PHYLOGENY AND MOLECULAR IDENTIFICATION OF *CRONOBACTER* STRAINS ISOLATED FROM SOUTH AFRICAN FOOD PRODUCTS

AMY STRYDOM

Thesis presented in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE IN FOOD SCIENCE

at the Stellenbosch University

Department of Food Science
Faculty of AgriSciences

Study Leader: Prof. R.C. Witthuhn
Co-study Leader: Dr. M. Cameron

March 2011

DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Amy Strydom

Date

ABSTRACT

The genus *Cronobacter* (*Enterobacter sakazakii*) contains opportunistic pathogens that can cause a severe form of neonatal meningitis, necrotising enterocolitis and septicaemia. *Cronobacter* infections have been reported in all age groups, however, immuno-compromised infants are more susceptible to these infections. Furthermore, *Cronobacter* strains have been reported to show differences in sensitivity to antibiotics and virulence. These differences led to the reclassification of *Cronobacter* and currently the genus contains five distinct species, namely *Cronobacter sakazakii, Cronobacter malonaticus, Cronobacter turicensis, Cronobacter dublinensis* and *Cronobacter muytjensii*. As this reclassification was only accepted recently, there are not many typing methods optimised for differentiation between the five *Cronobacter* species. Typing of *Cronobacter* strains are important as the species may be diverse regarding their virulence.

Cronobacter strains have been isolated from infant formula milk (IFM), the environment of an IFM processing facility and fresh produce in South Africa. However, little is known about the phylogeny and prevalence of these strains. The aim of this study was to classify 24 South African *Cronobacter* strains (previously identified as *E. sakazakii*) and to evaluate the phylogeny of the isolates based on the 16S ribosomal RNA (rRNA) and *rpo*A genes. All 24 South African strains were identified as *Cr. sakazakii* despite a wide variety of isolation sources. Other studies have also found that irrespective of the isolation source, the majority of *Cronobacter* strains are identified as *Cr. sakazakii*. The South African strains were found to be phylogenetically closely related. However, two distinct clusters separated at a 93 % confidence level were observed in the *Cr. sakazakii* group based on the 16S rRNA gene analysis.

Strains of *Cr. sakazakii*, *Cr. dublinensis*, *Cr. turicensis* and *Cr. muytjensii* were differentiated from each other with sequence data of the 16S rRNA and *rpo*A genes, but it was not possible to differentiate between *Cr. sakazakii* and *Cr. malonaticus*. The phylogram based on the *rpo*A gene sequences did separate *Cr. malonaticus* and *Cr. sakazakii* strains, however, the clusters were separated with a low bootstrap value of 70 %. Phylogenetic analysis based on the *rpo*A and 16S rRNA genes were, therefore, not sufficient to distinguish between all the *Cronobacter* species. The sequence data of these two genes can be used to differentiate between the *Cronobacter* strains when used in combination with malonate utilisation analysis.

A PCR-RFLP method was subsequently developed to facilitate the simultaneous differentiation between all five *Cronobacter* species. The PCR-RFLP assay was based on the amplification of the *rpo*B gene followed by the combined digestion with restriction endonucleases *Csp*6I and *Hin*P1I. Unique profiles for each of the five *Cronobacter* species were obtained and it was also possible to differentiate between Enterobacteriaceae and *Cronobacter* strains. Furthermore, two strains which were identified as *Cr. sakazakii* with sequencing based on the 16S rRNA and *rpo*A genes had PCR-RFLP profiles identical to that of *Cr. malonaticus*. Sequencing based on the *rpo*B gene and additional biochemical analysis with malonate broth confirmed the identities of these two strains as *Cr. malonaticus*. This PCR-RFLP assay is, therefore, an accurate typing method that ensures rapid differentiation between the five species of *Cronobacter*.

OPSOMMING

Die *Cronobacter* genus (*Enterobacter sakazakii*) bevat opportunistiese patogene wat 'n ernstige vorm van neonatale meningitis, enterokolitis en septisemie kan veroorsaak. *Cronobacter* infeksies is al in alle ouderdomsgroepe aangemeld, maar immuungekompromitteerde babas is die meeste vatbaar vir hierdie infeksies. Verder toon *Cronobacter* spesies verskille in virulensie en sensitiwiteit vir antibiotika. Hierdie verskille het gelei tot die herklassifikasie van *Cronobacter* en tans bestaan die genus uit vyf afsonderlike spesies, naamlik *Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter turicensis*, *Cronobacter dublinensis* en *Cronobacter muytjensii*. Aangesien hierdie herklassifikasie slegs onlangs aanvaar is, is daar nie baie metodes wat geskik is vir onderskeiding tussen die vyf *Cronobacter* spesies nie. Onderskeiding tussen *Cronobacter* spesies is belangrik omdat die spesies verskillend kan wees met betrekking tot hulle virulensie.

Cronobacter is geisoleer uit baba formule melk (BFM), die omgewing van 'n BFM fabriek en vars produkte in Suid-Afrika. Daar is egter nie baie bekend oor die filogenie en voorkoms van hierdie isolate nie. Die doel van hierdie studie was om 24 Suid-Afrikaanse *Cronobacter* stamme (voorheen geïdentifiseer as *E. sakazakii*) te klassifiseer en die filogenie van die isolate te evalueer gebaseer op die 16S ribosomale RNS (rRNS) en *rpo*A gene. Al 24 Suid-Afrikaanse stamme is geïdentifiseer as *Cr. sakazakii* ten spyte van 'n wye verskeidenheid isolasie bronne. Ander studies het ook gevind dat, ongeag die isolasie bron, die meerderheid van *Cronobacter* stamme as *Cr. sakazakii* geïdentifiseer word. In hierdie studie is gevind dat die Suid-Afrikaanse stamme filogeneties nou verwant is. Op grond van die 16S rRNA geen analise is die *Cr. sakazakii* stamme egter in twee afsonderlike groepe gedeel met 'n 93% vertrouens vlak.

Dit was moontlik om stamme van *Cr. sakazakii, Cr. dublinensis, Cr. turicensis* en *Cr. muytjensii* van mekaar te onderskei met die DNS volgorde data van die 16S rRNA en *rpo*A gene, maar geen onderskeid tussen *Cr. sakazakii* en *Cr. malonaticus* stamme was moontlik nie. Die filogram gebaseer op die *rpo*A DNS volgorde data het aparte takke vir *Cr. malonaticus* en *Cr. sakazakii* stamme getoon, maar die twee takke is met ‘n lae vertrouens waarde van slegs 70 % geskei. Filogenetiese analise gebaseer op die *rpo*A en 16S rRNA gene is dus nie voldoende om te onderskei tussen al die *Cronobacter* spesies nie. Die DNS volgorde data van hierdie twee gene sou egter gebruik kon word om te onderskei

tussen die *Cronobacter* spesies wanneer dit gebruik word in kombinasie met malonaatbenutting-analises.

'n Polimerase ketting reaksie (PKR) beperkings fragment lengte polimorfisme (BFLP) metode is ontwikkel om die gelyktydige onderskeiding tussen al vyf *Cronobacter* spesies te fasiliteer. Die PKR-BFLP metode is gebaseer op die vermeerdering van die *rpo*B geen gevolg deur die gesamentlike vertering met die beperkingsensieme, *Csp*6I en *Hin*P1I. Unieke profiele vir elk van die vyf *Cronobacter* spesies is verkry en dit was ook moontlik om tussen Enterobacteriaceae en *Cronobacter* spesies te onderskei. Verder het twee stamme wat as *Cr. sakazakii* geïdentifiseer is met DNS volgordebepaling van die 16S rRNA en *rpo*A gene, PKR-BFLP profiele identies aan dié van *Cr. malonaticus* getoon. DNS volgordebepaling van die *rpo*B geen en ‘n addisionele biochemiese toets met malonaat sop het die identiteit van hierdie twee stamme as *Cr. malonaticus* bevestig. Hierdie PKR-BFLP is dus 'n akkurate metode wat vinnige onderskeid tussen die vyf spesies van *Cronobacter* kan verseker.

CONTENTS

Language and style used in this thesis are in accordance with the requirements of the *International Journal of Food Science and Technology*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the following individuals and institutions for their valuable contributions to the successful completion of this research:

My study leaders, Prof. R.C. Witthuhn and Dr. M. Cameron, for their expert guidance, knowledge, support, positive criticism, skilled practical assistance and general awesomeness during this study, *I loved working with you*;

SAMPRO, FoodBev Seta, Milk SA, CGCSA, the University of Stellenbosch and the Ernst and Ethel Eriksen Trust for financial support;

Prof K. Jacobs for teaching me the art of drawing a tree, *who knew it would be that complicated?*;

Donna Cawthorn for advice and interest in my project, *even when you hated being reminded of Cronobacter*;

My fellow post-graduate students (especially the *mol* lab) for their friendship of six years, *I will dearly miss you next year*;

Dr. N. Brown, Mrs A. Lombaard and Mrs D. du Preez for always lending a helping hand and valuable advice, *you are not appreciated enough*;

The civilians: my parents; Alwyn and Elna Strydom, family and my close friends, Bernice, Caty, Cecilia, Evette, Ilona, Ilze, Mia and Nola, for their unconditional love and support, *even when they did not understand what I was doing*;

My Heavenly Father for giving me the aptitude, patience and strength to succeed.

Dit is die Here wat wysheid gee, uit sy mond kom die kennis en die insig.

Spreuke 2

for the glory of the Lord

CHAPTER 1

INTRODUCTION

Microbial foodborne diseases pose a considerable threat to human health and are a concern for food legislators, food manufacturers and consumers. The number of individuals that are highly susceptible to these foodborne diseases is increasing due to the ageing population and high numbers of HIV/AIDS infections. Furthermore, infants and children in developing countries are particularly vulnerable to foodborne infections due to reduced immunity caused by malnutrition (WHO, 2001; 2002).

Cronobacter (previously known as *Enterobacter sakazakii*) is an opportunistic pathogen that can cause neonatal meningitis, necrotizing enterocolitis and septicemia. These bacteria received increased attention as foodborne pathogens after an outbreak of meningitis in Tennessee in 2001 (Iversen & Forsythe, 2003). Urmenyi & Franklin (1961) reported on the first incidence of *Cronobacter* infections of two fatal cases of neonatal meningitis that occurred in England. The bacteria that caused these infections were referred to as yellow pigmented *Enterobacter cloacae*. Shortly thereafter the first evidence of a separate species was based on DNA analysis that indicated the yellow pigmented strains were less than 50 % related to other *E. cloacae* strains (Brenner, 1974). *Enterobacter sakazakii* was proposed as the new name for these isolates, although it was suggested that the isolates may comprise of more than one species. In 2008 *E. sakazakii* isolates were reclassified in the novel genus, *Cronobacter*. Currently there are five distinct species in the genus, namely *Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter turicensis*, *Cronobacter dublinensis* and *Cronobacter muytjensii* (Iversen *et al*., 2007; 2008).

Many characteristics of these pathogens are strain dependant and differences in specifically virulence have been documented. Only *Cronobacter* species associated with neonatal infections, namely *Cr. sakazakii, Cr. malonaticus* and *Cr. turicensis* have the genes encoding for a cation efflux system which allows bacteria to invade brain microvascular endothelial cells. *Cronobacter muytjensii* has been isolated from human bone marrow which would normally be sterile (Kucerova *et al*., 2010). As it is still unclear whether all of the species are virulent, the genus is currently classified as pathogenic (FAO/WHO, 2008).

The original reservoir of *Cronobacter* is still unknown but there are indications that these pathogens might be of plant origin. *Cronobacter* strains have been isolated from various food products such as mixed salad vegetables, meat, milk and cheese (Kahn *et al*., 1998; Iversen & Forsythe, 2003; Kim & Beuchat, 2005; Beuchat *et al*., 2009; El Sharoud *et al*., 2009). However, infant formula milk (IFM) is the only source that has been epidemiologically linked to disease outbreaks caused by *Cronobacter* (Muytjens et al., 1983; Pagotto & Farber, 2009). The risk of *Cronobacter* contamination is further increased as it has been reported that regularly used disinfectants are insufficient to kill *Cronobacter* cells imbedded in biofilms Kim *et al*., 2007) and that some strains survive refrigeration temperatures (Nazarowec-White *et al*., 1997).

Low contamination levels (1 cfu.100 g^{-1}) of *Cronobacter* can have a severe impact on health and the rapid detection and correct identification of these pathogens are important for food safety (Van Acker *et al*., 2001). The inactivation and inhibition, as well as thermal, osmotic and desiccation tolerance of these bacteria have been characterised and may assist in the development of risk management strategies for the production of safe products (Al-Holy *et al*., 2009; Chenu & Cox, 2009; Osaili & Forsythe, 2009). Despite the increased research interest in *Cronobacter*, there is limited information available on the prevalence and genetic diversity of these bacteria in South Africa. The aim of this study was to reclassify previously identified *E. sakazakii* (*Cronobacter* spp.) strains isolated from IFM, the production facility of IFM and fresh produce in South Africa and to determine the phylogenetic relatedness of these *Cronobacter* strains. Since there are indications that some of the *Cronobacter* species might not be virulent, a second aim of this study was to develop an accurate molecular identification method to differentiate between the five *Cronobacter* species.

References

Al-Holy, M., Castro, L.F. & Al-Qadiri, H. (2009). Inactivation of *Cronobacter* spp. (*Enterobacter sakazakii*) in infant formula using lactic acid, copper sulfate and monolaurin. *Letters in Applied Microbiology,* **50** (3), 246-251.

Beuchat, L.R., Kim, H., Gurtler, J.B., Lin, L.C., Ryu, J.H. & Richards, G.M. (2009). *Cronobacter sakazakii* in foods and factors affecting its survival, growth, and inactivation. *International Journal of Food Microbiology,* **136** (2), 204-213.

Brenner, D.J. (1974). DNA reassociation for the clinical differentiation of enteric bacteria, *Public Health Lab.,* 118–130.

Chenu, J.W. & Cox, J.M. (2009). *Cronobacter* ("*Enterobacter sakazakii*"): current status and future prospects. *Letters in Applied Microbiology,* **49** (2), 153-159.

El-Sharoud, W., O'Brien, S., Negredo, C., Iversen, C., Fanning, S. & Healy, B. (2009). Characterization of *Cronobacter* recovered from dried milk and related products. *BMC Microbiology,* **9**, 9.

Food and Agriculture Organization / World Health Organization (FAO/WHO) (2004). *Enterobacter sakazakii* and other microorganisms in powdered infant formula: Meeting Report, MRA series 6. Geneva, Switzerland. [www document]. URL http://www.who.int/foodsafety/micro/meetings/feb2004/en/. 6 March 2009.

Food and Agriculture Organization / World Health Organization (FAO/WHO) (2008). *Enterobacter sakazakii* (*Cronobacter* spp.) in powdered follow-up formulae: Meeting Report, MRA series 15. Rome. [www document]. URL http://www.who.int/foodsafety/micro/meetings/2008/en/. 11 May 2009.

Iversen, C. & Forsythe, S. (2003). Risk profile of *Enterobacter sakazakii*, an emergent pathogen associated with infant milk formula. *Trends in Food Science & Technology,* **14** (11), 443-454.

Iversen, C., Lehner, A., Mullane, N., Bidlas, E., Cleenwerck, I., Marugg, J., Fanning, S., Stephan, R. & Joosten, H. (2007). The taxonomy of *Enterobacter sakazakii*: proposal of a new genus *Cronobacter* gen. nov. and descriptions of *Cronobacter sakazakii* comb. nov. *Cronobacter sakazakii* subsp. *sakazakii*, comb. nov., *Cronobacter sakazakii* subsp. *malonaticus* subsp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov., *Cronobacter* genomospecies 1. *BMC Evolutionary Biology,* **64** (7), 1471-2148.

Iversen, C., Mullane, N., McCardell, B., Tall, B.D., Lehner, A., Fanning, S., Stephan, R. & Joosten, H. (2008). *Cronobacter* gen. nov., a new genus to accommodate the biogroups of *Enterobacter sakazakii*, and proposal of *Cronobacter sakazakii* gen. nov., comb. nov., *Cronobacter malonaticus* sp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov., *Cronobacter* genomospecies 1, and of three subspecies, *Cronobacter dublinensis* subsp. *dublinensis* subsp. nov., *Cronobacter dublinensis* subsp. *lausannensis* subsp. nov.

and *Cronobacter dublinensis* subsp. *lactardi* subsp. nov.. *International Journal of Systematic and Evolutionary Microbiology,* **58** (6), 1442-1447.

Khan, A.A, Jones, R.A., Cerniglia, C.E. (1998). Rapid method for the detection of genetically engineered microorganisms by polymerase chain reaction from soil and sediments. *Journal of Industrial Microbiology & Biotechnology*, **20**, 90-94.

Kim, H. & Beuchat, L.R. (2005). Survival and growth of *Enterobacter sakazakii* on fresh-cut fruits and vegetables and in unpasteurized juices as affected by storage temperature. *Journal of Food Protection,* **68** (12), 2541-2552.

Kim, H., Ryu, J.H. and Beuchat, L.R. (2007). Effectiveness of disinfectants in killing *Enterobacter sakazakii* in suspension, dried on the surface of stainless steel, and in a biofilm. *Applied Environmental Microbiology,* **73**, 1256–1265.

Kucerova, E., Clifton, S.W., Xia, X., Long, F., Porwollik, S., Fulton, L., Fronick, C., Minx, P., Kyung, K.,Warren, W., Fulton, R., Feng, D., Wollam, A., Shah, N., Bhonagiri, V., Nash, W.E., Hallsworth-Pepin, K., Wilson, R.K., McClelland, M. & Forsythe, S.J. (2010). Genome sequence of *Cronobacter sakazakii* BAA-894 and comparative genomic hybridisation analysis with other *Cronobacter* species. *PLoS ONE*. **5** (3) e9556. Doi: 10.1371/ journal.pone0009556.

Muytjens, H.L., Zanen, C.H., Sonderkamp, J.H., Kollee, L.A., Wachsmuth, K.,Farmer, J.J. (1983). Analysis of Eight Cases of Neonatal Meningitis and Sepsis. *Journal of Clinical Microbiology,* **18** (1), 115-120.

Nazarowec-White, M. & Farber, J.M. (1997). Thermal resistance of *Enterobacter sakazakii* in reconstituted dried-infant formula. *Letters in Applied Microbiology,* **24** (1), 9-13.

Osaili, T. & Forsythe, S. (2009). Desiccation resistance and persistence of *Cronobacter* species in infant formula. *International Journal of Food Microbiology,* **136** (2), 214-220.

Pagotto, F.J. & Farber, J.M. (2009). *Cronobacter* spp. (*Enterobacter sakazakii*): Advice, policy and research in Canada. *International Journal of Food Microbiology,* **136** (2), 238-245.

Urmenyi, A.M.C. & Franklin, A.W. (1961). Neonatal death from pigmented coliform infection. *The Lancet*, **1**, 313-315.

Van Acker, J., de Smet, F., Muyldermans, G., Bougatef, A., Naessens, A. & Lauwers, S. (2001). Outbreak of necrotizing enterocolitis associated with *Enterobacter sakazakii* in powdered milk formula. *Journal of Clinical Microbiology*, **39**, 293-297.

World Health Organisation (WHO) (2001). Infant and young child nutrition: global strategy for infant and young child feeding, EB109/12. [www document]. URL http://www.who.int/gb.pdf_files/WHA54/ea54r2.pdf. 6 August 2007.

World Health Organisation (WHO) (2002). Foodborne diseases. Fact sheet No. 124. [www document]. URL http://www.who.int/mediacentre/factsheet/fs124/en/. 14 July 2007.

CHAPTER 2

LITERATURE REVIEW

A. Background

The first report of members of the genus *Cronobacter* was in 1961 by Urmenyi & Franklin. In this study, two fatal cases of neonatal meningitis that occurred in England were investigated. Strains isolated from infants were described as yellow-pigmented *Enterobacter cloacae* at the Manchester Public Health Laboratory. There was, however, no conclusion on the origin of the bacteria. Another case of meningitis was ascribed to the yellow pigmented *E. cloacae* in 1965 (Jøker *et al*., 1965). The infection was cleared after antibiotic treatment that included chloramphenicol and ampicillin. The strains isolated from this case were compared to the strains from England and they differed only in gas production from glycerol, inositol and starch, as well as in malotone utilisation.

