strains. Although strain 658 is not a CC4 strain, it has been associated with meningitis infections and was linked with the consumption of powdered infant formula in the Tennessee (USA) outbreak in 2001 (Himelright *et al.* 2002).

An interesting observation was the detection of OMPs (MW range 160-150, 140-149, 138-136, 123-128, 24-27 and 14-13 kDa) at pH 6 specifically to CC4; all of these bands were missing uniformly from the non-CC4 isolates and could be important for survival in the host and environment (**Table 5.12**). An attempt was made to predict the function of these OMPs **(Tables 5.8 and 5.12).** The OMPs in MW ranges 160–15, 140–149, 138–136 and 69–66 kDa included homologues of *E. coli* YpjA and YuaQ, the autotransporter OMP, and the vitamin B12 transporter BtuB (**Table 5.12**);. However, none of these OMPs have been linked with bacterial virulence. The OMP profiles of non-CC4 isolates cultured at pH 6 showed greater variation compared to the profiles of CC4 isolates (**Figure 5.2**).

Many of the OMPs in cells cultured at pH 6 were found in both CC4 and non-CC4 isolates. This included protein bands with MWs in the following ranges: 267–183, 130–131, 59–53, 40–36, 35–30, and 15–19 kDa. These OMPs may by integral components of the OM in *C. sakazakii*. **Table 5.12**

shows the predicted function of these shared OMPs, but additional studies are needed to determine their roles in the virulence or environmental fitness of this important clinical lineage.

An attempt was also made to correlate the OMP profile at pH 6 and serotype of *C. sakazakii* isolates. The analysis indicated that overall there was no clear correlation of OMP profile and serotype. However, the interesting observation was that all of the CC4 isolates were O:2 serotype. Nevertheless, the O:2 is not a unique serotype for CC4 as a recent study by our group has indicated that it is a pre-dominant serotype in *C. sakazakii* (Ogrodzki and Forsythe, 2015).

5.5.2.2 **OMP profiles and molecular weights analysis in bacteria cultured at pH 3.5**

The pH of the neonatal stomach ranges from pH 2.5–4.3 (Hurrell *et al.* 2009). This study determined and compared the OMP profiles of *C. sakazakii* CC4 and non-CC4 strains cultured at pH 3.5. A number of OMPs were detected in both CC4 and non-CC4 strains cultured at pH 3.5, these included OMPs such as OmpA and OmpC. This is in agreement with previous studies by Riedel and Lehner (2007) who found that OMPs: OmpA and OmpC are differentially expressed in *C. sakazakii* strain under stress conditions like desiccation and osmotolerance stress.

Interestingly, OMP profiling of cells grown at pH 3.5 showed that most of the CC4 strains had more OMP bands than the non-CC4 strains. These included protein bands of MW 99-93, 61–65, 60-60.5, 50–53, 35–30 and 15-19 kDa**.** Further investigation of the OMPs detected pre-dominantly in CC4 is warranted to elucidate their significance towards virulence or environmental fitness of this important clinical lineage. There were no bands in any of the strains in the 140–267 kDa range, but there were many protein bands that were common to both CC4 and non-CC4 isolates cultured at pH 3.5 (**Table 5.12**). These included protein bands of MW 46-44, 24–27 and 23–20 kDa that could represent major OMPs in *C. sakazakii*.

There were some OMPs that were detected mainly in CC4 cells cultured at pH 3.5 (MW range 99-93, 61-65, 50-55, 35-30 and 15-19 kDa). The functions of these OMPs were predicted **(Tables 5.12).** The OMPs in the MW ranges 99–93, 61–65, 50–53, 35–30, and 15–19 kDa included homologues of the *E. coli* OM usher proteins, PcoP, OmpT and OmpX (**Table 5.12**). It is important to mention that the

C. sakazakii CC4 strains in this study included isolates from neonates with meningitis as well as isolates from milk powder factories. An attempt was also made to link the OMP profile at pH 3.5 and serotype, however no clear correlation was observed (**Figure 5.3**).