These yellow-pigmented bacteria were identified as *E. cloacae* on account of the phenotypic similarities, the only difference being pigment production. In 1974 the first evidence of a separate species was based on DNA analysis that showed the pigmented strains were less than 50 % related to the non-pigmented strains (Brenner, 1974). Additional evidence was based on antibiotic susceptibility and biochemical reactions. DNA-DNA hybridisation confirmed that the yellow-pigmented strains were a separate species as it was only 50 % related to *E. cloacae* and *Citrobacter diversus*. *Enterobacter sakazakii* was proposed as the new name for this novel species as it was phenotypically more similar to *E. cloacae* and only had a 41 % DNA homology with *Citrobacter freundii*, the type species for *Citrobacter* (Farmer *et al.,* 1980).

During the first classification of *E. sakazakii* it was proposed that the isolates may comprise of more than one species. The biochemical evaluation of 57 strains resulted in 15 different biogroups and it was suggested that biogroup 15 may be grouped in a separate species from biogroups 1 – 14 (Fig. 1) (Farmer *et al.,* 1980). These findings were compared to the partial 16S ribosomal RNA (rRNA) genotypes of 189 different strains with corresponding biochemical traits (Iversen *et al*., 2006). The molecular analysis gave rise to an additional biogroup (biogroup 16) and the 189 strains were divided into four clusters.

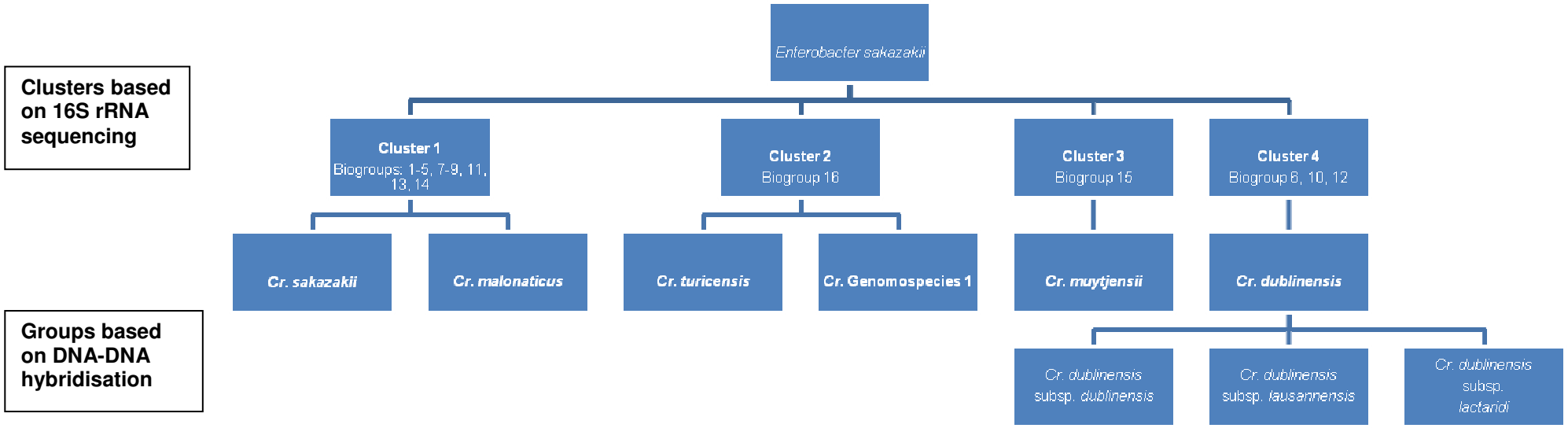


Figure 1 Distribution of biogroups and clusters in the taxonomy of *Cronobacter.*

These four clusters could be distinguished with inositol, dulcitol and indole tests (Iversen *et al*., 2006). The divergent biogroups reinforced the hypothesis that there could be more than one species and it was proposed to divide *E. sakazakii* into five separate genomospecies (Iversen *et al.,* 2007a). Finally in 2008 *Enterobacter sakazakii* was reclassified as a member of the novel genus, *Cronobacter* that contains five species and three subspecies (Iversen *et al*., 2008a) (Fig. 1).

B. Characteristics of *Cronobacter*

Species of the genus *Cronobacter* consists of Gram-negative rods that are 1 µm x 3 µm in size. These bacteria are facultative anaerobic and generally peritrichous. Enzymatic profiles confirmed the absence of phosphoamidase and indicated that α-glucosidase activity is unique to *Cronobacter* species (Muytjens *et al*., 1984). The activity of α-glucosidase has been implemented as a selective marker in differential chromogenic agar (Iversen *et al*., 2004a).

Species of this genus can grow at temperatures between 6° - 47 °C with an optimum growth temperature of 39 °C (Iversen & Forsythe, 2003). However, some strains are inhibited at temperatures above 44 °C (Nazarowec-White & Farber, 1997a; Iversen *et al*., 2004a) and some strains are capable of growing at 5 °C (Nazarowec-White & Farber, 1997b). Pigment production and colony size on tryptone soy agar (TSA) are greatly influenced by the incubation temperature. The yellow pigment production after 24 h of incubation is more pronounced at 25 °C than at 36 °C, with colony sizes of 1 – 2 mm and 2 – 3 mm, respectively (Farmer *et al.,* 1980).

Two colony types have been observed when isolates were streaked on TSA. Type A (Farmer *et al*., 1980) or matt (Iversen & Forsythe, 2003) is dry or mucoid, scalloped and rubbery when touched with a loop. Type B (Farmer *et al*., 1980) or glossy (Iversen & Forsythe, 2003) is smooth and often exhibits little pigment production. Sub-culturing of the different colony types showed that the matt colonies may spontaneously change to glossy colonies and it is very common to find both colony types in one culture (Farmer *et al*., 1980). Differences between environmental strains and clinical strains were also observed (Beuchat *et al*., 2009). The clinical strain produced mucoidal colonies on violet red bile glucose agar (VRBGA), whereas the environmental strains produced crinkled, matt colonies with a rubbery texture.

Biofilm formation

Members of the *Cronobacter* genus have been reported to form biofilms on a range of surfaces including silicon, glass, stainless steel, latex and polycarbonate (Iversen *et al*., 2004b). Attachment of the bacteria occurs more rapidly to hydrophobic substances such as plastic than to hydrophilic materials (Lehner *et al.,* 2005). The production of biofilms can be influenced by the nutrient availability, as well as the temperature of the growth medium (Kim *et al*., 2006). The importance of nutrient availability was demonstrated by the formation of biofilms on plastic in different media. The highest number of strains (77 %) produced biofilms in infant formula milk (IFM), whereas only 16 % produced biofilms in diluted tryptone soy broth (TSB) (Oh *et al*., 2007). The influence of temperature was illustrated as no biofilms were formed in any of the media at 12 °C (Kim *et al*., 2006).

The disinfectants used in hospitals, day-care centres and food service kitchens are inefficient in eliminating cells embedded in organic matrices (Kim *et al*., 2007). Therefore, formation of biofilms in hospital environments and on equipment may increase the risk of infections in infants and immuno-compromised adults. In addition, high density (10^7 cfu.cm^{-1}) biofilm formation in IFM was observed after 24 h on enteral feeding tubes. This greatly increases the risk for neonatal infection specifically in hospitals as enteral feeding tubes can remain *in situ* at 37 °C for several days and nutrients are administered to the infants every 2 - 3 h (Hurelle *et al*., 2009).

Thermal resistance

Thermal resistance studies indicate contradicting results for species of the genus *Cronobacter*. There is a general agreement that *Cronobacter* spp. are thermo-tolerant bacteria (Chenu & Cox, 2009), although *D*-values (the time required for a 10-fold reduction in the viable numbers of a bacterium at a given temperature) of 0.4 min (Breeuwer *et al.,* 2003), 2.6 min (Iversen *et al*., 2004b) and 4.2 min (Nazarowec-White & Farber, 1997a) have been reported at 58 °C. The thermal resistance of *Cronobacter* spp. has been shown to be strain dependent (Breeuwer *et al*., 2003; Edelson-Mammel & Buchanan, 2004; Arroyo *et al*., 2009), although some studies did not report any significant differences between different strains (Nazarowec-White & Farber, 1997a; Iversen *et al*., 2004b).

Heat shock, pH and water activity influence the thermal resistance of *Cronobacter* (Arroy *et al*., 2009). Contradicting results have been published regarding the effect that heat shock has on the thermal resistance of *Cronobacter* strains. After subjection of *Cronobacter* cells to heat shock for 15 min at 47 °C, the thermal resistance at 47 °C was enhanced (Chang *et al*., 2009). However, the sensitivity of *Cronobacter* cells to heat treatment increased after a heat shock for 1 h at 42.5 ° and 45 °C, respectively. The maximum heat resistance of these *Cronobacter* cells was observed after incubation at 20 °C. The thermal resistance of *Cronobacter* was approximately 10 times more at pH 7 than at pH 4. However, when the water activity was lowered from more than 0.99 to 0.96 the bacteria showed a 32-fold increase in thermal resistance at 4 °C and pH 4 (Arroyo *et al*., 2009).

The heat treatment applied during commercial pasteurisation is sufficient to destroy *Cronobacter* in IFM as no contamination was observed directly after pasteurisation in an IFM manufacturing facility (Proudy *et al*., 2008). It was, therefore, suggested that contamination occurs after the pasteurisation step, as *Cronobacter* have been isolated from the final products. This can possibly occur during the production process of IFM before or during spray drying (Iversen & Forsythe, 2003; Proudy *et al*., 2008). *Cronobacter* strains can survive spray drying at inlet temperatures of 160 °C and outlet temperatures of 90 °C (Arku *et al.,* 2008), confirming that this may be the point of contamination in an IFM processing facility.

Osmotic and desiccation tolerance

Members of *Cronobacter* have shown greater osmotic and desiccation tolerance than other bacteria like *E. coli* and species of *Salmonella* (Breeuwer *et al.,* 2003). *Cronobacter* strains can survive in substances with a low water activity, such as infant rice cereals (0.3 - 0.69 a_w) and IFM (0.25 - 0.5 a_w). The survival of *Cronobacter* spp. at lower temperatures seems better as higher numbers of the bacteria survived in cereals (0.63 - 0.83 a_w) at 4 °C as opposed to 21° or 31 °C (Gurtler & Beuchat, 2007; Lin & Beuchat, 2007).

Given that *Cronobacter* cells can survive for up to 2 years in a desiccated environment and multiply rapidly after hydration, it is important to understand the stress responses of *Cronobacter* in order to control contamination (Osaili & Forsythe, 2009). Cell

metabolism studies indicate the accumulation of trehalose in *Cronobacter* cells during the stationary phase. Trehalose is a highly soluble disaccharide of glucose which can possibly stabilise proteins and phospholipid membranes, thereby protecting the bacteria from dehydration (Breeuwer *et al*., 2003). Another stress response is the modification of proteins produced in *Cronobacter* cells during desiccation which can lead to oxidation of DNA and membrane components. Several proteins like the *Dps* and *Hns* proteins were shown to be expressed in *Cronobacter* cells exposed to desiccation. These proteins are involved in DNA repair and protection of proteins against oxidation. The production of higher levels of superoxide dismutase and alkylhydroperoxide reductase may also participate in the protection against oxidation (Riedel & Lehner, 2007).

Acid tolerance

Cronobacter has been described as moderately acid resistant enteric bacteria, as the acid resistance correlates with that of salmonellae (Gorden & Small, 1993). Substantial diversity in the acid resistance among 12 *Cronobacter* strains was observed, particularly when the strains were exposed to a very low pH (3.0) for 1 h. Despite this diversity, all the strains were inactivated when exposed to pH 3 for 6 h at 36 °C, while the strains had a higher resistance at pH 3.5 at 36 °C (Edelson-Mammel *et al*., 2006).

The acid resistance of *Cronobacter* in food products may vary. *Cronobacter* cells survived in acidic juices such as watermelon (pH 5.0), cantaloupe (pH 6.8) and tomato juices (pH 4.4) (Kim & Beuchat, 2005). However, these bacteria did not survive in strawberry and apple juices inoculated with 1000 cfu.mL^{-1} *Cronobacter* cells. These juices have acidity of pH 3.6 and pH 3.9, respectively and were incubated at 25 °C. In contrast *Cronobacter* strains have been isolated from fermented products with a higher acidity such as sobia, a fermented food product made from wheat and malt, with an average pH of 3.4 - 4.0 (Gassem, 2002). *Cronobacter* has also been isolated from cheese, such as domiati with a pH between 4.9 and 6.4 and ras with a pH of 5.8 (El Sharoud *et al.,* 2009).

Antibiotic susceptibility

Members of the genus *Cronobacter* appear to differ considerably in terms of their susceptibility to various antibiotics. All the *Cronobacter* strains tested by Farmer *et al.*

(1980) were resistant to penicillin, whereas some strains were susceptible to chloramphenicol and ampicillin and only 13 % of the strains were susceptible to cephalothin. In contrast *Cronobacter* strains were subsequently identified that were resistant to cephalothin and chloramphenicol, as well as ampicillin and tetracycline (Muytjens & van der Ros-van de Repe, 1982; Nazarowec-White & Farber, 1999). Recently the resistance of *Cronobacter* species to ampicillin, cephalothin and extended spectrum penicillin have been confirmed (Kuzina *et al*., 2001; Lai, 2001). A recent clinical case has been reported in which multiple antibiotics including ampicillin, gentamicin and cefotaxamine were ineffective in the treatment of a *Cronobacter* infection (Dennison & Morris, 2002). Ampicillin in combination with chloramphenicol or gentamicin is also inefficient in the treatment of *Cronobacter* infections as the pathogen seems to be increasingly resistant to these antibiotics (Lai, 2001).

Virulence

The genus *Cronobacter* contains opportunistic pathogens, causing illness in immuno-compromised individuals (Pagotto *et al.,* 2003). The strains in this genus display differences in pathogenicity and may have different virulence factors (Pagotto *et al.,* 2003; Healy *et al*., 2009; MacLean *et al*., 2009). *Cronobacter sakazakii, Cr. turicensis* and *Cr. malonaticus* are the only species which have been isolated from cases of neonatal meningitis (Kucerova *et al*., 2010). However, a strain belonging to *Cr. muytjensii* has been isolated from human bone marrow which would normally be sterile (Farmer *et al*., 1980). Since the bacterium was reclassified in 2008 as *Cronobacter* (Iversen *et al.,* 2008a), the World Health Organisation (WHO) classified all six species as pathogens (FAO/WHO, 2008). Little is known about the mechanism of infection or the different virulence factors of *Cronobacter* spp.

One virulence factor of *Cronobacter* is the O-antigen. These polysaccharide side chains are variable and are responsible for serological diversity among bacteria. Two serotypes of the *rfb* locus which are implicated in the synthesis of the O-antigen were identified in *Cronobacter* strains. This has important implications for the virulence of *Cronobacter* since the O-antigen is a major surface antigen present in Gram-negative bacteria (Mullane *et al.,* 2008a). The structure of the O-antigen in the endotoxin of *Cr. muytjensii* strain 3270 has recently been described. The O-polysaccharide produced

by this strain is a linear unbranched polymer consisting of a repeating pentasaccharide unit. The structure of this O-polysaccharide differs in size according to sugar composition and complexity of the structure when compared to the O-polysaccharide structures of other *Cronobacter sakazakii* strains. These differences create diversity between serotypes (Healy *et al*., 2009) and may possibly reveal that *Cronobacter* is serologically heterogeneous with respect to the O-antigens (MacLean *et al.,* 2009). Microarray analysis supports the observations that there are multiple O-antigen serotypes, not only between *Cronobacter* species, but also within *Cr. sakazakii*.

Another virulence factor of the *Cronobacter* species is the production of proteolytic enzymes. Cell deformation, particularly "rounding" of cells, is a result of the action of various proteases (Lockwood *et al.,* 1982) and *Cronobacter* strains have been found to cause this type of deformation of the tissue cells of mice (Pagotto *et al.,* 2003). In particular a zinc-containing metalloprotease were identified in *Cronobacter* cells which caused rounding of Chinese hamster ovary cells. This enzyme had collagenolytic (lysis of collagen) activity which may allow the pathogen to cross the blood-brain barrier or cause the extensive cell damage found in neonates with necrotising enterocolitis. It was found that all of the strains tested possessed the *zpx* gene which codes for this proteolytic enzyme (Kothary *et al*., 2007).

Additionally, *Cronobacter* strains have been found to produce an enterotoxin (Pagotto *et al.,* 2003). Purification and characterisation of this enterotoxin indicated its molecular mass as 66 kDA and that it is most active at pH 6. The enterotoxin proved to be highly stable as it was unaffected after incubation at 70 ℃ for 30 min and showed only a decrease in activity after 30 min incubation at 90 ℃ (Rhagav & Aggrawal, 2007). However, the importance of the enterotoxin is still unclear as the genes encoding the putative toxin and the protein itself remain unidentified (Chenu & Cox, 2009).

C. Disease report

Since the case of a *Cronobacter* infection in Tennessee was reported in 2002 (CDC, 2002), the number of well documented cases worldwide has increased. However, the surveillance of the infections and number of incidences in different age groups are not sufficient to provide the exact number of infections attributed to this pathogen. At least 111 cases have been reported in infants and children of which 26 were fatal (Nazarowec-White & Farber,

1997a; Iversen & Forsythe, 2003; Gurtler *et al*., 2005; Drudy *et al*., 2006; Mullane *et al*., 2008b). Only a few of these cases are well described and most of them occurred sporadically, making epidemiological investigations impossible (FAO/WHO, 2008).

In the case of IFM, the bacteria may exist in clumps rather than be spread out evenly throughout the product (Witthuhn *et al*., 2007). This may also lead to false negative results causing underestimation of contamination and the retail of contaminated products (FAO/WHO, 2008). The accurate determination of the occurrence of *Cronobacter* species is also greatly influenced by the low sensitivity and specificity of the detection methods for this genus. However, improvements have been made in the detection and identification methods that would aid in the accurate estimation of *Cronobacter* contamination (Druggan & Iversen, 2009).

These contributions will assist in the development of a reasonable risk assessment and consequent control of *Cronobacter*. While England, Wales, Scotland and Ireland has the most information about *Cronobacter* infections (FAO/WHO, 2008), countries such as Canada, Argentina and the Netherlands are making remarkable efforts to evaluate the risk and characteristics of *Cronobacter* spp. (Pagotto & Farber, 2009; Reij *et al*., 2009; Terragno *et al*., 2009).

Characteristics of disease

The genus *Cronobacter* has been associated with sporadic infections and outbreaks (FAO/WHO, 2004). The first known cases of *Cronobacter* infections were two cases of meningitis in neonates (Urmenyi & Franklin, 1961). These pathogens have been shown to cause a severe form of neonatal meningitis which is an acute inflammation of the membranes surrounding the brain and spinal cord (Nazarowec-White & Faber, 1997c). *Cronobacter* strains have also been isolated from infants associated with necrotising enterocolitis which is caused by infection of the intestines. Other symptoms of infections include septicaemia (Nazarowec-White & Faber, 1997c), bloody diarrhoea (Simmons *et al.,* 1989) and brain abscess (Naqvi *et al*., 1990). The mortality rate has been reported to vary from 10 % to 80 % with fatalities occurring just days after symptoms developed (Iversen *et al*., 2004a). Generally *Cronobacter* affects the central nervous system (Gallagher & Ball, 1991) and survivors often suffer from severe neurological problems after the infections (Muytjens *et al*., 1983; Naqvi *et al*., 1990; Lai, 2001).

Risk groups

Infections of *Cronobacter* have been reported in all age groups, though immuno-compromised and very young individuals are more susceptible to infection. Neonates with a low birth weight or who are immuno-compromised are at the greatest risk (FAO/WHO, 2008). Infections in healthy infants have also been reported (Adamson & Rogers, 1981) and it may be that the immature immune system and gastro-intestinal tract contribute to the high susceptibility of this group. Since IFM is a liquid it passes quickly through the stomach to the small intestines and, therefore, the pathogen is not as stressed as it would be in the case of a mature adult (Iversen & Forsythe, 2003). Reports of infections in adults are mostly in combination with other diseases such as the isolation of *Cronobacter* strains from a foot ulcer (Pribyl *et al*., 1985) and an infected wound (Dennison & Morris, 2002).

Cronobacter species pose a significant threat to immuno-compromised individuals, especially HIV/AIDS patients. This is even more intensified as HIV-positive mothers are encouraged to give IFM to their babies to prevent transmission of the HI-virus via breast-feeding (FAO/WHO, 2004). Developing countries that have a high number of premature babies and HIV-infected individuals are, therefore, likely to have more *Cronobacter* infections. High ambient temperatures in developing countries increase the risk of rapid growth of *Cronobacter* and insufficient surveillance systems prevent the documentation of infections (FAO/WHO, 2008).

Infectious dose

The infectious dose for *Cronobacter* has not been determined, although Health Canada is working on a dose-response relationship (Pagotto & Farber, 2009). The infectious dose will be influenced by the state of the bacteria, the immune system of the host and the environment in which the bacteria grew before infection. The proposed infectious dose value is 1 000 cfu.100 g^{-1} although Pagotto *et al.* (2003) found that 10 000 cfu per mouse was the lowest count to be lethal in a suckling mouse assay. Nevertheless, it will take up to 9 days at 8 °C in reconstituted IFM for the pathogen to reach 1 000 cfu.g^{-1}, whereas it may take only 17.9 h at room temperature with a contamination level of 0.36 cfu.100 g^{-1}. This model shows that it is unlikely that normal contamination levels would cause infection.

The more likely possibility is temperature abuse and/or cross contamination from preparation utensils (Iversen & Forsythe, 2003).

D. Importance in the food industry

Sources of *Cronobacter*

Species of *Cronobacter* have been described as ubiquitous bacteria (Cawthorn *et al*., 2008; El Sharoud *et al*., 2009). These pathogens have been isolated from a range of environmental, clinical, food and beverage sources. IFM is the only source that has been epidemiologically linked to disease outbreaks caused by *Cronobacter* (WHO/FAO, 2004) and research has specifically focused on the presence of *Cronobacter* strains in IFM processing facilities (Mullane *et al.,* 2007; Proudy *et al.,* 2008), raw materials (El-Sharoud *et al.,* 2009) and final products (Mullane *et al*., 2008b). Contamination of IFM can occur via an intrinsic route through the addition of contaminated raw materials after pasteurisation or the processing facility environment during packaging. External contamination can occur during reconstitution by using contaminated utensils (Mullane *et al*., 2008b).

In addition to IFM, *Cronobacter* has been isolated from numerous dry environments including dust, soil, grains, tea, herbs and spices. *Cronobacter* has also been isolated from water, vegetables, cheese and meat (Kahn *et al.,* 1998; Iversen & Forsythe, 2003; Kim & Beuchat, 2005; Beuchat *et al*., 2009; El Sharoud *et al.,* 2009). Additionally, *Cronobacter* has been isolated from ready-to-eat foods (Kandhai *et al*., 2004). The presence of *Cronobacter* in these products and subsequent contamination of households increase the potential risks for infections in immuno-compromised adults (Baumgartner *et al*., 2009). *Cronobacter* strains have not been isolated from cattle faeces, indicating that the pathogen is not carried by beef cattle (Molloy *et al*., 2009).

Clinical sources of *Cronobacter* are diverse and include blood, nose, throat, sputum, and bone marrow. Apart from isolation from infected patients, *Cronobacter* strains have also been isolated from the hospital environment, including a physician's stethoscope (Farmer *et al.,* 1980) and utensils used to prepare IFM in a hospital nursery (Simmons *et al*., 1989; Bar-Oz *et al*., 2001). The possibility of cross-contamination in hospitals is supported by evidence that a contaminated brush used to clean feeding bottles were the source of three cases of infections (Smeets *et al.,* 1998).

The original habitat and mode of transmission of *Cronobacter* are still unknown (Nazarowec-White & Farber, 1997b), however, it has been suggested that since the pathogen does not occur naturally in animals and humans, the principal sources of food contamination is soil, water and vegetables (Iversen & Forsythe, 2003). The yellow pigment production, production of a gum-like extracellular polysaccharide and the ability to persist in a desiccated state suggests an environmental niche associated with plants. *Cronobacter* strains that were evaluated for root colonising properties showed solubilisation of mineral phosphate and the production of indole acetic acid. These characteristics are often found in plant-associated bacteria and rhizosphere microorganisms indicating that the natural habitat of *Cronobacter* may be of plant origin (Schmid *et al*., 2009).

Inactivation and inhibition in IFM

The inactivation and inhibition of *Cronobacter* has been predominantly studied in IFM since the product was identified as the primary vehicle for infection (Muytjens *et al*., 1983). IFM is not a sterile product and it is recommended to keep the reconstituted milk at low temperatures before consumption. Sterilisation of IFM is only possible by irradiation, but the high levels required to inactivate *Cronobacter* influence the organoleptic properties of the product (FAO/WHO, 2004).

Natural antimicrobial agents may be used as additional control measures in the final products (Al-Holy *et al*., 2009). The addition of caprylic acid to IFM was proposed as this antimicrobial agent was found to inhibit *Cronobacter* strains at lower temperatures. After treatment with 10 mM caprylic acid for 20 min at 55 °C and 30 mM caprylic acid for 10 min at 55 °C no *Cronobacter* cells were recovered (Jang & Rhee, 2009). Lactic acid (0.2 %) in combination with copper (II) sulphate (50 µg ml^{-1}) resulted in the complete elimination of *Cronobacter* strains after a 6 h treatment at 21 °C. The lactic acid chelates the copper ions, allowing it to penetrate the cytoplasmic membrane and become toxic to the bacteria. Unfortunately the organoleptic and nutritional properties of IFM using these compounds were not evaluated (Al Holy *et al*., 2009). Bovine lactoferrin related compounds may be useful for the inhibition of *Cronobacter* spp. in foods as growth was inhibited by lactoferrin at 1 $mg.ml^{-1}$ (Wakabayashi *et al*., 2008). In contrast, stressed *Cronobacter* cells were not affected by bovine lactoferrin. Cells were grown in 0.2 % peptone water and reconstituted IFM and challenged with bovine lactoferrin and nisin. Desiccated cells in peptone water

were more susceptible to lactoferrin than undesiccated cells, whereas the undesiccated cells were more susceptible to nisin. Both antimicrobial agents had no inhibitory effect on *Cronobacter* strains in reconstituted IFM at 37 ℃. Bovine lactoferrin may, therefore, not be a suitable antimicrobial agent in IFM for the inhibition of *Cronobacter* (Al-Nabulsi *et al*., 2009).

Regulatory aspects

The Codex Alimentarius Commission (CAC) provides regulations relative to IFM in the *Recommended International Code for Hygienic Practice for Foods for Infants and Children* (CAC, 1979) stating that good manufacturing practices should be followed and clear labelling should be applied. IFM is not required to be sterile and previously *Cronobacter* spp. fell under the specifications for coliforms. These specifications stipulated that four out of five control samples should contain less than 3 cfu.g^{-1} and a maximum of one out of five samples should contain between 3 and 20 cfu.g^{-1}. Since infections occurred under these limitations the CAC determined new specifications for *Cronobacter* spp. The revised regulations require that the product should specifically be tested for the presence of species of *Cronobacter*. A test should comprise of 30 samples of which none may be positive for *Cronobacter* strains, where a positive result is seen as 1 cfu.100 g^{-1} sample (CAC, 2008). The European Union set microbiological standards for *Cronobacter* as negative in 30 x 10 g samples. These guidelines are more stringent and will influence the import of products into European countries (EC, 2005).

E. Detection of *Cronobacter*

Isolation

The rapid isolation and identification of *Cronobacter* is important for the appropriate response to cases of contamination or infection. The detection protocols for *Cronobacter* strains should have a high specificity and sensitivity to prevent false positive and false negative results (Druggan & Iversen, 2009). The current Food and Drug Administration (FDA) culturing method for the detection of *Cronobacter* is based on the enumeration of Enterobacteriaceae in dried foods and IFM. It includes pre-enrichment in a non-selective

broth, enrichment in Enterobacteriaceae enrichment broth (EE), culturing on VRBGA, and sub-culturing onto TSA. Confirmation of presumptive *Cronobacter* strains are done with an oxidase test (oxidase negative) (FDA, 2002). This method has been found to have low sensitivity and relatively low specificity (Cawthorn *et al*., 2008; Iversen *et al*., 2008b).

Alternative methods were developed for the detection and enumeration of members of the genus *Cronobacter* (Iversen *et al*., 2004a; Druggan & Iversen, 2009; Lampel & Chen, 2009). The pre-enrichment step included in these methods recovers injured cells and should increase the sensitivity of the detection method. Buffered peptone water, proposed by the International Organisation for Standardisation (ISO) appear to be more effective than distilled water prescribed in the FDA method (Druggan & Iversen, 2009). Selective enrichment should increase the number of *Cronobacter* cells relatively to the competitive bacteria. In some cases this step can reduce the sensitivity of the method as some *Cronobacter* strains have been found to be sensitive to selective agents including dyes, bile salts, detergents and some antibiotics (Joosten *et al*., 2008; Druggan & Iversen, 2009). The use of antibiotics to increase specificity of detection methods have also been discarded by Druggan & Iversen (2009) and presently vancomycin is the only antibiotic that is used to inhibit Gram-positive bacteria. These findings led to the development of a screening broth, *Cronobacter* screening broth (CSB) that does not inhibit the growth of other Enterobacteriaceae (Iversen *et al*., 2008b). However, the incubation temperature of 42 ℃ and inclusion of sucrose give *Cronobacter* strains advantage over competitors that cannot grow at this temperature or utilise sucrose. The combination of this broth and chromogenic agar based on 5-bromo-4-chloro-3-indolyl-α,D-glugopyranoside (XαGlc) has superior specificity and sensitivity compared to other existing methods (Druggan & Iversen, 2009).

Media that have been proposed as alternatives for VRBGA and TSA include fluorogenic, chromogenic and dual chromogenic media. *Enterobacter sakazakii* Isolation Agar (ESIA®) for example has very good specificity, however, the incubation of 45 ℃ and inclusion of crystal violet reduces the sensitivity. Modified Druggan-Forsythe-Iversen Agar (mDFI), is an improvement on Druggan-Forsythe-Iversen Agar (DFI) and address the limitations of both DFI and ESIA® (Iversen *et al.,* 2008b). A higher concentration of XαGlc, a reduced concentration of sodium deoxycholate and incubation at 42 ℃ increased the sensitivity of mDFI for the detection of *Cronobacter*.

The FDA is currently in the process of validating a new detection protocol for *Cronobacter* that will subsequently be adopted into the FDA's *Bacteriological Analytical Manual* (Druggan & Iversen, 2009; Lampel & Chen, 2009). The proposed protocol includes incubation at 36 ± 1 °C in buffered peptone water after which the culture is streaked on DFI and R&F agar (R&F Laboratories, Downers Grove, IL: http://wwwrf-labs.com/). Single colonies are subjected to a real-time PCR assay developed by Seo & Brackett (2005). RAPID ID 32E is also used as a confirmation step and the complete analysis can be accomplished in 24 to 48 h.

Identification

Colonies on isolation media that are presumed to be *Cronobacter* are traditionally confirmed with biochemical galleries (Iversen *et al.,* 2004a; Drudy *et al*., 2006; Fanjat *et al*., 2007). Biochemical galleries such as the API 20E are regularly used to identify organisms. However, numerous reports have indicated that identification of *Cronobacter* strains with biochemical galleries was often inaccurate. Iversen *et al.* (2004a) reported false-negative and false-positive results with ID 32E, whereas Drudy *et al.* (2006) identified 98 % of 57 isolates correctly. More recently the VITEK 2, ID 32E version 2.0 and ID 32E version 3.0 systems were evaluated for the identification of *Cronobacter*. It was found that the newest version of the ID 32E 3.0 and VITEK 2 systems had a 100 % sensitivity and the ID 32E version 2.0 only had a 71.4 % sensitivity (Fanjat *et al.,* 2007).

Alternative methods for the identification of *Cronobacter* have also been explored (Hoffman *et al*., 2008; Lin *et al.,* 2009). Gas chromatography with flame ionisation detection was used to analyse the cellular fatty acid methyl esters of 30 *Cronobacter* strains. The resulting fatty acid profiles had good repeatability and could be useful for identification purposes (Hoffmann *et al.,* 2008). Detection by Fourier transform infrared (FTI) spectroscopy was also used to discriminate *Cronobacter* from *Enterobacter cloacae*, *Escherichia coli* and *Klebsiella pneumoniae*. Subtle compositional differences were detected with FTI in the carbohydrates of the cell membranes between the *Cronobacter* strains and other species (Lin *et al*., 2009).

Molecular methods for the identification of *Cronobacter* have been extensively researched (Malorny & Wagner, 2005; Seo & Brackett, 2005; Lehner *et al*., 2006; Liu *et al*., 2006a; 2006b; Mohan Nair & Venkitanarayanan, 2006; Mullane *et al*., 2006;

Cawthorn *et al*., 2008). Iversen *et al*. (2007b) developed *dnaG* based reverse transcriptase-PCR (RT-PCR) and 1,6-α-glucosidase (*gluA*) based conventional PCR assays. These PCR systems were evaluated with 312 Enterobacteriaceae strains, including 210 *Cronobacter* strains, making it the most extensively evaluated methods (Iversen *et al*., 2007b). All the *Cronobacter* strains tested positive with both PCR assays.

F. Phylogeny

The reclassification of *Enterobacter sakazakii* to *Cronobacter* was based on a polyphasic approach that included DNA-DNA hybridisation, amplified fragment length polymorphisms (AFLP), automated ribotyping, full length 16S rRNA gene sequencing and phenotypic analysis (Iversen *et al*., 2007a; 2008a). A total of 210 strains, previously described as *Enterobacter sakazakii* were divided into 16 biogroups based on indole production, methyl red test, Voges-Proskauer, ornithine decarboxylase motility, malonate utilisation and acid production from *i*-inositol, dulcitol and methylglucoside. Defining characteristics of each biogroup corresponded with previous findings and included indole, dulcitol and inositol tests (Iversen *et al*., 2006; 2007a). Sequence analysis based on 16S rRNA of these strains resulted in four clusters (Table 1). The majority of strains were grouped in cluster 1 together with the *Enterobacter sakazakii* type strain, ATCC 29544^{T}. Automated ribotyping of the 210 strains resulted in four groups largely corresponding with the four 16S rRNA clusters. The ribotyping results showed a similarity pattern of less than 62 % between the *Enterobacter sakazakii* strains and other Enterobacteriaceae. Subsequent fluorescent-AFLP (f-AFLP) analysis divided the strains into 6 groups that corresponded with the 16S rRNA clusters, as clusters 1 and 2 were each divided into two groups (Table 1) (Iversen *et al*., 2007a).

DNA-DNA hybridisation is considered to be the “gold standard” method to evaluate relatedness between bacterial species (Stackebrandt *et al*., 2002). The recommended cut-off point for species delineation is regarded at a DNA homology of more than or equal to 70 % between two strains (Wayne *et al*., 1987). Representative strains of each of the four 16S rRNA clusters were subjected to DNA-DNA hybridisation and these strains were divided into five groups which had DNA-homology values of less than 70 %. Based on the combination of the genetic and phenotypic data, four *Cronobacter* species were proposed, *Cronobacter sakazakii, Cronobacter turicensis, Cronobacter dublinensis* and

Cronobacter muytjensii, an additional *Cronobacter* genomospecies 1 and a subspecies namely *Cr. sakazakii* subsp. *malonaticus.* This subspecies grouped separately from *Cr. sakazakii* strains with f-AFLP and ribotyping analysis, but had a 99.6 % similarity based on 16S rRNA with the *Cr. sakazakii* type strain, ATCC 29544^T (Iversen *et al*., 2007a). This subspecies was accepted as a distinct species, namely *Cronobacter malonaticus* after subsequent DNA-DNA hybridisation indicated that the *Cr. malonaticus* strains had DNA homology values of less than 70 % with the other *Cronobacter* species (Iversen *et al*., 2008a).

There is a high level of similarity between *Cr. sakazakii* and *Cr. malonaticus* and sequence analysis based on 16S rRNA is not sufficient to distinguish between these two species (Iversen *et al*., 2007a; 2008a; Kuhnert *et al*., 2009). Biochemical differentiation between the two species can be accomplished by testing for the utilisation of malonate, although a small number of *Cr. sakazakii* strains does utilise malonate (Iversen *et al*., 2008a). Controversial results regarding these two species have been found when the phenotypic data of 150 isolates was compared to ribotyping results (Miled-Bennour *et al*., 2010). According to biochemical analysis strain 05CHPL02 was identified as *Cr. sakazakii* and strain 05CPL53 as *Cr. malonaticus*. However, the ribotyping results placed the *Cr. sakazakii* strain closer to the non-sakazakii strains and the *Cr. malonaticus* strain was grouped with the *Cr. sakazakii* strains (Miled-Bennour *et al*., 2010). A higher resolution between *Cr. sakazakii* and *Cr. malonaticus* were obtained with multilocus sequence typing (MLST) based on seven genes. The strains of these two species were clearly phylogenetic distinct, supporting the organisation of *Cr. sakazakii* and *Cr. malonaticus* in two distinct species (Baldwin *et al*., 2009).

Species description

The genus *Cronobacter* consists of five distinct species and *Cr. sakazakii* is the type species of the genus. *Cronobacter sakazakii* was named in honour of the renowned microbiologist Riichi Sakazaki and has been the dominant species in terms of isolation frequency. The strains of *Cr. sakazakii* are allocated in biogroups 1-4, 7, 8, 11 and 13 (Iversen *et al*., 2007a) and the type strain of this species (DSM 4485^T; ATCC 29544^T; NCTC 11467^T) was isolated from a child's throat (Farmer *et al*., 1980).

Table 1 Differentiation of *Cronobacter* strains based on 16S rRNA sequence analysis, f-AFLP and ribotyping

Isolate number	Strain	16S rRNA	f-AFLP	Ribotyping
756	*Cr. sakazakii*	Cluster 1	Group 1	Group 1
E775	*Cr. sakazakii*	Cluster 1	Group 1	Group 1
E768	*Cr. sakazakii*	Cluster 1	Group 1	Group 1
CDC 9369-75	*Cr. sakazakii*	Cluster 1	Group 1	Group 1
ATCC 29004	*Cr. sakazakii*	Cluster 1	Group 1	Group 1
NCTC 8155	*Cr. sakazakii*	Cluster 1	Group 1	Group 1
E807	*Cr. sakazakii*	Cluster 1	Group 1	ND
ATCC 29544	*Cr. sakazakii*	Cluster 1	Group 1	Group 1
ATCC 12868	*Cr. sakazakii*	Cluster 1	Group 1	Group 1
E607	*Cr. sakazakii*	Cluster 1	Group 1	Group 1
E811	*Cr. sakazakii*	Cluster 1	Group 1	ND
CDC 3128-77	*Cr. sakazakii*	Cluster 1	Group 1	Group 1
E327	*Cr. sakazakii*	Cluster 1	Group 1	ND
E266	*Cr. sakazakii*	Cluster 1	Group 1	Group 1
E274	*Cr. sakazakii*	Cluster 1	Group 1	Group 1
E765	*Cr. malonaticus*	Cluster 1	Group 1a	ND
LMG 23826	*Cr. malonaticus*	Cluster 1	Group 1a	Group 1
CDC 1716-77	*Cr. malonaticus*	Cluster 1	Group 1a	Group 1
LMG 23827	*Cr. turicensis*	Cluster 2	Group 2	Group 2
E694	*Cr. turicensis*	Cluster 2	Group 2	Group 2
E625	*Cr. turicensis*	Cluster 2	Group 2	Group 2
E609	*Cr. turicensis*	Cluster 2	Group 2	Group 2
E680	*Cr. genomospecies 1*	Cluster 2	Group 2a	Group 4
NCTC 9529	*Cr. genomospecies 2*	Cluster 2	Group 2a	Group 3
E769	*Cr. muytjensii*	Cluster 3	Group 3	Group 3
E616	*Cr. muytjensii*	Cluster 3	Group 3	Group 3
CDC 3523-75	*Cr. muytjensii*	Cluster 3	Group 3	Group 3
E488	*Cr. muytjensii*	Cluster 3	Group 3	Group 3
ATCC 51329	*Cr. muytjensii*	Cluster 3	Group 3	Group 3
LMG 23824	*Cr. dublinensis* subsp. *lausannensis*	Cluster 4	Group 4	Group 4
NCTC 9844	*Cr. dublinensis* subsp. *lausannensis*	Cluster 4	Group 4	Group 4
NCTC 9846	*Cr. dublinensis*	Cluster 4	Group 4	Group 4
LMG 23825	*Cr. dublinensis* subsp. *lactaridi*	Cluster 4	Group 4	Group 4
E465	*Cr. dublinensis*	Cluster 4	Group 4	Group 4
CDC 5960-70	*Cr. dublinensis* subsp. *lactaridi*	Cluster 4	Group 4	Group 4
LMG 23823	*Cr. dublinensis* subsp. *dublinensis*	Cluster 4	Group 4	Group 4

ND: not determined

(Adapted from Iversen *et al*. 2007a)

Cronobacter sakazakii is generally indole, dulcitol and malonate negative, but methyl-α-D-glucopyranoside positive (Iversen *et al*., 2007a). DNA-DNA hybridisation analysis of *Cr. sakazakii* with the other *Cronobacter* species resulted in a maximum similarity of 61 % with strains of *Cr. malonaticus* and a minimum value of 16 % with *Cr. dublinensis* (Iversen et al., 2008a).