These findings suggested that CC4 strains might be more acid-tolerant than non-CC4 isolates. The MW analysis data was in agreement with the acid resistance assays conducted in the present study **(Section 4.5.4.1).** Specifically, the acid resistance assays indicated that the CC4 isolates were more acid tolerant at pH 3.5 than the non-CC4 strains (**Figure 4.5; Figure 4.6**). This was an interesting finding and suggested that when the cells are grown in highly acidic conditions (i.e. pH 3.5), the CC4 isolates may express more OMPs than the non-CC4 isolates, which might help them to survive in strongly acidic conditions found in the stomachs of neonates. The immune systems of neonates are not fully developed, and their intestinal microflora is less dense than in older children. Therefore, if neonates ingest large numbers of *Cronobacter* cells, the host intestinal flora may be overwhelmed and unable to overcome the pathogens. Once the pathogens invade the host's intestinal cells, the immature immune system cannot protect the infant from systemic infection. The infectious dose of *C. sakazakii* for neonates has not been determined yet.

Due to time limitations, it was not possible to characterise these OMPs. However, it is expected that characterising these CC4 OMPs may reveal interesting information. It remains to be seen whether the OMPs that were identified as predominant proteins in CC4 strains in the present OMP analysis at pH 3.5 are the same as these hypothetical proteins found in *E.coli*.

In the present study, an attempt was also made to perform the BLAST search of the genes encoding OMPs previously described in *E. coli* (**Section 5.4.1**), however none of them indicated any pattern for their presence/absence in CC4 and non-CC4.

To conclude, the present study showed that the *C. sakazakii* OMP profile findings largely correlated with the MLST. Interestingly, more OMPs were detected in *C. sakazakii* CC4 at highly acidic conditions which correlated with acid resistance assay suggesting that *C. sakazakii* CC4 might be more acid tolerant than non-CC4 strains. Additional studies using 2D electrophoreses and other proteomics-based approaches are needed to characterise the OMPs that were detected predominantly

in CC4 strains at low pH using 2-D gel and other proteomics based approaches in order to elucidate their significance towards virulence of this important clonal lineage.

CHAPTER 6: Conclusion and future directions

6.1 Conclusion

Cronobacter spp., previously known as *Enterobacter sakazakii*, are opportunistic pathogens. These bacteria have been associated with infections that affect people of all ages, but they are particularly known for their association with neonatal meningitis, necrotizing enterocolitis (NEC), and bacteraemia. In recent decades, following numerous deadly outbreaks of *Cronobacter*-associated illnesses in neonatal intensive care units (Masood *et al.* 2015), efforts have been made to identify and control these bacteria. In the *Cronobacter* MLST database (http://www.pubMLST.org/cronobacter), more than one-third of all *C. sakazakii* isolates that were isolated over a 50-year period are categorised as strains in the CC4 lineage (Joseph *et al*. 2012c). The *Cronobacter* MLST scheme has identified over 200 sequence types (STs) in this genus; however, *C. sakazakii* ST4 and its single/double loci variants are the main strains that are associated with neonatal infections, especially neonatal meningitis (Joseph and Forsythe, 2011). The studies described in this thesis aimed to characterise *C. sakazakii* CC4 isolates and to identify the differences in CC4 vs. non-CC4 isolates that impact their environmental persistence. Accordingly, the physiological characteristics of the bacteria were analysed and the genomes were screened for genetic traits that were suggested previously to be associated with the environmental fitness of *C. sakazakii*.

The present study had three specific aims:

- Investigation of the molecular epidemiology and clonality of environmental *Cronobacter* isolates with an emphasis on *C. sakazakii* CC4 .
- Determination of the physiological and virulence-related factors that affect the persistence of neonatal meningitis *C. sakazakii* strains in different environments and hosts
- Profiling of bacterial outer membrane proteins (OMPs) using genomic determination and SDS-PAGE analysis.

- **Investigation of the molecular epidemiology and clonality of environmental *Cronobacter* isolates with an emphasis on *C. sakazakii* CC4**

In order to analyse the diversity of *Cronobacter* spp. isolated from the environment, an MLST scheme was applied to three strain collections that included 85 isolates that were identified previously as *E. sakazakii* **(Section 3.5).** These 85 isolates included 20 strains reported by Muytjens *et al*. (1988) that were originally collected between 1988 and 2009 from 14 countries (**Section 3.5)**; 52 strains reported by Craven *et al*. (2010), including 3 strains that could not be profiled using PFGE (**Section 3.5.4)**; and 13 strains from Jacobs *et al*. (2011) (**Section 3.5.3)**. The *Cronobacter* spp. strains that were identified in this study included *C. sakazakii, C. malonaticus*, *C. turicensis* and *C. muytjensii* isolates. Interestingly, the results of the present study indicated that *C. sakazakii* CC4 strains are ubiquitous in the environment and that *C. sakazakii* ST4 (20/85 isolates) and ST1 (16/85 strains) are the main sequence types. If the single locus variant ST97 is included as an ST4 strain, 21/85 isolates are ST4 strains.

Another important finding of the MLST study was that *C. sakazakii* ST4 can be found in many locations in milk powder factories, including in the tanker bay, on workers' shoes, on the roof, on roller-dryers, in the spray-drying area, and in milk powder itself. The predominance of *C. sakazakii* CC4 in dairy factory environments is not surprising, as CC4 strains are more frequently associated with *Cronobacter* spp. infections and outbreaks compared to non-CC4 strains. The *C. sakazakii* CC4 lineage, including ST4, is the predominant lineage of *Cronobacter* spp. associated with neonatal meningitis (Joseph and Forsythe, 2011; Hariri *et al*. 2012). The use of MLST as an identification tool revealed that one strain isolated from PIF in India by Muytjens *et al*. (1988) was re-identified as *E. hormaechei* strain **(Section 3.5.2)**. This study also showed correlations between two molecular identification methods, PFGE and MLST **(Section 3.5.5 and 3.5.6).**

- **Determination of the physiological and virulence-related factors that affect the persistence of neonatal meningitis *C. sakazakii* strains in different environments and hosts. There were two aims for the physiological characterisation of *C. sakazakii*.**

1. To investigate the diversity of the physiological traits of *C. sakazakii*, laboratory studies and genomic studies were performed and the data were compared in different strains. The aim was to gain a better understanding of the pathogenicity and persistence of the organism in various environments. Studies of selected clinical and environmental strains of *C. sakazakii* can help us better understand bacterial pathogenicity. Accordingly, the initial studies in my thesis compared the physiological and virulence-related traits of 13 representative strains, 7 that were CC4 and 6 that were non-ST4 (2 ST1, 2 ST8, and 2 ST12).
2. To investigate the presence of virulence-associated traits identified in previous studies, genomic studies were performed and the data were compared in *C. sakazakii* CC4 versus non-CC4 isolates. Thus, the later studies in my thesis compared the genomic data of 38 representative strains, 23 that were CC4 and 15 that were non-ST4 (3 ST1 strains, 3 ST8 strains, 2 ST12 strains, 2 ST3 strains, and 1 strain each belonging to ST40, ST16, ST125, ST31, and ST45).

All of the *C. sakazakii* CC4 and non-CC4 strains were recovered after desiccation. The recovery of the strains after desiccation differed according to the composition of the culture media **(Section 4.5.2).** Sequenced *C. sakazakii* CC4 and non-CC4 strains were analysed for the unique presence of desiccation-related or osmotolerance genes. The frequent isolation of *C. sakazakii* CC4 from PIF and from other dry and desiccated environments indicates that it can survive in conditions with very low water activity. All *C. sakazakii* CC4 and non-CC4 strains had most of the osmotolerant genes that were investigated. There was some variation, but the variation did not seem to correlate with the results of the desiccation stress assay **(Section 4.5.1.4).**

The increased stress tolerance of *C. sakazakii* i.e. its thermotolerance and its resistance to desiccation enhances its survival in the environment of a PIF manufacturing factory. This increases its chances of contaminating the PIF, thereby increasing the risk that neonates will be exposed to this pathogen.