Cronobacter malonaticus is the closest related to *Cr. sakazakii* of all the *Cronobacter* species (Kuhnert *et al*., 2009) and is characterised by the utilisation of malonate (Iversen *et al*., 2008a). This species is negative for indole and dulcitol utilisation and include biogroups 5, 9 and 14. The type strain (DSM 18702^T; CDC 1058-77^T; LMG 23826^T) was originally isolated from a breast abscess (Iversen *et al*., 2007a). Sequence analysis based on 16S rRNA showed no distinction between *Cr. sakazakii* and *Cr. malonaticus,* but DNA-DNA hybridisation resulted in DNA homology values between 54 and 60 %. DNA-homology between two *Cr. malonaticus* strains was found to be 95.6 %, confirming that the strains from *Cr. malonaticus* form a separate species (Iversen *et al*., 2008a).

Cronobacter turicensis is derived from biogroup 16 identified by Iversen *et al*. (2006). The type strain (DSM 18703^T; LMG 23827^T; z3032^T) was isolated from an individual suffering from neonatal meningitis in 2005 (Mange *et al*., 2006). The strains from this species are indole negative, but malonate and dulcitol positive. It is interesting to note that this species consists of all the strains in biogroup 16, except the strains NCTC 9529 and E680. DNA-DNA hybridisation of NCTC 9529 with the *Cr. turicensis* type strain resulted in DNA homology of 55 % and, therefore, these two strains (NCTC 9529 and E680) are currently designated as *Cronobacter* genomospecies 1 (Iversen *et al*., 2008a). However, no distinction can be made with phenotypic analysis and the two strains have a 16S rRNA similarity of 99.6 % with the *Cr. turicensis* strains. Analysis of these two strains with f-AFLP resulted in patterns that had less than 50 % similarity with the *Cr. turicensis* strains. Additionally, ribotyping did not group these two strains with the other *Cr. turicensis* strains (Table 1) (Iversen *et al*., 2007a).

Cronobacter muytjensii is the only *Cronobacter* species that is negative for the utilisation of 1-0-methyl-α-D-glucopyranoside. This species is also positive for indole, dulcitol and malonate utilisation. *Cronobacter muytjensii* consists of strains in biogroup 15 (Farmer *et al*., 1980) and is represented by the type strain ATCC 51329^T (CIP 103581^T) (Iversen *et al*., 2007a). Sequencing based on 16S rRNA resulted in similarity values above

97 % between *Cr. muytjensii* and the other *Cronobacter* species. Automated ribotyping and f-AFLP, however, showed these strains to be separate from the other *Cronobacter* species (Table 1). DNA-DNA hybridisation of *Cr. muytjensii* and *Cr. sakazakii* resulted in 31 to 53 % DNA homology values.

Cronobacter dublinensis consists of three subspecies, namely *Cr. dublinensis* subsp. *dublinensis*, *Cr. dublinensis* subsp. *lausannensis* and *Cr. dublinensis* subsp. *lactaridi.* *Cronobacter dublinensis* subsp. *dublinensis* (DSM 18707^T LMG 23825^T; E464^T) contains strains from biogroup 6 and the type strain was isolated from an environmental sample in a milk processing facility. Similarly the type strain of *Cr. dublinensis* subsp. *lactaridi* (DSM 18705^T; LMG 23823^T; DES 187^T) was isolated from a dried milk product manufacturing facility (biogroup 12). *Cronobacter dublinensis* subsp. *lausannensis* (DSM 18706^T; LMG 23824^T; E515^T) consists of strains in biogroup 10 and the type strain of this species was isolated from the basin of a water fountain (Iversen *et al*., 2008a). The strains from these three subspecies were grouped together with sequence analysis based on 16S rRNA, f-AFLP and ribotyping (Iversen *et al*., 2007a). The DNA-DNA relatedness between *Cr. sakazakii* ATCC 29544^T and strains from all of the *Cr. dublinensis* subspecies were less than 55 %. *Cronobacter dublinensis* subsp. *dublinensis* DSM 18705^T and *Cr. dublinensis* subsp. *lausannensis* NCTC 9844 had a DNA-DNA hybridisation value of 95.2 %, whereas *Cr. dublinensis* subsp. *lausannensis* NCTC 9844 had a value of 77.4 % when compared with *Cr. dublinensis* subsp. *lactaridi* CDC 5960-70. Based on these results the three biogroups were designated as three subspecies (Iversen *et al*., 2008a). These subspecies are generally dulcitol negative and indole production is variable (Iversen *et al*., 2007a).

Typing of *Cronobacter*

Recent studies on the identification of the five *Cronobacter* species is based on biochemical tests to differentiate between the strains, as well as identification based on a molecular approach (Healy *et al*., 2009; Terragno *et al*., 2009). Extensive phenotypic analysis was done on 23 isolates originating from three brands of IFM in Argentina. The biochemical tests included the production of acid from carbohydrates, gas production from glucose and the Voges-Proskauer test. The utilisation of malonate, as well as citrate as a sole carbon source was also evaluated. Based on the phenotypic analysis 22 isolates

were identified as *Cr. sakazakii* and one isolate as *Cr. malonaticus*. The strains were also subjected to pulsed field gel electrophoresis (PFGE) analysis using the restriction enzyme *Xba*I. The phenotypic analysis corresponded with the PFGE results as the single *Cr. malonaticus* strain had a distinct *Xba*I pattern and 7 different patterns could be distinguished for the *Cr. sakazakii* strains (Terragno *et al*., 2009).

Phenotypic analysis was used to classify a collection of 150 *Enterobacter sakazakii* strains. These strains originated from IFM production facilities and final products, as well as clinical samples. The phenotypic analysis included indole production, malonate utilisation and acid production from methyl-α-D-glucopyranoside and dulcitol. The majority of strains were identified as *Cr. sakazakii* (82.5 %), with 8 % of the strains designated as *Cr. malonaticus*, 5 % as *Cr. muytjensii*, 3 % as *Cr. dublinensis* and 1.5 % as *Cr. turicensis*. Genetic typing data for these strains based on PFGE, ribotyping and 16S rRNA sequencing indicated a high level of diversity between the 150 strains and compared well with the phenotypic results. However, discrepancies were found between the phenotypic groupings and the 16S rRNA data of *Cr. malonaticus* and *Cr. sakazakii* strains. This was expected as limited resolution of the 16S rRNA gene between these two species is not uncommon (Miled-Bennour *et al*., 2010).

Genetic characterisation of *Cronobacter*

Microarray-based comparative genomic indexing (CGI) was used to analyse 78 *Cronobacter* strains isolated from food, environmental and clinical samples. The array consisted of 276 open reading frames, targeting most of the functional gene categories in the genome of *Cr. sakazakii* strain BAA-894. In total, 200 of the 276 DNA coding sequences were present in all the *Cronobacter* strains. The gene categories with the most variable genes were the extracellular structures and the cell wall/membrane biogenesis. Species-specific genes which were present in all the *Cr. sakazakii* strains, but variable in the other species, were identified and can possibly serve as molecular markers for the identification of particular species in the genus *Cronobacter* (Healy *et al*., 2009).

The genome of *Cr. sakazakii* strain BAA-894 was sequenced and described as a 4.4 Mb chromosome with two plasmids of 31 kb and 131 kb. Comparative genomic hybridisation (CGH) was undertaken on representatives of the five *Cronobacter* species, excluding *Cronobacter* genomospecies 1. In total, 4 382 genes were examined of which

43 % were common to all the *Cronobacter* strains. Genes that are associated with virulence factors were of particular interest. All the species had the *ompA* gene which is associated with the invasive ability of the *E. coli* strains responsible for neonatal meningitis (Prasadarao *et al*., 1996). However, the genes encoding for a cation efflux system which allows bacteria to invade brain microvascular endothelial cells (Franke *et al*., 2003) were only present in *Cronobacter* species associated with neonatal infections. In addition, 15 gene clusters including putative prophages and prophage fragments were absent in more than half of the tested strains. Putative virulence genes were identified in most of these clusters. This indicates that the acquisition of genes via integration or phages, as well as specific gene loss played a major role in the evolution of *Cronobacter* and diversity among the species (Kucerova *et al*., 2010).

Multilocus sequence analysis (MLSA) was used to evaluate the similarity of the *recN*, *rpoA* and *thdF* genes of different Enterobacteriaceae focussing on the genus *Cronobacter*. Based on the *recN* gene, *Cr. malonaticus* and *Cr. sakazakii* showed the highest similarity and *Cr. dublinensis* and *Cr. muytjensii* was shown to be least similar with respect to the rest of the genus. The *rpoA* gene proved to be useful in the identification and differentiation of species in the Enterobacteriaceae family. The generation of phylogenetic data with these genes supported the reclassification of the *Cronobacter* spp. and provided data for phylogenetic and taxonomic analysis and identification (Kuhnert *et al*., 2009).

The use of genes other than the 16S rRNA is common in PCR assays for the typing of bacterial strains. The *rpo*B gene which encodes for the bacterial RNA polymerase β-subunit has been evaluated for its suitability to distinguish between species (Mollet *et al.,* 1997). The levels of divergence in Enterobacteriaceae strains between the sequences of the *rpo*B gene were significantly higher than the levels of divergence between the 16S rRNA sequences. A conventional PCR assay targeting the *rpo*B gene was the first species-specific PCR assay for the *Cronobacter* genus is (Stoop *et al.,* 2009). In this assay six primer pairs, namely Cturf/Cturr, Cdubf/Cdubr, Cmuyf/Cmuyr, Cgenomof/Cgenomor, Cmalf/Cmalr and Csakf/Csakr were used to differentiate between 57 strains. The first four primer pairs had a 100 % specificity, but the Cmalf/Cmalr and Csakf/Csakr primer pairs had to be used in a two-step procedure since the *rpo*B gene sequences of *Cr. malonaticus* and *Cr. sakazakii* are very closely related. The strains that tested positive with the Csakf/Csakr primers were tested in a follow-up PCR with the

Cmalf/Cmalr primer pair. *Cronobacter malonaticus* strains would test positive with the latter PCR (Stoop *et al*., 2009). The *rpo*B gene is, therefore, useful in the differentiation between *Cronobacter* species.

G. Conclusion

Differentiation between the various *Cronobacter* species has been the focus of research since these bacteria have been identified as opportunistic pathogens readily isolated from IFM. The inactivation, inhibition, thermal, osmotic and desiccation tolerance of these bacteria have been characterised to create risk management strategies for the production of hygienic products. This was aided by the improvements made in the isolation and detection of *Cronobacter* strains. Furthermore, the reclassification of this genus has played an important role in understanding the phylogeny of these pathogens.

However, there still remain many questions regarding differences in the characteristics of the five species. Although all five *Cronobacter* species are classified as pathogens it has yet to be shown that they are all virulent. The differentiation on a species level has become more important for this reason. The few typing methods available for the *Cronobacter* species are either time consuming or inaccurate and, therefore, other genetic typing methods should be evaluated for the accurate distinction between the species of *Cronobacter*.

References

Adamson, D. H., & Rogers, J. R. (1981). *Enterobacter sakazakii* meningitis with sepsis. *Clinical Microbiology Newsletter*, **3**, 19-20.

Al-Holy, M., Castro, L.F. & Al-Qadiri, H. (2009). Inactivation of *Cronobacter* spp. (*Enterobacter sakazakii*) in infant formula using lactic acid, copper sulfate and monolaurin. *Letters in Applied Microbiology,* **50** (3), 246-251.

Al-Nabulsi, A., Osaili, T.M., Al-Holy, M., Shaker, R.R., Ayyash, M.M., Olaimat, A.N. & Holley, R.A. (2009). Influence of desiccation on the sensitivity of *Cronobacter* spp. to lactoferrin or nisin in broth and powdered infant formula. *International Journal of Food Microbiology,* **136** (2), 221-226.

Arku, B., Mullane, N., Fox, E., Fanning, S. & Jordan, K. (2008). *Enterobacter sakazakii* survives spray drying. *International Journal of Dairy Technology,* **61** (1), 102-108.

Arroyo, C., Condon, S. & Pagan, R. (2009). Thermobacteriological characterisation of *Enterobacter sakazakii*. *International Journal of Food Microbiology,* **136** (1), 110-118.

Baldwin, A., Loughlin, M., Caubilla-Barron, J., Kucerova, E., Manning, G., Dowson, C. & Forsythe, S. (2009). Multilocus sequence typing of *Cronobacter sakazakii* and *Cronobacter malonaticus* reveals stable clonal structures with clinical significance which do not correlate with biotypes. *BMC Microbiology,* **9**, 223.

Bar-Oz, B., Preminger, A., Peleg, O., Block, C. & Arad, I. (2001). *Enterobacter sakazakii* infection in the newborn. *Acta Paediatrica*, **90**, 356-358.

Baumgartner, A., Grand, M., Liniger, M. & Iversen, C. (2009). Detection and frequency of *Cronobacter* spp. (*Enterobacter sakazakii*) in different categories of ready-to-eat foods other than infant formula. *International Journal of Food Microbiology,* **136** (2), 189-192.

Beuchat, L.R., Kim, H., Gurtler, J.B., Lin, L.C., Ryu, J.H. & Richards, G.M. (2009). *Cronobacter sakazakii* in foods and factors affecting its survival, growth, and inactivation. *International Journal of Food Microbiology,* **136** (2), 204-213.

Breeuwer, P., Lardeau, A., Peterz, M. & Joosten, H.M. (2003). Desiccation and heat tolerance of *Enterobacter sakazakii*. *Journal of Applied Microbiology,* **95** (5), 967-973.

Brenner, D.J. (1974). DNA reassociation for the clinical differentiation of enteric bacteria, *Public Health Lab.,* 118–130.

Cawthorn, D.M., Botha, S. & Witthuhn, R.C. (2008). Evaluation of different methods for the detection and identification of *Enterobacter sakazakii* isolated from South African infant formula milks and the processing environment. *International Journal of Food Microbiology,* **127** (1-2), 129-138.

Chenu, J.W. & Cox, J.M. (2009). *Cronobacter* ("*Enterobacter sakazakii*"): current status and future prospects. *Letters in Applied Microbiology,* **49** (2), 153-159.

Chang, C., Chiang, M., Chou, C. (2009). The effect of temperature and length of heat shock treatment on the thermal tolerance and cell leakage of *Cronobacter sakazakii* BCRC 13988. *International Journal of Food Microbiology,* **134** (3), 184-189.

CDC (Centers for Disease Control and Prevention). 2002. *Enterobacter sakazakii* infections associated with the use of powdered infant formula - Tennessee, 2001. *Morbidity and Mortality Weekly Report*, 51: 297-300.

Codex Alimentarius Commission (CAC) (1979). *Recommended international code of hygienic practice for foods for infants and children* (CAC/RCP 21-1979). [www document]. URL http://fao.org/codex/standard/en/CXP021e.pdf. 16 September 2006.

Codex Alimentarius Commission (CAC) (2008). Code of Hygienic Practice for Powdered Formulae for Infants and Young Children (Cac/Rcp 66-2008). [Www Document]. Url http://fao.org/codex/standard/en/cxp066e.pdf. 19 February 2009.

Dennison S.K. & Morris J. (2002). Multiresistant *Enterobacter sakazakii* wound infection in an adult, *Infect. Med.*. 533–535.

Drudy, D., O'Rourke, M., Murphy, M., Mullane, N.R., O'Mahony, R., Kelly, L., Fischer, M., Sanjaq, S., Shannon, P., Wall, P., O'Mahony, M., Whyte, P. & Fanning, S. (2006). Characterization of a collection of *Enterobacter sakazakii* isolates from environmental and food sources. *International Journal of Food Microbiology,* **110** (2), 127-134.

Druggan, P. & Iversen, C. (2009). Culture media for the isolation of *Cronobacter* spp. *International Journal of Food Microbiology,* **136** (2), 169-178.

Edelson-Mammel, S. & Buchanan, R.L. (2004). Thermal inactivation of *Enterobacter sakazakii* in rehydrated infant formula. *Journal of Food Protection,* **67** (1), 60-63.

Edelson-Mammel, S., Porteous, M.K. & Buchanan, R.L. (2006). Acid Resistance of Twelve Strains of *Enterobacter sakazakii*, and the Impact of Habituating the Cells to an Acidic Environment. *Journal of Food Science,* **71** (6), 201-207.

El-Sharoud, W., O'Brien, S., Negredo, C., Iversen, C., Fanning, S. & Healy, B. (2009). Characterization of *Cronobacter* recovered from dried milk and related products. *BMC Microbiology,* **9**, 9.

European Commission (EC) (2005). Commission regulation (EC) number 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. *Official Journal of the European Union*, **L338**, 1-26.

Fanjat, N., Leclercq, A., Joosten, H. & Robichon, D. (2007). Comparison of the phenotyping methods ID 32E and VITEK 2 compact GN with 16S rRNA gene sequencing for the identification of *Enterobacter sakazakii*. *Journal of Clinical Microbiology,* **45** (6), 2048-2050.

Farmer, J.J., Asbury, M.A., Hickman, F.W., Brenner, D.J. & the *Enterobacteriaceae* Study Group (1980). *Enterobacter sakazakii*, new species of *Enterobacteriaceae* isolated

from clinical specimens. *International Journal of Systematic Bacteriology*, **30**, 569-584.

Felske, A.,Franke, S., Grass, G., Rensing, C. & Nies, D.H. (2003). Molecular analysis of the copper-transporting efflux system CusCFBA of *Escherichia coli*. *Journal of Bacteriology* **185,** 3804–12.

Food and Agriculture Organization / World Health Organization (FAO/WHO) (2004). *Enterobacter sakazakii* and other microorganisms in powdered infant formula: Meeting Report, MRA series 6. Geneva, Switzerland. [www document]. URL http://www.who.int/foodsafety/micro/meetings/feb2004/en/. 6 March 2009.

Food and Agriculture Organization / World Health Organization (FAO/WHO) (2008). *Enterobacter sakazakii* (*Cronobacter* spp.) in powdered follow-up formulae: Meeting Report, MRA series 15. Rome. [www document]. URL http://www.who.int/foodsafety/micro/meetings/2008/en/. 11 May 2009.

Food and Drug Administration (FDA) (2002). Isolation and enumeration of *Enterobacter sakazakii* from dehydrated powdered infant formula. [www document]. URL http://www.cfsan.fda.gov/~comm/mmesakaz.html. 29 July 2006.

Franke, S., Grass, G., Rensing, C. & Nies, D. H. (2003). Molecular analysis of the copper-transporting efflux system CusCFBA of *Escherichia coli*. *Journal of Bacteriology*, **185**, 3804–12.