Most of the *C. sakazakii* CC4 and non-CC4 strains remained viable after being treated with 60°C, 80°C, or 100°C heat for 1 hour. In this study, all of the CC4 strains remained viable after exposure to 100°C heat for 1 hour except for strain 1587 (ST4) **(Section 4.5.3.2).** In contrast, none of the non-CC4 strains survived these conditions except for strain 520 (ST12) **(Section 4.5.3.2).** These results suggested that CC4 strains were more tolerant than non-CC4 strains to 100°C heat. This should be confirmed in a study that includes more strains. The heat tolerance of CC4 may contribute to its predominance in milk factories and in PIF (Sonbol *et al.* 2013). The thermotolerance regions of the *Cronobacter* genomes in the PubMLST database were analysed, but no significant variations were found in the genes of CC4 vs. non-CC4 strains. This region showed significant variation between CC4 and non-CC4 isolates; in fact, this region was absent in a non-CC4, expect strain 1 (ST8) that had all of the genes and in strain 696 (ST12) that had *orfABCDE* and *orfOP*; most of this region was present in *C. sakazakii* CC4 genomes. However there did not appear to be a link between the presence of this region and the results of the heat tolerance assay**(Section 4.5.3.3).**

One of the first steps in bacterial pathogenesis is its survival in the acidic environment of the stomach, since it must survive in order for the bacteria to colonise the host. All of the *C. sakazakii* strains survived at pH 3.5, which is similar to the pH of the neonatal stomach **(Section 4.5.4).** Thus, neonates are vulnerable to these bacterial pathogens. In *Salmonella enterica*, the expression of *ompR*, the envelope stress response gene, is regulated by environmental stress and specifically by acidic pH (Bang *et al.* 2000). A recent report found that *ompR* mediates acid tolerance in *C. sakazakii* (Alvarez-Ordóñez *et al.* 2014). In the present study, the *ompR* gene was detected in all *C. sakazakii* genomes, both CC4 and non-CC4 strains. Studying the sequence variation of the *ompR* gene in the *C. sakazakii* genome will be important for understanding differences in the response of this stress gene to acidic environments in CC4 vs. non-CC4 strains.

Most of the *C sakazakii* CC4 and non-CC4 strains were found to be serum-resistant and to replicate in serum. Only strain 1 (ST8) was serum-sensitive. *In silico* analysis of the plasmid-borne *cpa* gene, which is encoded by pESA3, showed that the majority (33/38) of *C. sakazakii* strains have this gene and therefore the ability to survive in blood. However, strains that lacked the *cpa* gene, including ST12 strains 696 and 520, were also serum-resistant in our laboratory assay. Moreover, *C sakazakii*

can survive and even multiply inside macrophages. This allows bacteraemia to develop and spread throughout the body via intracellular reproduction **(Section 4.5.5).**

The strains in this study showed variable motility rates. Most of the CC4 strains were motile, whereas there were both motile and non-motile non-CC4 strains. An analysis of the genes that encode the flagellar proteins that are responsible for bacterial motility showed that all of the *C. sakazakii* strains had the *flg* (ESA_02264–77) gene cluster, whereas the *fli* (ESA_01248–61) flagellar genes were variably present in the 38 *C. sakazakii* genomes **(Section 4.5.6.2).** These genes showed no significant variations between CC4 and non-CC4 isolates. All of the strains had detectable α and β haemolytic activity and protease activity, these activities could make these bacteria more harmful to neonates. All of the *C. sakazakii* CC4 and non-CC4 genomes also had the haemolysis (*hly*) genes and the *zpx* gene **(Section 4.5.7.2).** There were no clear differences in these genes in CC4 vs. non-CC4 isolates. A number of genes involved in key traits, such as the carotene genes, cellulose production genes, and biofilm genes, were analysed in 38 genomes. These genes are scattered across the *C. sakazakii* genome, and there were no patterns in terms of their presence or absence in the CC4 and non-CC4 strains

Analysis of bacterial growth on milk agar plates showed two types of colonies, mucoid and leathery. Genomic analysis showed that there was no variation in most of the capsule genes and, when there was variation, it did not correlate with the capsule phenotype.

Three types of capsule-associated genes were analysed in this study:

1) The colanic acid synthesis gene cluster (ESA_01155-01175; *wzABCKM*), which was present in most of the CC4 and non-CC4 isolates.
2) The capsular polysaccharide assembly and export operon-encoded (*kps*) genes (ESA_03349 to 03359). There was a variation of the *kpsC* (ESA_03352) gene in both *C. sakazakii* CC4 and non-CC4 strains. Region 2 in the *kps* operon was present only in ST1 and ST8 strains but absent from CC4 and other non-CC4 strains.

3) The main gene that regulates colanic acid assembly and export, *rcsA* (ESA_01247), was present in most of the CC4 and non-CC4 isolates. Strains 680 (ST8) and 520 (ST12) were missing this gene.

There might be a correlation between capsules and desiccation, heat tolerance, and acid resistance in *C. sakazakii*, as the capsule genes are present in all of the strains. The following strains were isolated from clinical cases such as NECII cases and severe meningitis cases like strain 696 (ST12); strain 5 (ST8); and strains 721, 1220, and 1221 (ST4).

Using BLAST analysis, all of the capsule genes that were studied except for one region, region 2 on the *kps* were found has variation among the STs in *C. sakazakii* genome. Therefore, further analysis to characterise these gene was needed to identify the role of these proteins in the capsule formation.

- **Profiling of bacterial outer membrane proteins (OMPs) using genomic determination and SDS-PAGE analysis.**

In Gram-negative bacteria, the production of OMPs is often regulated by environmental conditions. Moreover, OMPs play essential roles in bacterial pathogenicity by improving environmental adaptations (Zhang *et al.* 2002). This study included the following main aims:

1) Bioinformatics analyses of selected OMPs that are associated with environmental survival **(Section 5.4.1).**
2) Determination of whether proteins previously reported to be involved in desiccation and osmotolerance were present in CC4 and non-CC4 strains **(Section 5.4.1).**
3) OMP profile analysis of CC4 and non-CC4 strains **(Section 5.5.1 and 5.5.2)**

- **OMP analysis revealed the following:**

1. Bioinformatics analysis of the OMPs of some well-characterised bacteria showed that *Cronobacter* has some OMPs that are similar to identified proteins, suggesting that they may have a similar function. Further analysis is required to identify the proteins using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry.

2. Analysis of proteins that were reported previously to be involved in osmotolerance and in the response to desiccation conditions did not show any variations in CC4 vs. non-CC4 strains.
3. The OMPs were generally distinct for different STs but showed similarities in strains of the same ST (especially for ST4 strains). Cluster analysis of the OMP profiles revealed that the CC4 strains clustered together, suggesting that this lineage might share OMPs that are involved in bacterial survival, persistence, and adaptions to changing environmental conditions. Additional 2D-PAGE and protein expression analysis is needed to validate this result. The OMP profiles from cells cultured in acidic conditions included most of the OMPs present in cells cultured at pH 6, suggesting that *Cronobacter* adapted to acidic stress conditions despite having fewer OMPs.

   Also, the result of this study suggested that CC4 strains may be more acid-tolerant than non-CC4 isolates. The MW analysis was in agreement with the acid resistance assays conducted in the present study **(Section 4.5.3.1).** The acid resistance assays indicated that the CC4 isolates were more acid tolerant at pH 3.5 than non-CC4 (**Figure 4.5; Figure 4.6**). This is an interesting finding suggesting that when grown on highly acidic conditions i.e. pH 3.5, CC4 isolates express more OMPs than non-CC4.