Gallagher, P.G. & Ball, W.S. (1991). Cerebral infarctions due to CNS infection with *Enterobacter sakazakii*. *Pediatric Radiology*, **21**, 135-136.

Gassem, M.A.A. (2002). A microbiological study of Sobia: a fermented beverage in the Western province of Saudi Arabia. *World Journal of Microbiology & Biotechnology* **18**: 173–177.

Gorden, J. & Small, P.L.C. (1993). Acid resistance in enteric bacteria. *Infection and Immunity*, **61**, 364-367.

Gurtler, J.B. & Beuchat, L.R. (2007). Survival of *Enterobacter sakazakii* in powdered infant formula as affected by composition, water activity, and temperature. *Journal of Food Protection,* **70** (7), 1579-1586.

Gurtler, J.B., Kornacki, J.L. & Beuchat, L.R. (2005). *Enterobacter sakazakii*: a coliform of increased concern to infant health. *International Journal of Food Microbiology*, **104**, 1-34.

Healy, B., Huynh, S., Mullane, N., O'Brien, S., Iversen, C., Lehner, A., Stephan, R., Parker, C.T. & Fanning, S. (2009). Microarray-based comparative genomic indexing of the *Cronobacter* genus (*Enterobacter sakazakii*). *International Journal of Food Microbiology,* **136** (2), 159-164.

Hoffmann, M., Keys, C.E., Song, K.Y., Brown, E.W., Fry, F.S. & Whittaker, P. (2008). Evaluation of multiple strains of *Enterobacter sakazakii* using fatty acid profiles. *Food Chemistry,* **107** (4), 1623-1628.

Hurrelle, E., Kucerova, E., Loughlin, M., Caubilla-Barron, S., Forsythe, S.J. (2009). Biofilm formation on enteral feeding tubes by *Cronobacter sakazakii*, *Salmonella* serovars and other Enterobacteriaceae. *International Journal of Food Microbiology,* **136** (2), 227-31.

Iversen, C. & Forsythe, S. (2003). Risk profile of *Enterobacter sakazakii*, an emergent pathogen associated with infant milk formula. *Trends in Food Science & Technology,* **14** (11), 443-454.

Iversen, C., Druggan, P. & Forsythe, S. (2004a). A selective differential medium for *Enterobacter sakazakii*, a preliminary study. *International Journal of Food Microbiology,* **96** (2), 133-139.

Iversen, C., Lane, M. & Forsythe, S. J. (2004b). The growth profile, thermotolerance and biofilm formation of *Enterobacter sakazakii* grown in infant formula milk. *Letter Applied Microbiology*, **38**, 378–382.

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

Iversen, C., Lehner, A., Mullane, N., Bidlas, E., Cleenwerck, I., Marugg, J., Fanning, S., Stephan, R. & Joosten, H. (2007a). The taxonomy of *Enterobacter sakazakii*: proposal of a new genus *Cronobacter* gen. nov. and descriptions of *Cronobacter sakazakii* comb. nov. *Cronobacter sakazakii* subsp. *sakazakii*, comb. nov., *Cronobacter sakazakii* subsp. *malonaticus* subsp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov., *Cronobacter* genomospecies 1. *BMC Evolutionary Biology,* **64** (7), 1471-2148.

Iversen, C., Lehner, A., Mullane, N., Marugg, J., Fanning, S., Stephan, R. & Joosten, H. (2007b). Identification of '*Cronobacter*' spp. [*Enterobacter sakazakii*]. *Journal of Clinical Microbiology,* **45** (11), 3814-3816.

Iversen, C., Mullane, N., McCardell, B., Tall, B.D., Lehner, A., Fanning, S., Stephan, R. & Joosten, H. (2008a). *Cronobacter* gen. nov., a new genus to accommodate the biogroups of *Enterobacter sakazakii*, and proposal of *Cronobacter sakazakii* gen. nov., comb. nov., *Cronobacter malonaticus* sp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov., *Cronobacter* genomospecies 1, and of three subspecies, *Cronobacter dublinensis* subsp. *dublinensis* subsp. nov., *Cronobacter dublinensis* subsp. *lausannensis* subsp. nov. and *Cronobacter dublinensis* subsp. *lactardi* subsp. nov.. *International Journal of Systematic and Evolutionary Microbiology,* **58** (6), 1442-1447.

Iversen, C., Druggan, P., Schumacher, S., Lehner, A., Feer, C., Gschwend, K., Joosten, H. & Stephan, R. (2008b). Development of a novel screening method for the isolation of '*Cronobacter*' spp. [*Enterobacter sakazakii*]. *Applied and Environmental Microbiology,* **74** (8), 2550-2553.

Jang, H.I. & Rhee, S.M. (2009). Inhibitory effect of caprylic acid and mild heat on *Cronobacter* spp. (*Enterobacter sakazakii*) in reconstituted infant formula and determination of injury by flow cytometry. *International Journal of Food Microbiology,* **133** (1), 113-120.

Joker, R. N., Norholm, T. & Siboni, K. E. (1965). A case of neonatal meningitis caused by a yellow *Enterobacter*. *Danish Medical Bulletin,* 128–130.

Joosten, H., Marugg, J., Stephan, R., Klijn, A., Jackson, T. & Iversen, C. (2008). A rapid and reliable alternative to ISO 21528-1:2004 for detection of Enterobacteriaceae. *International Journal of Food Microbiology,* **125** (3), 344-346.

Kandhai, C., Reij, M.W., Gorris, L.G.M., Guillaume-Gentil, O. & Schothorst, M. (2004). Occurrence of *Enterobacter sakazakii* in food production environments and households. *Lancet,* **363** (9402), 39-40.

Khan, A.A, Jones, R.A., Cerniglia, C.E. (1998). Rapid method for the detection of genetically engineered microorganisms by polymerase chain reaction from soil and sediments. *Journal of Industrial Microbiology & Biotechnology*, **20**, 90-94.

Kim, H. & Beuchat, L.R. (2005). Survival and growth of *Enterobacter sakazakii* on fresh-cut fruits and vegetables and in unpasteurized juices as affected by storage temperature. *Journal of Food Protection,* **68** (12), 2541-2552.

Kim, H., Ryu, J.-H., Beuchat, L.R., (2006). Attachment and biofilm formation by *Enterobacter sakazakii* on stainless steel and enteral feeding tubes. *Applied Environmental Microbiology,* **73**, 5846–5856.

Kim, H., Ryu, J.H. and Beuchat, L.R. (2007). Effectiveness of disinfectants in killing *Enterobacter sakazakii* in suspension, dried on the surface of stainless steel, and in a biofilm. *Applied Environmental Microbiology,* **73**, 1256–1265.

Kothary, M.H., McCardell, B.A., Frazar, C.D., Deer, D. & Tall, B.D. (2007). Characterization of the zinc-containing metalloprotease encoded by zpx and development of a species-specific detection method for *Enterobacter sakazakii*. *Applied and Environmental Microbiology,* **73** (13), 4142-4151.

Kucerova, E., Clifton, S.W., Xia, X., Long, F., Porwollik, S., Fulton, L., Fronick, C., Minx, P., Kyung, K.,Warren, W., Fulton, R., Feng, D., Wollam, A., Shah, N., Bhonagiri, V., Nash, W.E., Hallsworth-Pepin, K., Wilson, R.K., McClelland, M. & Forsythe, S.J. (2010). Genome sequence of *Cronobacter sakazakii* BAA-894 and comparative genomic hybridisation analysis with other *Cronobacter* species. *PLoS ONE*. **5** (3) e9556.

Kuhnert, P., Korczak, B.M., Stephan, R., Joosten, H. & Iversen, C. (2009). Phylogeny and prediction of genetic similarity of *Cronobacter* and related taxa by multilocus sequence analysis (MLSA). *International Journal of Food Microbiology,* **136** (2), 152-158.

Kuzina, L.V., Peloquin, J.J., Vacek, D.C. & Miller, T.A. (2001). Isolation and identification of bacteria associated with adult laboratory Mexican fruit flies,*Anastrepha ludens*. *Current Microbiology*, **42**, 290-294.

Lai, K.K. (2001). *Enterobacter sakazakii* infections among neonates, infants, children, and adults: case reports and a review of the literature. *Medicine Baltimore*, **80**, 113–122.

Lampel, K.A. & Chen, Y. (2009). Method for the isolation and detection of *Enterobacter sakazakii* (*Cronobacter*) from powdered infant formula. *International Journal of Food Microbiology,* **136** (2), 179-184.

Lehner, A., Riedel, K., Eberl, L., Breeuwer, P., Diep, B. & Stephan, R. (2005). Biofilm formation, extracellular polysaccharide production, and cell-to-cell signaling in various *Enterobacter sakazakii* strains: aspects promoting environmental persistence. *Journal of Food Protection,* **68** (11), 2287-2294.

Lehner, A., Riedel, K., Rattei, T., Ruepp, A., Frishman, D., Breeuwer, P., Diep, B., Eberl, L. & Stephan, R. (2006). Molecular characterization of the α-glucosidase activity in *Enterobacter sakazakii* reveals the presence of a putative gene cluster for palatinose metabolism. *Systematic and Applied Microbiology,* **29** (8), 609-625.

Lin, L.C. & Beuchat, L.R. (2007). Survival and growth of *Enterobacter sakazakii* in infant cereal as affected by composition, reconstitution liquid, and storage temperature. *Journal of Food Protection,* **70** (6), 1410-1422.

Lin, M., Al-Holy, M., Al-Qadiri, H., Kong, F., Rasco, B.A. & Setiady, D. (2009). Detection and discrimination of *Enterobacter sakazakii* (*Cronobacter* spp.) by mid-infrared spectroscopy and multivariate statistical analyses. *Journal of Food Safety,* **29** (4), 531-545.

Liu, Y., Cai, X., Zhang, X., Gao, Q., Yang, X., Zheng, Z., Luo, M. & Huang, X. (2006a). Real time PCR using TaqMan and SYBR Green for detection of *Enterobacter sakazakii* in infant formula. *Journal of Microbiological Methods,* **65** (1), 21-31.

Liu, Y., Gao, Q., Zhang, X., Hou, Y., Yang, J. & Huang, X. (2006b). PCR and oligonucleotide array for detection of *Enterobacter sakazakii* in infant formula. *Molecular and Cellular Probes,* **20** (1), 11-17.

Lockwood, D.E.; Kreger, A.S.; Richardson, S.H. Detection of toxins produced by *Vibrio fluvialis* (1982). *Infect. Immun.*, ***35***, 702-708.

MacLean, L.L., Pagotto, F., Farber, J.M. & Perry, M.B. (2009). The structure of the O-antigen in the endotoxin of the emerging food pathogen *Cronobacter* (*Enterobacter*) *muytjensii* strain 3270. *Carbohydrate Research,* **344** (5), 667-671.

Malorny, B. & Wagner, M. (2005). Detection of *Enterobacter sakazakii* strains by real-time PCR. *Journal of Food Protection,* **68** (8), 1623-1627.

Mange, J.P., Stephan, R., Borel, N., Wild, P., Kim, K.S., Pospischil, A. & Lehner, A. (2006). Adhesive properties of *Enterobacter sakazakii* to human epithelial and brain microvascular endothelial cells. *BMS Microbiology.* **6** (58).

Miled-Bennour, R., Ells, C., Pagotto, F.J., Farber, J.M., Kerouanton, A., Meheut, T., Colin, P., Joosten, H., Leclercq, A. & Besse, N.G. (2010). Genotypic and phenotypic characterisation of a collection of *Cronobacter* (*Enterobacter sakazakii*) isolates. *International Journal of Food Microbiol*ogy, **139,** 116–125.

Mohan Nair, M.K. & Venkitanarayanan, K.S. (2006). Cloning and sequencing of the *omp*A gene of *Enterobacter sakazakii* and development of an *omp*A-targeted PCR for rapid

detection of *Enterobacter sakazakii* in infant formula. *Applied and Environmental Microbiology,* **72** (4), 2539-2546.

Mollet, C., Drancourt, M. & Raoult, D. (1997). *rpoB* sequence analysis as a novel basis for bacterial identification. *Molecular Microbiology*. **25** (5), 1005-1011.

Molloy, C., Cagney, C., O'Brien, S., Iversen, C., Fanning, S. & Duffy, G. (2009). Surveillance and characterisation by Pulsed-Field Gel Electrophoresis of *Cronobacter* spp. in farming and domestic environments, food production animals and retail foods. *International Journal of Food Microbiology,* **136** (2), 198-203.

Mullane, N.R., Murray, J., Drudy, D., Prentice, N., Whyte, P., Wall, P.G., Parton, A. & Fanning, S. (2006). Detection of *Enterobacter sakazakii* in dried infant milk formula by cationic-magnetic-bead capture. *Applied and Environmental Microbiology,* **72** (9), 6325-6330.

Mullane, N.R., Whyte, P., Wall, P.G., Quinn, T. & Fanning, S. (2007). Application of pulsed-field gel electrophoresis to characterise and trace the prevalence of *Enterobacter sakazakii* in an infant formula processing facility. *International Journal of Food Microbiology,* **116** (1), 73-81.

Mullane, N., O'Gaora, P., Nally, J.E., Iversen, C., Whyte, P., Wall, P.G. & Fanning, S. (2008a). Molecular analysis of the *Enterobacter sakazakii* O-antigen gene locus. *Applied and Environmental Microbiology,* **74** (12), 3783-3794.

Mullane, N., Healy, B., Meade, J., Whyte, P., Wall, P.G. & Fanning, S. (2008b). Dissemination of *Cronobacter* spp. [*Enterobacter sakazakii*] in a powdered milk protein manufacturing facility. *Applied and Environmental Microbiology,* **74** (19), 5913-5917.

Muytjens, H.L. & van der Ros-van der Repe, J. (1982). Comparative studies of 13 β-Lactam Antibiotics. *Antimicrobial Agents and Chemotherapy,* **21** (6), 6925-934.

Muytjens, H.L., Zanen, C.H., Sonderkamp, J.H., Kollee, L.A., Wachsmuth, K.,Farmer, J.J. (1983). Analysis of Eight Cases of Neonatal Meningitis and Sepsis. *Journal of Clinical Microbiology,* **18** (1), 115-120.

Muytjens, H.L., van der Ros-van de Repe, J., van Druten, H. (1984). Enzymatic Profiles of *Enterobacter sakazakii* and related species with Special Reference to the ox-Glucosidase Reaction and Reproducibility of the Test System. *Journal of Clinical Microbiology,* **20** (4), 684-686.

Naqvi, S.H., Maxwell, M.A. & Schwalbe, R.S. (1990). Nosocomial bacteremia caused by *Enterobacter sakazakii* and *Leuconostoc mesenteroides* resulting from extrinsic contamination of infant formula. Journal of *Pediatric Infectious Diseases,* 9, 447–449.

Nazarowec-White, M. & Farber, J.M. 1995, *Biological characterization of Enterobacter sakazakii*, Microbiol. Res. Div., Banting Building, Health Canada, Ottawa, Ont. K1A 0L2, Canada.

Nazarowec-White, M. & Farber, J.M. (1997a). Thermal resistance of *Enterobacter sakazakii* in reconstituted dried-infant formula. *Letters in Applied Microbiology,* **24** (1), 9-13.

Nazarowec-White, M. & Farber, J.M. (1997b). Incidence, survival, and growth of *Enterobacter sakazakii* in infant formula. *Journal of Food Protection,* **60** (3), 226-230.

Nazarowec-White, M. & Farber, J.M. (1997c). *Enterobacter sakazakii*: a review. *International Journal of Food Microbiology,* **34** (2), 103-113.

Nazarowec-White, M., and J. M. Farber. (1999). Phenotypic and genotypic typing of food and clinical isolates of *Enterobacter sakazakii*. *Journal of Medical Microbiol*ogy, **48**, 559–567.

Oh, S.W., Chen, P.C., & Kang, D.H. (2007). Biofilm formation by *Enterobacter sakazakii* grown in artificial broth and infant milk formula on plastic surface. *Journal of Rapid Methods and Automation in Microbiology,* **15** (4), 311-319.

Osaili, T. & Forsythe, S. (2009). Desiccation resistance and persistence of *Cronobacter* species in infant formula. *International Journal of Food Microbiology,* **136** (2), 214-220.

Pagotto, F.J. & Farber, J.M. (2009). *Cronobacter* spp. (*Enterobacter sakazakii*): Advice, policy and research in Canada. *International Journal of Food Microbiology,* **136** (2), 238-245.

Pagotto, F.J., Nazarowec-White, M., Bidawid, S. & Farber, J.M. (2003). *Enterobacter sakazakii*: infectivity and enterotoxin production in vitro and in vivo. *Journal of Food Protection,* **66** (3), 370-375.

Prasadarao, N.V., Wass, C.A., Weiser, J.N., Stins, M.F., & Huang. S.H.. (1996). Outer membrane protein A of Escherichia coli contributes to invasion of brain microvascular endothelial cells. *Infection and Immunity,* **64**, 146–53.

Pribyl, C., Salzer, R., Beskin, J., Haddad, R.J., Pollock, B., Beville, R., Holmes. B., Mogabgab, W.J. (1985). Aztreozam in the Treatment of Serious Orthopedic Infections. *The American Journal of Medicine,* **78**, 51-56.

Proudy, I., Bougle, D., Leclercq, R. & Vergnaud, M. (2008). Tracing of *Enterobacter sakazakii* isolates in infant milk formula processing by BOX-PCR genotyping. *Journal of Applied Microbiology,* **105** (2), 550-558.

Raghav, M. & Aggarwal, P.K. (2007). Purification and characterisation of *Enterobacter sakazakii* enterotoxin. *Canadian Journal of Microbiology,* 53, 750-755.

Reij, M.W., Jongenburger, I., Gkogka, E., Gorris, L.G.M. & Zwietering, M.H. (2009). Perspective on the risk to infants in the Netherlands associated with *Cronobacter* spp. occurring in powdered infant formula. *International Journal of Food Microbiology,* **136** (2), 232-237.

Riedel, K. & Lehner, A. (2007). Identification of proteins involved in osmotic stress response of *Enterobacter sakazakii* by proteonomics. *Proteonomics,* **7**, 1217-1231.

Schmid, M., Iversen, C., Gontia, I., Stephan, R., Hofmann, A., Hartmann, A., Jha, B., Eberl, L., Riedel, K. & Lehner, A. (2009). Evidence for a plant-associated natural habitat for *Cronobacter* spp. *Research in Microbiology,* **160** (8), 608-614.

Seo, K.H. & Brackett, R.E. (2005). Rapid, specific detection of *Enterobacter sakazakii* in infant formula using a real-time PCR assay. *Journal of Food Protection,* **68** (1), 59-63.

Simmons, B.P., Gelfand, M.S., Haas, M., Metts, L. & Ferguson, J. (1989). *Enterobacter sakazakii* infections in neonates associated with intrinsic contamination of a powdered infant formula. *Infection Control and Hospital Epidemiology*, **10**, 398-401.

Smeets, L. C., A. Voss, H. L. Muytjens, J. F. G. M. Meis, and W. J. G. Melchers. 1998. Genetische karakterisatie van *Enterobacter sakazakii*-isolaten van Nederlandse patienten met neonatale meningitis. *Ned. Tijdschr. Med. Microbiol*. **6**, 113–115.

Stackebrandt, E., Frederiksen, W., Garrity, G. M. & 10 other authors (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. International Journal of Systematic Evolutionary Microbiology **52**, 1043–1047.

Stoop, B., Lehner, A., Iversen, C., Fanning, S. & Stephan, R. (2009). Development and evaluation of rpoB based PCR systems to differentiate the six proposed species within the genus *Cronobacter*. *International Journal of Food Microbiology,* **136** (2), 165-168.

Terragno, R., Salve, A., Pichel, M., Epszteyn, S., Brengi, S. & Binsztein, N. (2009). Characterization and subtyping of *Cronobacter* spp. from imported powdered infant formulae in Argentina. *International Journal of Food Microbiology,* **136** (2), 193-197.

Urmenyi, A.M.C. & Franklin, A.W. (1961). Neonatal death from pigmented coliform infection. *The Lancet*, **1**, 313-315.