6.2 Summary

The MLST analysis in the first part of this thesis revealed the geographic and temporal diversity of *C. sakazakii* isolates. The results of this study increased our understanding of the distribution of important strains of the organism and this may help devise strategies for controlling the spread of pathogenic organisms in the dairy industry. Based on the MLST results, the laboratory experiments and genomic analysis were performed on three collections of environmental strains. CC4 was identified as the predominant lineage in the environment isolates, therefore this study focused on characterizing CC4 physiological characteristics and on genes related to its survival in stressful environments. The genomes of sequenced isolates were screened for genes that are associated with environmental fitness traits, namely the *rpoS* and the acid stress response, *ompR* genes, plus genes associated with thermotolerance, the osmotic stress response, biofilm formation, and capsule formation. The *in silico* analysis of genes related to environmental fitness showed that both *C. sakazakii* CC4 and non-CC4 strains had most of these genes. This suggests that both groups may have equal peristalses mechanisms, as many of the genes were about the same in both groups. The SDS-PAGE OMP profile analysis was performed to identify traits that were specific to *C. sakazakii* CC4. In fact, CC4 and non-CC4 strains have dissimilar OMP profiles, with one exception in ST1 658.

- **The impact of this research in the context of what is known about *C. sakazakii***
  - The expansion of the *Cronobacter* PubMLST database will make it possible to conduct larger and more detailed genomic analysis studies and to identify targets for typing schemes.
  - The findings increase our understanding of the distribution of important strains of the organism, which will help devise ways to control the spread of pathogenic organisms in the dairy industry.
  - The findings raise awareness about the importance of using effective control measures and improved reconstitution practices to ensure the safety of PIF and dairy products.
  - The genus population study adds to our general knowledge of this bacterium, which can cause irreparable damage to a newborn baby's brain.

- Continuing this research will help clarify the destructive pathogenic mechanisms of *Cronobacter* spp., and this information may help improve control measures.
- The findings open up new lines of research that will help us further understand this opportunistic food-borne pathogen.
- The results show that *C. sakazakii* CC4 strains are heat-tolerant at 100°C.
- The OMP results identified a unique CC4 OMP profile.

6.3 Future directions

This work is just the beginning. Indeed, as of October 2015, the *Cronobacter* MLST database included data related to > 1007 isolates and 214 full sequenced genome along with information about the sources and the geographic and temporal diversity of the genus. The genomes are now available in the PubMLST database for further analysis. These *Cronobacter* strains have been isolated from over 36 different countries and from various environments. The results presented in this thesis open up new avenues of research that will help us further investigate this opportunistic food-borne bacterium and, hopefully, help us eliminate it as a cause of infections. Future research should include:

- Gene expression studies that investigate which genes are differentially expressed in *C. sakazakii* CC4 strains after exposure to stressful conditions such as desiccation, heat, and osmotic shock. Such studies may help identify genes that are expressed exclusively in CC4 strains or that are overexpressed in CC4 genomes in hostile environmental conditions.
- Mutation (knock-out) and functional characterisation of selected physiological genes. First, identification and bioinformatics analysis can be used to identify genes that are involved in bacterial survival in the host and in different environments as well as genes involved in virulence. Examples include the genes responsible for capsule production synthesis and capsule regulation. The results may help elucidate the mechanisms underlying bacterial persistence, attachment, invasion, and spreading in the infected host. This might also observe important changes in virulence-associated behaviour and phenotypic traits in cultures of different cell lines.
- Isolation and identification of bacterial OMPs using 2D-PAGE followed by mass spectrometry. Using data about predicted integral OMPs that are annotated in the SWISS-PROT database, it may be possible to identify unique CC4 OMPs.

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