Wakabayashi, H., Yamauchi, K. & Takase, M. (2008). Inhibitory effects of bovine lactoferrin and lactoferricin B on *Enterobacter sakazakii*. *Biocontrol Science,* **13** (1), 29-32.

Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. International Journal of Systematic Bacteriology **37**, 463–464.

Witthuhn, R.C., Kemp, F. & Britz, T.J. (2007). Isolation and PCR detection of *Enterobacter sakazakii* in South African food products, specifically infant formula milks. *World Journal of Microbiology & Biotechnology,* **23** (2), 151-157.

CHAPTER 3

PHYLOGENETIC ANALYSIS OF *CRONOBACTER* ISOLATES BASED ON THE *rpo*A AND 16S rRNA GENES

Abstract

The reclassification of the genus *Cronobacter* (previously known as *Enterobacter sakazakii*) was based on a polyphasic analysis that led to the description of five species. These bacteria are opportunistic pathogens that can cause neonatal meningitis and other infections in immuno-compromised individuals. *Cronobacter* strains have been reported to show differences in sensitivity to antibiotics, heat and chemicals, as well as differences in virulence. The aim of this study was to classify *Cronobacter* strains isolated from infant formula milk, the food processing environment and fresh produce in South Africa and to evaluate the phylogenetic placement of these isolates based on the *rpo*A and 16S ribosomal RNA (rRNA) gene sequences. All the South African strains were identified as *Cr. sakazakii* despite the wide variety of isolation sources. No relation between the phylogenetic placement and strain origin could be determined. Strains from *Cr. sakazakii*, *Cr. dublinensis*, *Cr. turicensis* and *Cr. muytjensii* could be differentiated from each other, but it was not possible to differentiate between *Cr. sakazakii* and *Cr. malonaticus* based on the *rpo*A and 16S rRNA gene sequences. However, sequence data of these two genes can be used to differentiate between the members of the genus *Cronobacter* when used in combination with biochemical analysis based on the utilisation of malonate.

Introduction

The genus *Cronobacter* (previously known as *Enterobacter sakazakii*) consists of five species and three subspecies, namely *Cronobacter sakazakii, Cronobacter malonaticus, Cronobacter muytjensii, Cronobacter dublinensis* subsp. *dublinensis, Cr. dublinensis* subsp. *lausannensis, Cr. dublinensis* subsp. *lactaridi* and *Cronobacter turicensis*. Additionally, three *Cronobacter* strains were grouped together as *Cronobacter* genomospecies 1 (Iversen *et al*., 2008). The existence of multiple species in the *E. sakazakii* group had already been suggested in 1980, as 15 different biogroups were identified among these strains (Farmer *et al*., 1980). The reclassification of *Cronobacter* was based on a polyphasic approach which included DNA-DNA hybridisation, amplified

fragment length polymorphisms (AFLP), automated ribotyping, full length 16S ribosomal RNA (rRNA) gene sequencing and phenotypic analysis (Iversen *et al*., 2007; 2008). The description of the five new *Cronobacter* species was supported by two independent multi-locus sequencing data sets based on seven and three genes, respectively (Baldwin *et al*., 2009; Kuhnert *et al*., 2009). However, some discrepancies have been reported concerning the identification of some *Cronobacter* strains at the species level, where the results of phenotypic analysis differed from that obtained with sequencing based on the 16S rRNA gene (Miled-Bennour *et al*., 2010).

Cronobacter strains have been reported to show differences in sensitivity to chemical reagents, heat, antibiotics (Kuzina *et al*., 2001; Lai, 2001; Arroyo *et al*., 2009), as well as differences in virulence factors (Pagotto *et al.,* 2003; Healy *et al*., 2009; MacLean *et al*., 2009). Recently, Kucerova *et al*. (2010) reported that only strains from *Cr. sakazakii*, *Cr. malonaticus* and *Cr. turicensis* have been associated with neonatal infections. Additionally, only these three species possess a gene cluster encoding for a cation efflux system which allows bacteria to invade brain microvascular endothelial cells (Franke *et al*., 2003). *Cronobacter sakazakii* strains isolated from a hospital in France showed that all the strains associated with fatal *Cronobacter* infections were grouped together in one pulsetype (Caubilla-Barron *et al*., 2007). This indicates that there may be variation between the *Cr. sakazakii* strains regarding virulence potential.

Members of the genus *Cronobacter* can cause a severe form of neonatal meningitis, necrotising enterocolitis and septicaemia (Nazorowec-White & Farber, 1997; Van Acker *et al*., 2001). These opportunistic pathogens have been associated with sporadic infections and disease outbreaks (FAO/WHO, 2004; 2008). The number of *Cronobacter* related infections in South Africa is unknown, but these bacteria may pose a significant risk to the high number of HIV-positive individuals. Between 2005 and 2008 *Cronobacter* strains (*E. sakazakii*) have been isolated from an infant formula milk (IFM) processing facility and final products, as well as fresh produce in South Africa (Cawthorn *et al*., 2008; Mofokeng *et al*., 2010). These results and the recent reclassification of the genus have led to the present study in which the aim is to identify the different *Cronobacter* spp. and to determine the phylogenetic relationship between the recently isolated strains.

Materials and methods

Bacterial isolates

Bacterial isolates previously identified as *E. sakazakii* (*Cronobacter*) were evaluated in this study. The 24 isolates were recently isolated from various food and environmental sources in South Africa, including IFM products, the environment of an IFM production facility and fresh produce. The fresh produce included raw milk, potatoes, beetroot, carrots and mincemeat. The type strains *Cr. malonaticus* DSM 18702^T, *Cr. sakazakii* DSM 4485^T, *Cr. turicensis* DSM 18703^T, *Cr. dublinensis* DSM 18705^T (obtained from the Deutche Sammeling von Microorganismen und Zelkulturen) and *Cr. muytjensii* ATCC 51329^T (obtained from the American Type Culture Collection) were also included in this study. Bacteria were inoculated in tryptone soy broth (TSB) (Merck, Cape Town South Africa) and incubated at 37 °C for 24 h. Confirmation of *Cronobacter* spp. was done by streaking the incubated broth on Chromocult® agar, followed by incubation at 42 °C for 24 h. Blue-green colonies were regarded as *Cronobacter* positive.

DNA isolations

DNA was isolated using either the TZ-method described by Wang & Levin (2006) or a modified phenol-chloroform method described by van Elsas *et al*. (1997). For the TZ-extraction method cells were harvested by centrifuging 250 µL of the 24 h TSB cultures at 8 000 *g* for 5 min. The pellets were suspended in 250 µL double strength TZ (2 x TZ) (Wang & Levin, 2006), which consisted of 5 mg.mL^{-1} sodium azide (Merck) and 4 % (v/v) Triton X-100 (Merck) in 0.1 M Tris-HCl (Fluka) at pH 8. Cells were lysed by placing samples in a water bath at 100 °C for 10 min. The debris was pelleted by centrifugation at 10 000 *g* for 5 min and the supernatant was used as DNA template.

For the phenol-chloroform method bacterial samples were prepared by centrifuging (Eppendorf Centrifuge 5415D, Germany) 2 ml of the 24 h TSB cultures at 8 000 *g* for 10 min. Glass beads (0.6 g) were added to the cell pellets and 800 µL phosphate buffer (1 part 120 mM NaH_2PO_4 (Merck) to 9 parts 120 mM Na_2HPO_4 (Merck); pH 8), 700 µL phenol (Fluka, supplied by Sigma-Aldrich) and 100 µL of 20 % (m/v) sodium dodecyl sulphate (SDS) (Merck) were added to the samples. The samples were then vortexed for 1 to 2 min followed by incubation for 20 min at 60 °C. The vortexing and incubation step was repeated three times, after which the samples were centrifuged for 5 min at 4000 *g*. Phenol (600 µL) was added to each supernatant, the samples were vortexed and centrifuged for 5 min at 8 000 *g*. The water phase was removed and 600 µL

phenol:chloroform:isoamylalcohol (25:24:1) was added and the samples were centrifuged (5 min at 8 000 *g*) until the interphase was clean. The waterphase was removed, the volume of the supernatant noted and DNA was precipitated by adding 0.1 volume 3 M sodium acetate (NaOAc) (pH 5.5) (Saarchem, supplied by Merck) and 0.6 volume isopropanol (Saarchem) to the samples. The samples were stored overnight at -20 °C followed by centrifugation for 10 min at 12 000 *g*. The resulting pellet was washed with 70 % (v/v) ethanol after which the samples were centrifuged for 5 min at 12 000 *g*. The pellet was air-dried and 100 µL of 1 x TE (10 mM Tris (Fluka), 1 mM EDTA (Merck); pH 8) buffer was added. Samples were treated with 1 µL RNase for 1 h at 37 °C and stored at -20 °C.

Phylogenetic analysis

Phylogenetic evaluation of *Cronobacter* strains was based on two genes, 16S rRNA and *rpo*A. In order to compare the South African strains to strains from various sources and origin, 15 *Cronobacter* strains representing *Cr*. *sakazakii*, *Cr*. *muytjensii*, *Cr*. *dublinensis* and *Cr*. *turicensis* were obtained from GenBank (Table 1). Apart from the *Cr. malonaticus* type strain DSM 18702^T, no other strain from this species had both the *rpo*A and 16S rRNA gene sequences available and no additional *Cr. malonaticus* strains could be included in this study.

16S rRNA gene sequencing

The 16S rRNA gene was amplified with the primers F8 (5'- CAC GGA TCC AGA CTT TGA TYM TGG CTC AG -3') and R1512 (5'- GTG AAG CTT ACG GYT AGC TTG TTA CGA CTT -3') (Felske *et al*., 1997), resulting in a 1.5 kilobase (kb) fragment. The PCR reaction mixture (50 µL total volume) contained 1 µL (5 U) *Taq* DNA polymerase (Super-Therm, supplied by Southern Cross Biotechnologies, Cape Town, South Africa), 5 µL 10 X buffer (Super-Therm), 3 µL (1.5 mM) $MgCl_2$ (Super-Therm), 2 µL (400 nM) of each primer, 2 µL (400 nM) dNTPs (AB gene, supplied by Southern Cross Biotechnologies), 2 µL 99% (v/v) DMSO (Merck) and 2 µL DNA template. Thermal cycling parameters (Eppendorf mastercycler personal, Eppendorf, Germany) were as follows: initial denaturation at 92 °C for 3 min; 35 cycles of denaturation at 92 °C for 30 s, annealing at 54 °C for 30 s, and elongation at 68 °C for 60 s; and final elongation at 72 ºC for 7 min (Felske *et al*., 1997).

Table 1 16S rRNA gene and *rpo*A gene sequences of *Cronobacter* strains obtained from GenBank

Strain	Species designation	Source	Accession 16S rRNA gene*	Accession *rpoA*[#]
E266	*Cr. sakazakii*	NI	EU569598	AY03190
E274	*Cr. sakazakii*	Environment Malaysia	EU569599	EF059822
E456	*Cr. muytjensii*	NI	EU569593	EF059837
E464	*Cr. dublinensis* subsp. *lactaridi*	Environment Zimbabwe	EU569579	EF059838
E604	*Cr. sakazakii*	Clinical Canada	EU569608	EF059846
E616	*Cr. muytjensii*	NI	EU569592	EF059849
E627	*Cr. sakazakii*	NI	EU569609	EF059856
E681	*Cr. turicensis*	NI	EU569622	EF059862
E694	*Cr. turicensis*	NI	EU569620	EF059864
E769	*Cr muytjensii*	NI	EU569590	EF059872
E775	*Cr. sakazakii*	Food Russia	EU569601	EF059873
E791	*Cr. dublinensis*	Clinical USA	EU569576	EF059874
E793	*Cr. muytjensii*	Clinical USA	EU569591	EF059875
E798	*Cr. dublinensis*	Unknown UK	EU569578	EF059878
E828	*Cr. sakazakii*	Clinical USA	EU569611	EF059882

* Iversen *et al*. (2007); [#] Kuhnert *et al*. (2009); NI = not indicated

*rpo*A *gene sequencing*

The *rpo*A gene was amplified using the primers rpoA_entero-L (ATG CAG GGT TCT GTG ACA GAG) and rpoA_entero-R (GGT GGC CAR TTT TCY AGG CGC) (Kuhnert *et al*., 2010), resulting in a 960 basepair (bp) fragment. The PCR reaction mixture (30 µL total volume) contained 0.5 µL (2.5 U) *Taq* DNA polymerase (Super-Therm), 3 µL 10 X buffer (Super-Therm), 1.5 µL (1.25 mM) $MgCl_2$ (Super-Therm), 0.5 µL (167 nM) of each primer, 2 µL (667 mM) dNTPs (AB gene) and 1 µL DNA template. Thermal cycling parameters (Eppendorf mastercycler) were as follows: initial denaturation at 92 ℃ for 3 min; 35 cycles of denaturation at 92 ℃ for 30 s, annealing at 54 ℃ for 30 s, and elongation at 68 ℃ for 60 s; and final elongation at 72 ºC for 7 min.

Sequence analysis

Sequencing of the PCR products was performed using the ABI 3100 Genetic Analyser (Applied Biosystems, Foster City, USA). The closest recognised relatives of the isolates were determined by comparing the generated sequences to sequences available in GenBank using the BLASTn search option (Altschul *et al*., 1997).

The *rpo*A and 16S rRNA gene sequences of *Cronobacter* strains obtained from GenBank were added to the sequences generated for the South African strains and aligned with MAFFT (http://mafft.cbrc.jp/alignment/software/). Phylogenetic analysis was done on each set of sequences using the phylogenetic analysis using parsimony (PAUP) software with 1000 bootstraps and the neighbour-joining algorithm. Phylograms were constructed from the sequences of each gene, as well as from a combination of the two genes. A Templeton Nonparametric Wilcoxon Signed Ranked Test (Kellogg *et al*., 1996; Swofford, 2001) was performed to determine whether the two data sets could be combined. The type strain of *Enterobacter aerogenes*, DSM 30053^T, was used as an outgroup in the analysis since the *rpo*A and 16S rRNA gene sequences of this strain were related, but genetically distinct from the *Cronobacter* species.

Results and discussion

Reclassification of the South African strains

All the South African *Cronobacter* isolates showed blue-green colonies on the Chromocult® agar, testing positive for *Cronobacter*. The *rpo*A and 16S rRNA genes of the 24 South African strains were successfully amplified resulting in fragments of 960 bp and 1.5 kb,

respectively. Furthermore, the South African strains were all identified as *Cr. sakazakii* based on both the 16S rRNA gene and *rpo*A gene sequences with the BLASTn search function in Genbank (Table 2). This study supports the finding that *Cr. sakazakii* is the dominant species in terms of isolation frequency (Baldwin *et al*., 2009; Kuhnert *et al*., 2009). Iversen *et al*. (2007) also reported on the dominance of this species as *Cr sakazakii* consists of 12 of the 16 biogroups identified among the *Cronobacter* strains. Furthermore, in a separate study 82 % of 150 isolates were identified as *Cr. sakazakii* (Miled-Benour *et al*., 2010). The absence of the four other *Cronobacter* species amongst the South African isolates was not expected as the strains were isolated from a wide variety of sources. Since only 24 isolates were used in this study, it is possible that strains from the other *Cronobacter* species will be identified when more isolates of these bacteria are obtained in South Africa.

Phylogeny and genetic relatedness

The phylogeny of the genus *Cronobacter* was evaluated based on the two genes, 16S rRNA and *rpo*A. The 16S rRNA gene is most frequently used in phylogenetic studies but has been found to be too conserved to differentiate between some strains in the Enterobacteriaceae family (Mollet *et al*., 1997). The 16S rRNA gene sequences of many *Cronobacter* strains have been deposited in GenBank and were, therefore, available for comparisons in this study. The *rpo*A gene encodes for the α-subunit of the RNA polymerase and has proved to be useful in the identification and differentiation of species in the Enterobacteriaceae family (Kuhnert *et al*., 2009). This gene has also been sequenced for numerous *Cronobacter* strains and these sequences have also been made available on GenBank.

Phylogeny based on the 16S rRNA gene

The phylogram based on the 16S rRNA gene sequences showed clear separation between the *Cronobacter* isolates and the outgroup strain, *E. aerogenes* (Fig. 1). All the South African strains were grouped amongst the *Cr. sakazakii* strains from GenBank in two clusters (clusters 1 and 2). A total of 10 strains isolated from South Africa were grouped with the *Cr. sakazakii* type strain DSM 4485^T in cluster 1. All the strains that were isolated from fresh produce were grouped in this cluster, as well as four strains that were isolated from dried milk related sources (IFM and powdered milk). Cluster 2 contained all the

Table 2 Sources of *Cronobacter* strains isolated from South Africa and their closest relatives in GenBank

Strain	Source	16S rRNA[a] gene (% sequence similarity)[c]	*rpoA*[b] (% sequence similarity)[c]
DSM 18705[T]	Milk processing facility	*Cr. dublinensis* (97)	*Cr. dublinensis* (100)
DSM 18703[T]	Neonatal meningitis	*Cr. turicensis* (97)	*Cr. turicensis* (99)
DSM 18702[T]	Breast abscess	*Cr. malonaticus* (97)	*Cr. malonaticus* (99)
ATCC 51329[T]	NI	*Cr. muytjensii* (97)	*Cr. muytjensii* (98)
DSM 4485[T]	Child's throat	*Cr. sakazakii* (97)	*Cr. sakazakii* (99)
E14	IFM environment	*Cr. sakazakii* (98)	*Cr. sakazakii* (99)
E36	IFM environment	*Cr. sakazakii* (97)	*Cr. sakazakii* (99)
E48	IFM environment	*Cr. sakazakii* (99)	*Cr. sakazakii* (99)
E68	IFM environment	*Cr. sakazakii* (99)	*Cr. sakazakii* (99)
Scoop	IFM environment	*Cr. sakazakii* (96)	*Cr. sakazakii* (99)
23	IFM	*Cr. sakazakii* (99)	*Cr. sakazakii* (99)
E71	IFM environment	*Cr. sakazakii* (96)	*Cr. sakazakii* (99)
Esak 1039	Wine effluent	*Cr. sakazakii* (98)	*Cr. sakazakii* (99)
B3	Beetroot	*Cr. sakazakii* (99)	*Cr. sakazakii* (99)
C2	Carrot	*Cr. sakazakii* (99)	*Cr. sakazakii* (99)
C3	Carrot	*Cr. sakazakii* (99)	*Cr. sakazakii* (99)
C5	Carrot	*Cr. sakazakii* (99)	*Cr. sakazakii* (99)
CI	Industrial isolate, Canada	*Cr. sakazakii* (99)	*Cr. sakazakii* (99)
M3	Mince meat	*Cr. sakazakii* (99)	*Cr. sakazakii* (98)
Mon	Montreal hospital, Canada	*Cr. sakazakii* (99)	*Cr. sakazakii* (99)
P04	Potato	*Cr. sakazakii* (99)	*Cr. sakazakii* (99)
P1	IFM	*Cr. sakazakii* (99)	*Cr. sakazakii* (99)
PM1	Powdered milk	*Cr. sakazakii* (99)	*Cr. sakazakii* (99)
PM2	Powdered milk	*Cr. sakazakii* (99)	*Cr. sakazakii* (99)

DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen; ATCC, American Type Culture Collection; IFM, Infant formula milk; NI, not indicated.

[a] Based on DNA sequencing of a 1.5 kb fragment of the 16S rRNA gene.

[b] Based on DNA sequencing of a 960 bp fragment of the *rpo*A gene.

[c] Percentage similarity of the isolate to the closest relative in the NCBI database.

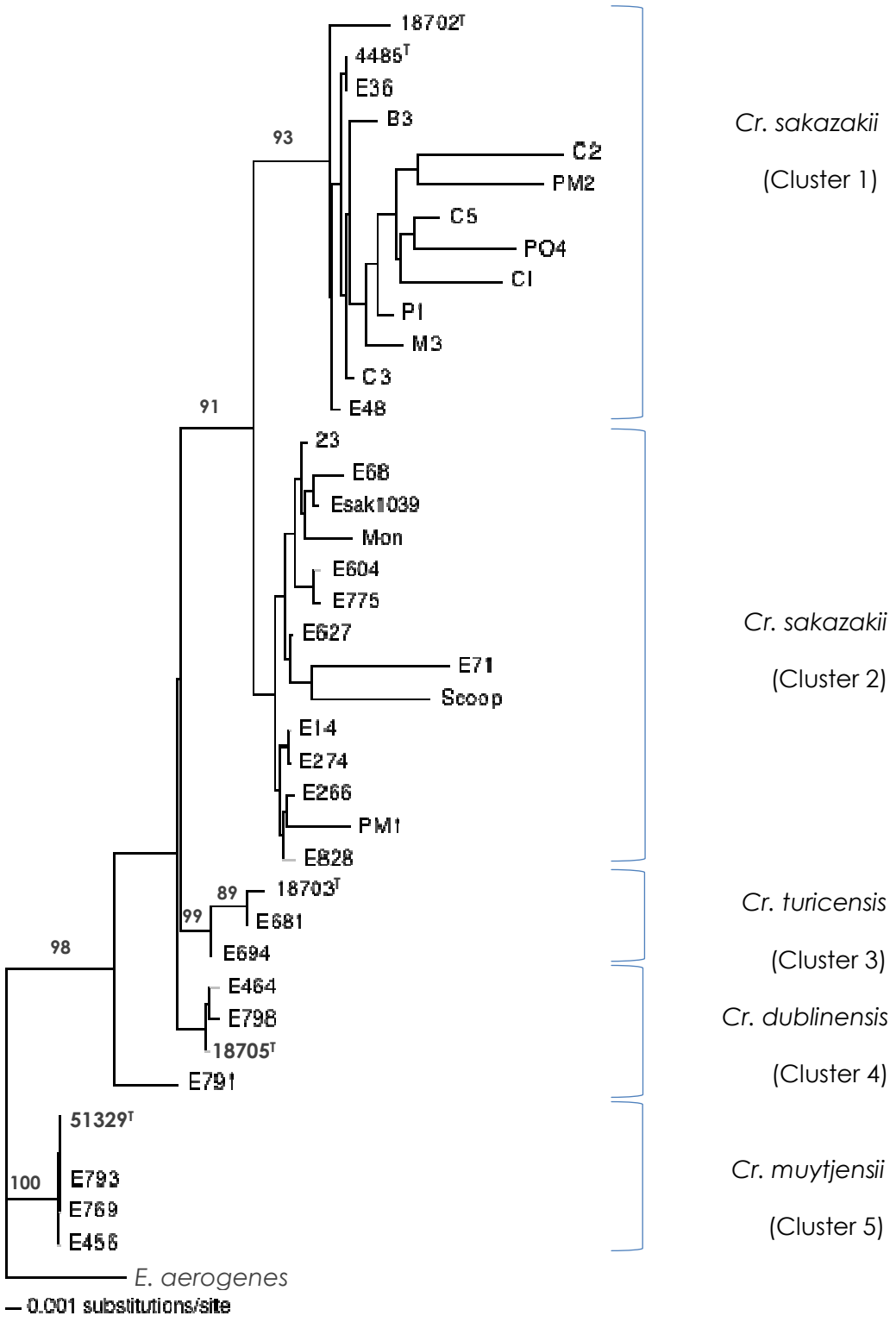


Figure 1 Phylogram based on 16S rRNA gene sequences using neighbour-joining analysis and 1000 bootstraps.

strains from GenBank and the remaining eight South African strains. The strains in cluster 2 were isolated from clinical, environmental and food sources, including wine effluent and IFM. It is interesting to note that all the fresh produce strains were grouped with the *Cr. sakazakii* type strain, which is a clinical strain. This supports the idea that fresh produce can be a source of infection for immuno-compromised adults, especially since *Cronobacter* has been shown to survive refrigeration temperatures (Nazarowec-White & Farber, 1997). No correlation could be made between strain grouping and origin as the strains from different sources were distributed throughout the two clusters.

These results confirm findings from other studies that evaluated the phylogenetic relationship of *Cronobacter* isolates in showing that there are differences between the *Cr. sakazakii* strains as confirmed by the formation of clusters 1 and 2 (Fig. 1) and that these strains do not form clear groups based on isolation source. Miled-Bennour *et al*. (2010) reported similar results after characterising of 64 *Cronobacter* isolates, using 16S rRNA gene sequencing, ribotyping and pulsed field gel electrophoresis. Three subgroups showing diversity in the genus were identified for the *Cr. sakazakii* strains in this study. Although these 64 *Cronobacter* isolates had a high genetic diversity it was not possible to trace a route of contamination. This indicates how difficult it can be to develop and maintain surveillance systems for these pathogens (Miled-Bennour *et al*., 2010). A phylogram from a study by Kuhnert *et al*. (2009) based on the *rec*N gene sequences of *Cronobacter* isolates indicated significant differences between *Cr. sakazakii* strains. Three *Cr. sakazakii* strains only had a 0.88 % similarity with the rest of this species compared to the more than 0.9 % similarity between strains from the rest of the *Cronobacter* genus. Based on multi-locus sequence analysis of seven loci, 9 sequence types were assigned to 60 *Cr. sakazakii* strains (Baldwin *et al*., 2009). The sequence analysis showed that *Cr. sakazakii* strains isolated from different sources and countries did grouped together. One sequence type was dominated by clinical strains (7 out of 8 strains) that were isolated from four different countries. This merits further investigation as there was strong evidence that this group may represent a specific virulence type of *Cr. sakazakii* (Baldwin *et al*., 2009).

No differentiation was observed in cluster 1 between the *Cr. malonaticus* type strain and the *Cr. sakazakii* strains. The high level of similarity between *Cr. sakazakii* and *Cr. malonaticus* have been noted on numerous occasions and sequence analysis based on the 16S rRNA gene have been found unsuitable for differentiation between these two species (Iversen *et al*., 2007; 2008; Kuhnert *et al*., 2009). Malonate utilisation can be used

for biochemical differentiation between the two species, although controversial results have been reported when biochemical analyses of 150 isolates were compared to ribotyping (Miled-Bennour *et al*., 2010). Since no differentiation between these two species was accomplished in this study there may be isolates that have been incorrectly identified as *Cr. sakazakii* and not *Cr. malonaticus*.

All the isolates of *Cr*. *muytjensii* formed a distinct cluster (cluster 5) from all the other *Cronobacter* species with a bootstrap value of 100 %. The strains in the *Cr. muytjensii* cluster did not show a high level of divergence, although they were isolated from different countries and sources.

The *Cr*. *turicensis* strains also formed a distinct group in cluster 3, separate from all the other isolates with a confidence value of 99 %. In the *Cr. turicensis* cluster strain E694 grouped separately from the type strain (DSM 18703^T) and strain E681 with a bootstrap value of 89 %.

Two isolates of *Cr*. *dublinensis* formed a distinct cluster (cluster 4) with the *Cr*. *dublinensis* type strain (*Cr*. *dublinensis* subsp. *dublinensis* DSM 18705^T), basal to the *Cr*. *sakazakii* and *Cr*. *turicensis* grouping. One isolate (E791) of *Cr*. *dublinensis* was basal to the groupings of *Cr*. *sakazakii*, *Cr*. *turicensis* and *Cr*. *dublinensis*. The different subspecies of *Cr. dublinensis* may have been represented by this grouping as strain E464 was identified as *Cr. dublinensis* subsp. *lactaridi* by Iversen *et al*. (2008). Strains E791 and E798 may represent the other subspecies *Cr*. *dublinensis* subsp. *lausannensis* or *Cr*. *dublinensis* subsp. *dublinensis*.

*Phylogeny based on rpo*A

In the phylogram based on the *rpo*A gene, the sequence of *E. aerogenes* had sufficient genetic differences to distinguish this strain from the *Cronobacter* isolates (Fig. 2). Unlike the analysis based on the 16S rRNA gene, the majority of the *Cr. sakazakii* strains all grouped in cluster 1 with a 93 % confidence. The South African strains C3 and C5 isolated from carrots were grouped with strains E68 and M3 that were isolated from the environment of an IFM production facility and mincemeat, respectively. A clinical strain from Canada (E604) and a strain isolated from a food sample in Russia (E775) grouped together with two strains from South Africa that were isolated from IFM and the IFM production environment. The remaining 16 *Cr*. *sakazakii* strains, with the exception of

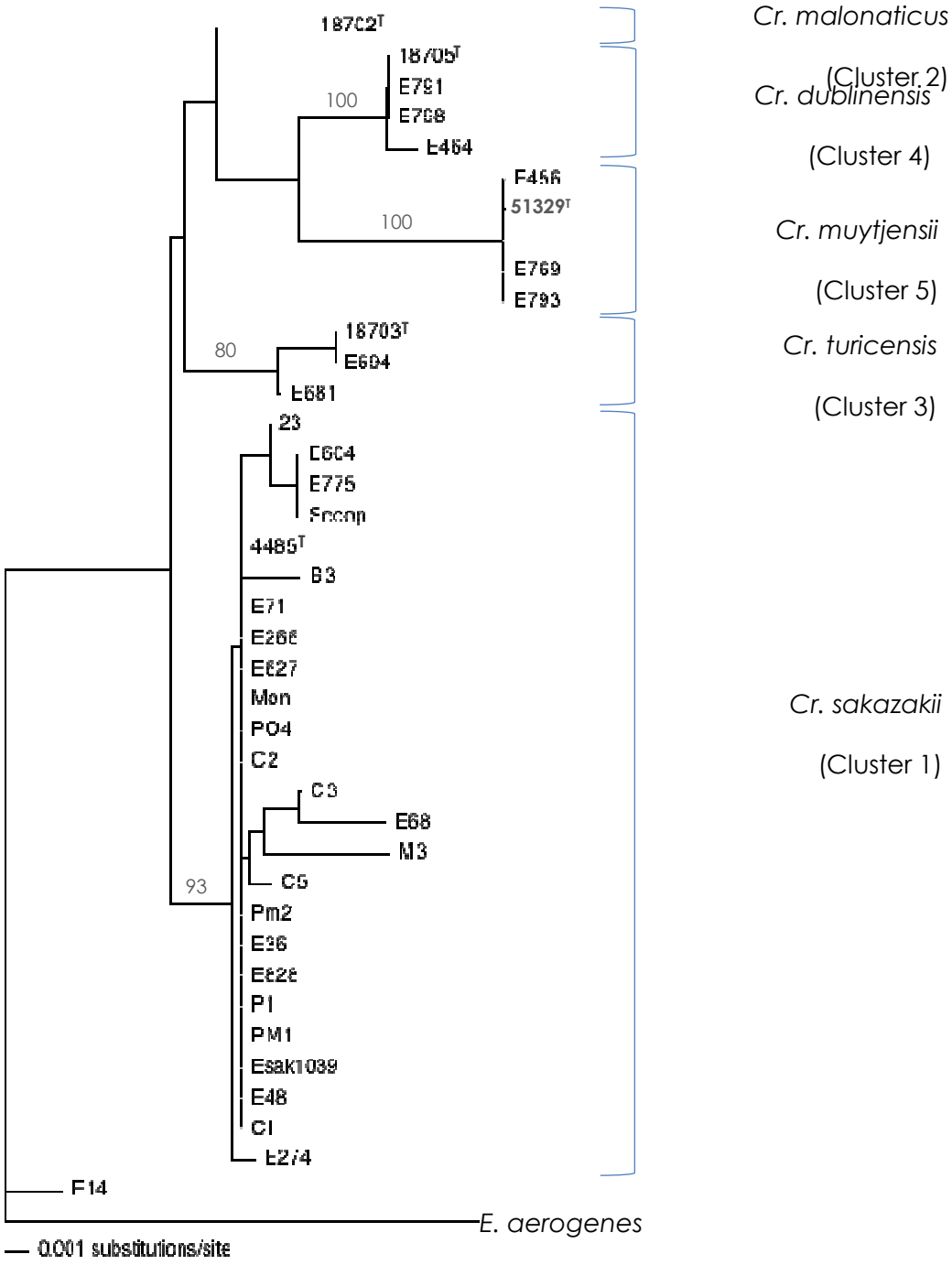


Figure 2 Phylogram based on *rpo*A gene sequences using neighbour-joining analysis and 1000 bootstraps.

strains E274 and E14, showed no differences in their *rpo*A sequences. Strain E274, from the environment in Malaysia, was grouped basal to the other *Cr*. *sakazakii* strains with a low confidence percentage of 55 %. Strain E14 showed the highest genetic diversity based on the *rpo*A gene as it was separate from all the other *Cronobacter* strains. Based on the phylogenetic analysis this strain does not seem to be *Cronobacter*, although it was identified as *Cr. sakazakii* based on both the *rpo*A and 16S rRNA gene sequences using the BLASTn search option in GenBank. Strains E274 and E14 were grouped together in the 16S rRNA phylogram in cluster 2 (Fig. 1) and seem to be distant from the other *Cr*. *sakazakii* strains.

The *Cr. malonaticus* type strain grouped separately from the *Cr. sakazakii* strains in cluster 2, but with a low bootstrap value of 70 %. The absence of additional *Cr*. *malonaticus* strains may have influenced this lack of differentiation between *Cr. sakazakii* and *Cr. malonaticus*. The *rpo*A sequences of 48 *Cronobacter* isolates have also been used to evaluate the genetic relatedness of this genus (Kuhnert *et al*., 2009). The results from this study indicated that the *rpo*A gene sequences were divergent enough to enable differentiation between all five *Cronobacter* species.

Cronobacter muytjensii strains were grouped in cluster 5 distinct from the other *Cronobacter* isolates with 100 % bootstrap value. No genetic variation for the *rpo*A gene sequences for these *Cr*. *muytjensii* strains was observed.

The strains belonging to *Cr. turicensis* were grouped separately from the rest of the *Cronobacter* isolates in cluster 3 with a bootstrap value of 80 %. The *Cr*. *turicensis* type strain (DSM 18703^{T}) and strain E694 was separate from strain E681 as in the case of the 16S rRNA gene phylogram analysis. The *rec*N gene sequences analysed by Kuhnert *et al*. (2009) also indicated the separation of strain E681 from the *Cr*. *turicensis* type strain and strain E694. These results indicate that there may be differences between the *Cr*. *turicensis* strains, especially since the differences are displayed by multiple genes. This might be related to the distinction of some strains from biogroup 16 (identified as *Cr*. *turicensis*) which were classified as *Cronobacter* genomospecies 1. However, no distinction can be made with phenotypic analysis between strains from *Cr*. *turicensis* and strains classified as *Cronobacter* genomospecies 1 (Iversen *et al*., 2008).

The strains identified as *Cr. dublinensis* were clearly separated in cluster 4 from the other *Cronobacter* isolates with a 100 % confidence. This grouping of the *Cr*. *dublinensis* strains based on the *rpo*A gene was more distinct than in the 16S rRNA gene phylogram.

Additionally, strain E791 that was separated from cluster 4 in Fig.1, grouped with the rest of the *Cr. dublinensis* strains. No genetic variation was observed based on the *rpo*A gene between this strain and the other *Cr. dublinensis* strains in cluster 4 (Fig 2). This indicates that the differences between these *Cr. dublinensis* strains are located in the 16S rRNA gene and this could be the reason why the *rpo*A gene showed a more distinct *Cr. dublinensis* cluster.

Phylogeny based on rpoA and 16S rRNA genes

The tree topologies resulting from the analysis of the *rpo*A and 16S rRNA gene datasets appeared to be similar to each other. This was confirmed with the Templeton Nonparametric Wilcoxon Signed Ranked Test (Kellogg *et al*., 1996) and the datasets were combined for further phylogenetic analysis. The resulting phylogram based on the combined sequences of the *rpo*A and 16S rRNA gene sequences served as a good indication of the genetic relatedness between the *Cronobacter* species, relatively to the outgroup *E. aerogenes* (Fig 3). All the species were clustered separately with high bootstrap values except for *Cr. malonaticus* (DSM 18702^T), which could not be differentiated from *Cr. sakazakii* in cluster 1. The *Cr. sakazakii* strains were divided into two clusters (cluster 1 and 2), although both contained strains from clinical, environmental and food sources. The strains identified as *Cr. muytjensii* was shown to be least similar to the rest of the species in cluster 5 with a bootstrap value of 100 %. This confirms findings of a study based on the *rpo*A, *rec*N and *thd*F genes (Kuhnert *et al*., 2009). *Cronobacter turicensis* strains formed a group basal to *Cr. sakazakii* in cluster 3 with a confidence level of 99 %. Additionally, *Cr. dublinensis* strains (cluster 4) were separated from the rest of the *Cronobacter* isolates with a bootstrap value of only 79 %.

The phylogeny of the *Cronobacter* genus based on the *rpo*A and 16S rRNA genes indicated that the five species were genetically closely related. However, the genus seems to be in the process of speciation as some strains show genetic variation in specific genes, but similarity in other genes. This was found in the case of strain E791, which was separated from the other *Cr. dublinensis* strains using the 16S rRNA gene analysis, but grouped with the *Cr. dublinensis* species in the *rpo*A based phylogram. Furthermore, strain E274 grouped basal to the rest of the *Cr. sakazakii* strains and strain E14 was separated from all the other *Cronobacter* isolates based on the phylogram using the *rpo*A gene. Both

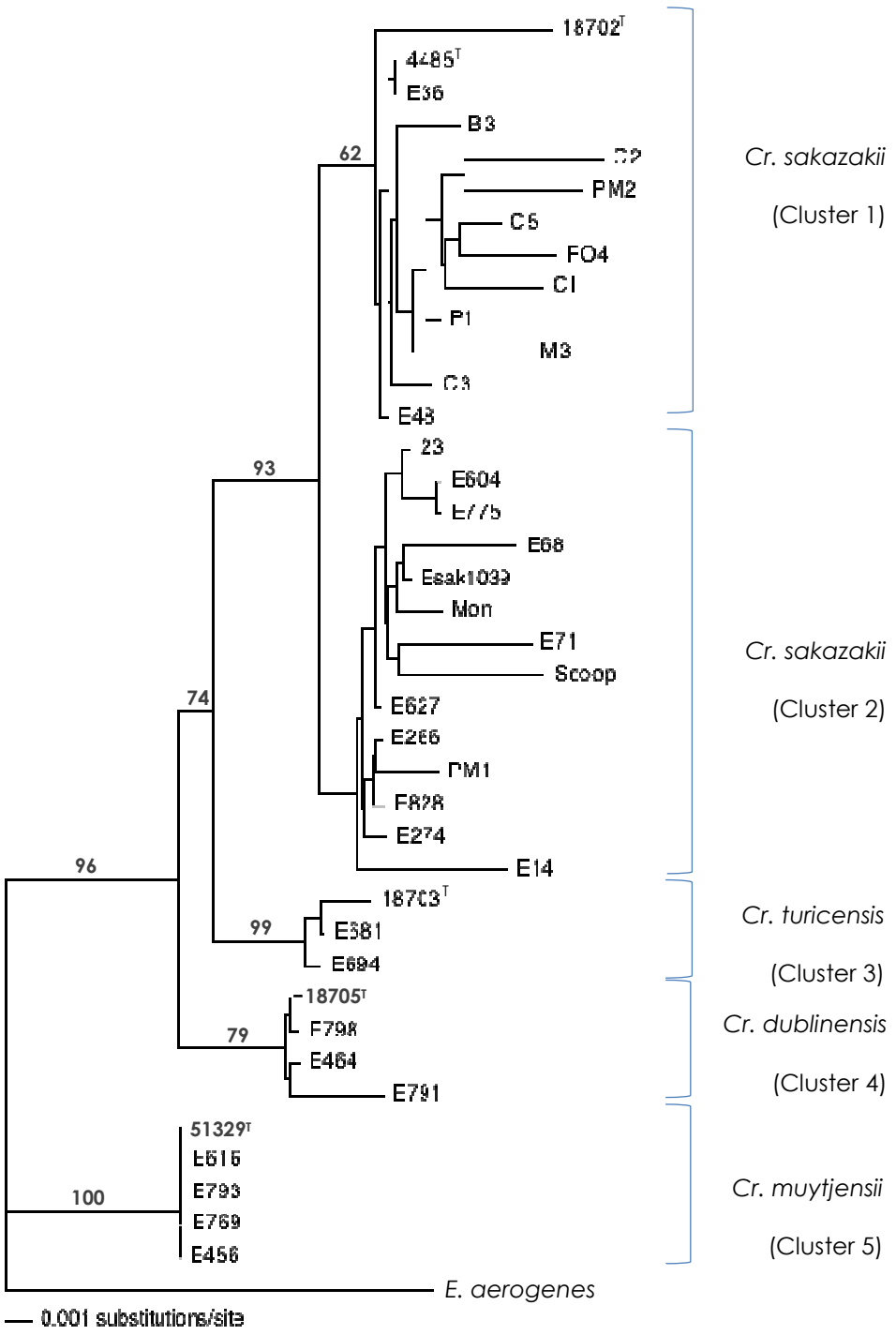


Figure 3 Phylogram based on the combined sequences of the *rpo*A and 16S rRNA genes using neighbour-joining analysis and 1000 bootstraps.

of these strains were grouped with the other *Cr*. *sakazakii* strains in the phylograms based on the 16S rRNA gene and the combined sequences of the *rpo*A and 16S rRNA genes.

Baldwin *et al*. (2009) evaluated the relationship between *Cr. sakazakii* and *Cr. malonaticus* sequences of seven loci. The results of this multi-locus sequencing analysis indicated that *Cr. sakazakii* showed a more clonal recombinating population. This type of recombination is an indication of bacteria that evolve through accumulation of point mutations. The speciation in the *Cronobacter* genus can also partially be clarified by comparative genomic hybridisation (CGH) on more than 4000 genes of *Cronobacter* species (Kucerova *et al*., 2010). A total of 15 gene clusters including putative prophages and prophage fragments were absent in more than half of the tested strains. Additionally, putative virulence genes were identified in most of these clusters. This indicates that the acquisition of genes via integration or phages, as well as specific gene loss played a major role in the evolution of *Cronobacter* and diversity among its species (Kucerova *et al*., 2010).

Conclusions

Insight into different characteristics of pathogens is a prerequisite for improving the reliability of risk assessment and the acquisition of data regarding the diversity of strains is essential for the development of risk management strategies. The recent reclassification of *Cronobacter* has involved the characterisation of more than 150 *Cronobacter* isolates and aided in the understanding of the phylogenetic relationship of these pathogens. The presence of these pathogens in South Africa is a cause of concern for both immuno-compromised adults and infants as South Africa has considerable population of HIV-infected individuals.

Phylogenetically, the species of *Cronobacter* are very closely related based on the *rpo*A and 16S rRNA genes. These two genes were not divergent enough to sufficiently distinguish between all the *Cronobacter* species and it is, therefore, advisable to use multiple genes when further evaluating the phylogeny of this genus. When used in combination with a biochemical analysis based on malonate utilisation, these two genes can be used to differentiate between the *Cronobacter* species. It is, however, necessary to develop a rapid and accurate typing method for the five different *Cronobacter* species.

References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, Z., Miller, W. & Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acid Research*, **25**, 3389-3402.

Arroyo, C., Condon, S. & Pagan, R. (2009). Thermobacteriological characterization of *Enterobacter sakazakii*. *International Journal of Food Microbiology,* **136** (1), 110-118.

Baldwin, A., Loughlin, M., Caubilla-Barron, J., Kucerova, E., Manning, G., Dowson, C. & Forsythe, S. (2009). Multilocus sequence typing of *Cronobacter sakazakii* and *Cronobacter malonaticus* reveals stable clonal structures with clinical significance which do not correlate with biotypes. *BMC Microbiology,* **9**, 223.

Caubilla-Barron J., Hurrell E., Townsend S., Cheetham P., Loc-Carrillo C., Fayet O., Prere M. F., Forsythe S.J. (2007). Genotypic and phenotypic analysis of *Enterobacter sakazakii* strains from an outbreak resulting in fatalities in a neonatal intensive care unit in France**.** *Journal of Clinical Microbiology*, **45**, 3979-3985.

Cawthorn, D.M., Botha, S. & Witthuhn, R.C. (2008). Evaluation of different methods for the detection and identification of *Enterobacter sakazakii* isolated from South African infant formula milks and the processing environment. *International Journal of Food Microbiology,* **127** (1-2), 129-138.

Farmer, J.J., Asbury, M.A., Hickman, F.W., Brenner, D.J. & the Enterobacteriaceae Study Group (1980). *Enterobacter sakazakii*, new species of Enterobacteriaceae isolated from clinical specimens. *International Journal of Systematic Bacteriology*, **30**, 569-584.

Felske, A., Rheims, H., Wolterink, A., Stackebrandt, E. & Akkermans, A.D.L. (1997). Ribosome analysis reveals prominent activity of an uncultured member of the class *Acinetobacteria* in grassland soils. *Microbiology*, **143**, 2983-2989.

Food and Agriculture Organization / World Health Organization (FAO/WHO) (2004). *Enterobacter sakazakii* and other microorganisms in powdered infant formula: Meeting Report, MRA series 6. Geneva, Switzerland. [www document]. URL http://www.who.int/foodsafety/micro/meetings/feb2004/en/. 6 March 2009.

Food and Agriculture Organization / World Health Organization (FAO/WHO) (2008). *Enterobacter sakazakii* (*Cronobacter* spp.) in powdered follow-up formulae: Meeting Report, MRA series 15. Rome. [www document]. URL http://www.who.int/foodsafety/micro/meetings/2008/en/. 11 May 2009.

Franke, S., Grass, G., Rensing, C. & Nies, D. H. (2003). Molecular analysis of the copper-transporting efflux system CusCFBA of *Escherichia coli*. *Journal of Bacteriology*, **185**, 3804–12.

Healy, B., Huynh, S., Mullane, N., O'Brien, S., Iversen, C., Lehner, A., Stephan, R., Parker, C.T. & Fanning, S. (2009). Microarray-based comparative genomic indexing of the *Cronobacter* genus (*Enterobacter sakazakii*). *International Journal of Food Microbiology,* **136** (2), 159-164.

Iversen, C., Lehner, A., Mullane, N., Bidlas, E., Cleenwerck, I., Marugg, J., Fanning, S., Stephan, R. & Joosten, H. (2007). The taxonomy of *Enterobacter sakazakii*: proposal of a new genus *Cronobacter* gen. nov. and descriptions of *Cronobacter sakazakii* comb. nov. *Cronobacter sakazakii* subsp. *sakazakii*, comb. nov., *Cronobacter sakazakii* subsp. *malonaticus* subsp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov., *Cronobacter* genomospecies 1. *BMC Evolutionary Biology,* **64** (7), 1471-2148.

Iversen, C., Mullane, N., McCardell, B., Tall, B.D., Lehner, A., Fanning, S., Stephan, R. & Joosten, H. (2008). *Cronobacter* gen. nov., a new genus to accommodate the biogroups of *Enterobacter sakazakii*, and proposal of *Cronobacter sakazakii* gen. nov., comb. nov., *Cronobacter malonaticus* sp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov., *Cronobacter* genomospecies 1, and of three subspecies, *Cronobacter dublinensis* subsp. *dublinensis* subsp. nov., *Cronobacter dublinensis* subsp. *lausannensis* subsp. nov. and *Cronobacter dublinensis* subsp. *lactardi* subsp. nov.. *International Journal of Systematic and Evolutionary Microbiology,* **58** (6), 1442-1447.

Kellogg, E.A., Appels, R. & Mason-Gamer, R.J. (1996). When genes tell different stories: the diploid genera of *Triticeae*. Systematic Bot, **21**, 321-347.

Kucerova, E., Clifton, S.W., Xia, X., Long, F., Porwollik, S., Fulton, L., Fronick, C., Minx, P., Kyung, K.,Warren, W., Fulton, R., Feng, D., Wollam, A., Shah, N., Bhonagiri, V., Nash, W.E., Hallsworth-Pepin, K., Wilson, R.K., McClelland, M. & Forsythe, S.J. (2010). Genome sequence of *Cronobacter sakazakii* BAA-894 and comparative genomic hybridisation analysis with other *Cronobacter* species. *PLoS ONE*. **5** (3) e9556. Doi: 10.1371/ journal.pone0009556.

Kuhnert, P., Korczak, B.M., Stephan, R., Joosten, H. & Iversen, C. (2009). Phylogeny and prediction of genetic similarity of *Cronobacter* and related taxa by multilocus

sequence analysis (MLSA). *International Journal of Food Microbiology,* **136** (2), 152-158.

Kuzina, L.V., Peloquin, J.J., Vacek, D.C. & Miller, T.A. (2001). Isolation and identification of bacteria associated with adult laboratory Mexican fruit flies, *Anastrepha ludens*. *Current Microbiology*, **42**, 290-294.

Lai, K.K. (2001). *Enterobacter sakazakii* infections among neonates, infants, children, and adults: case reports and a review of the literature. *Medicine Baltimore*, **80**, 113–122.

MacLean, L.L., Pagotto, F., Farber, J.M. & Perry, M.B. (2009). The structure of the O-antigen in the endotoxin of the emerging food pathogen *Cronobacter* (*Enterobacter*) *muytjensii* strain 3270. *Carbohydrate Research,* **344** (5), 667-671.

Miled-Bennour, R., Ells, C., Pagotto, F.J., Farber, J.M., Kerouanton, A., Meheut, T., Colin, P., Joosten, H., Leclercq, A. & Besse, N.G. (2010). Genotypic and phenotypic characterisation of a collection of *Cronobacter* (*Enterobacter sakazakii*) isolates. *International Journal of Food Microbiol*ogy, **139,** 116–125.

Mofokeng, L., Cawthorn, D. M., Witthuhn, C.R., Anelich, L. E. M. & Jooste, P. J. (2010). Characterization of *Cronobacter* species (*Enterobacter Sakazakii*) isolated from various South African food sources. *Journal of Food Safety*, 272, 1745-4565.

Mollet, C., Drancourt, M. & Raoult, D. (1997). *rpoB* sequence analysis as a novel basis for bacterial identification. *Molecular Microbiology*. **25** (5), 1005-1011.

Nazarowec-White, M. & Farber, J.M. (1997). *Enterobacter sakazakii*: a review. *International Journal of Food Microbiology,* **34** (2), 103-113.

Pagotto, F.J., Nazarowec-White, M., Bidawid, S. & Farber, J.M. (2003). *Enterobacter sakazakii*: infectivity and enterotoxin production in vitro and in vivo. *Journal of Food Protection,* **66** (3), 370-375.

Swofford, D.L. (2001). PAUP: phylogenetic analysis using parsimony (and other methods). Version 4.0. Sinauer Associates, Sunderland, MA, USA

Van Elsas, J. D.; Mäntynen, V.; Wolters, A.C. (1997). Soil DNA extraction and assessment of the fate of *Mycobacterium chlorophenolicum* strain PCP-1 in different soil by 16S ribosomal RNA gene sequence based most-probable-number PCR and immunofluorescence. *Biol Fertil Soils*. **24**,188-195.

Van Acker, J., Smet, F., Muyldermans, G., Bougatef, A., Naessens, A. & Lauwers, S. (2001). Outbreak of necrotizing enterocolitis associated with *Enterobacter sakazakii* in powdered milk formula. *Journal of Clinical Microbiology,* **39** (1), 293-297.

Wang, S. & Levin, R.E. (2006). Discrimination of viable *Vibrio vulnificus* cells from dead cells in real-time PCR. *Journal of Microbiological Methods*, **64**, 1-8.

CHAPTER 4

PCR-RFLP ANALYSIS OF THE *rpo*B GENE TO DISTINGUISH THE FIVE SPECIES OF *CRONOBACTER*

Abstract

Members of the genus *Cronobacter* (previously known as *Enterobacter sakazakii*) are opportunistic pathogens associated with life-threatening infections in immuno-compromised individuals. Polyphasic analysis has facilitated the classification of the novel genus *Cronobacter* containing five species. However, since this recent reclassification there are not many typing methods optimised for differentiation between the five *Cronobacter* species. This differentiation between the species is of importance as there are indications that the species may be diverse regarding their virulence. The aim of this study was to develop a PCR-RFLP protocol to differentiate between the five *Cronobacter* species. The *rpo*B gene of 49 Enterobacteriaceae strains, including 33 *Cronobacter* strains was amplified using conventional PCR, followed by digestion of these PCR products with restriction endonucleases *Mbo*I, *Hin*P1I and *Csp*6I. The PCR-RFLP analysis with single digestions of each of the restriction endonucleases did not distinguish between all five *Cronobacter* species. This study describes the successful differentiation of the five *Cronobacter* species based on the amplification of the *rpo*B gene followed by the combined digestion with restriction endonucleases *Csp*6I and *Hin*P1I. Unique profiles for each of the five *Cronobacter* species were obtained and it was also possible to differentiate between Enterobacteriaceae and *Cronobacter* strains. This PCR-RFLP assay is an accurate typing method that ensures rapid differentiation between the five species of *Cronobacter*.

Introduction

The genus *Cronobacter* contains facultative anaerobic, Gram-negative rods that belong to the family Enterobacteriaceae (Nazarowec-White & Farber, 1997). Previously known as *Enterobacter sakazakii* this genus currently consists of five species, namely *Cronobacter sakazakii, Cronobacter malonaticus, Cronobacter muytjensii, Cronobacter dublinensis* and *Cronobacter turicensis*. Additionally, three *Cronobacter* strains were grouped together and designated as *Cronobacter* genomospecies 1 (Iversen *et al*., 2008). *Cronobacter* species are opportunistic pathogens that have been associated with sporadic infections and

outbreaks (FAO/WHO, 2004; 2008). These bacteria may cause a severe form of neonatal meningitis, necrotising enterocolitis and septicaemia (Nazarowec-White & Farber, 1997; Van Acker *et al*., 2001). The mortality rates of these infections can vary between 10 and 80 % with fatalities occurring just days after infection (Iversen *et al*., 2004a).

Species of the genus *Cronobacter* have been reported to differ in sensitivity to chemical reagents, heat and antibiotics (Farmer *et al.,* 1980; Kuzina *et al*., 2001; Lai, 2001; Arroyo *et al*., 2009) and specifically vary in the type of virulence factors expressed by these bacteria (Pagotto *et al.,* 2003; Healy *et al*., 2009; MacLean *et al*., 2009). Recently, Kucerova *et al*. (2010) reported that only strains from three of the *Cronobacter* species, namely *Cr. sakazakii*, *Cr. malonaticus* and *Cr. turicensis* have been associated with neonatal infections. For these reasons, it is imperative to distinguish between the different species of *Cronobacter*.

Typing methods for differentiation between the members of *Cronobacter* are not readily reported in literature and isolates are identified with direct sequence analysis of the 16S ribosomal RNA (rRNA) gene. However, due to the close relationship between *Cr. sakazakii* and *Cr. malonaticus* it is not possible to distinguish between these two species using 16S rRNA sequence data (Iversen *et al*., 2007). Alternative genes such as the *rpo*B gene can be used for the identification of *Cronobacter* species (Stoop *et al*., 2009). This universal gene encodes for the bacterial RNA polymerase β-subunit and has been evaluated for its suitability in species identification. Sequence analysis of strains belonging to the Enterobacteriaceae family indicated that the *rpo*B gene was more diverse than the 16S rRNA gene (Mollet *et al.,* 1997). The *rpo*B gene can, therefore, potentially be used as a diagnostic tool to identify and differentiate between *Cronobacter* strains and may provide distinction between *Cr. sakazakii* and *Cr. malonaticus*, the two most closely related species in this genus (Kuhnert *et al*., 2009).

The use of conventional PCR assays for the differentiation between bacterial strains can be enhanced by using restriction endonucleases to digest the PCR fragments, followed by electrophoretic analysis of the restriction fragment length polymorphisms (RFLP). The aim of this study was to develop a PCR-RFLP assay based on the *rpo*B gene to differentiate between the five *Cronobacter* species. Three restriction endonucleases, namely *Mbo*I, *Csp*6I and *Hin*P1I were used singly to digest the *rpo*B PCR fragments. Additionally, *Csp*6I and *Hin*P1I were used in a combined digestion reaction. The specificity

of the newly developed PCR-RFLP method was evaluated by typing *Cronobacter* isolates, obtained from various environmental and food sources in South Africa.

Materials and methods

Bacterial isolates

Bacterial isolates from the Enterobacteriaceae family, including 28 *Enterobacter sakazakii* (*Cronobacter*) strains and 16 closely related strains were evaluated in this study. Isolates were obtained from various food and environmental sources in South Africa, including infant formula milk (IFM) products, the IFM production facility and fresh produce. The fresh produce strains were isolated from beetroot, carrots, potatoes, raw milk and mincemeat. The type strains *Cr. sakazakii* DSM 4485^T, *Cr. malonaticus* DSM 18702^T, *Cr. turicensis* DSM 18703^T, *Cr. dublinensis* DSM 18705^T (obtained from the Deutsche Sammeling von Mikroorganismen und Zelkulturen) and *Cr. muytjensii* ATCC 51329^T (obtained from the American Type Culture Collection) were also included in this study. Bacterial cultures were inoculated into tryptone soy broth (TSB) (Merck, Cape Town, South Africa) and incubated at 37 °C for 24 h. Identification of *Cronobacter* spp. was done by streaking the incubated broth on Chromocult® (Merck) followed by incubation at 42 °C for 24 h. Blue-green colonies were regarded as *Cronobacter* positive. Additionally, malonate broth (Sigma-Aldrich, Kempton Park, South Africa) was used to differentiate between *Cr. sakazakii* and *Cr. malonaticus* isolates. The 49 isolates were inoculated into the malonate broth and incubated at 37 °C for 24 h. A colour change in the broth from green to blue indicated a positive result for the utilisation of malonate and these strains were recorded as *Cr. malonaticus*.

DNA isolations

DNA was isolated using either a modified phenol-chloroform DNA-extraction method (van Elsas *et al*., 1997) or the TZ-method described by Wang & Levin (2006). For the phenol-chloroform method bacterial samples were prepared by centrifuging (Eppendorf Centrifuge 5415D) 2 ml of the 24 h TSB cultures at 8 000 *g* for 10 min. The supernatants were discarded and 0.6 g glass beads were added to the cell pellets. Subsequently 800 µL phosphate buffer (1 part 120 mM NaH_2PO_4 (Merck) to 9 parts 120 mM Na_2HPO_4 (Merck), pH 8), 700 µL phenol (Fluka, supplied by Sigma-Aldrich) and 100 µL 20 % (m/v) sodium dodecyl sulphate (SDS) (Merck) were added to the samples. The samples were then

subjected to vortexing for 1 to 2 min followed by incubation for 20 min at 60 °C, and this was repeated three times. After the third incubation step the samples were centrifuged for 5 min at 4 000 *g*. Phenol (600 µL) was added to each supernatant, the samples were vortexed and centrifuged for 5 min at 8 000 *g*. The water phase was removed and 600 µL phenol:chloroform:isoamylalcohol (25:24:1) was added. These samples were mixed and centrifuged for 5 min at 8 000 *g*, the water phase removed and repeated until the interphase was clean. The volume of supernatant was noted and the DNA was precipitated by adding 0.1 volume 3 M sodium acetate (NaOAc) (pH 5.5) (Saarchem, supplied by Merck) and 0.6 volume isopropanol (Saarchem). The samples were stored overnight at -20 °C. After centrifugation (10 min at 12 000 *g*), the resulting pellet was washed with 70 % (v/v) ethanol, followed by centrifugation for 5 min at 12 000 *g*. The pellet was air-dried and 100 µL of 1 x TE (10 mM Tris (Fluka), 1 mM EDTA (Merck), pH 8) buffer was added. Samples were treated with 1 µL RNase for 1 h at 37 °C and stored at -20 °C.

The TZ-method was performed on 250 µL of the 24 h TSB cultures after the cells were harvested by centrifugation at 8 000 *g* for 5 min. The pellets were resuspended in 250 µL double strength TZ (2 x TZ) (Wang & Levin, 2006) which consisted of 5 $mg.mL^{-1}$ sodium azide (Merck) and 4 % (v/v) Triton X-100 (Merck) in 0.1 M Tris-HCl (Fluka) at pH 8. Samples were boiled for 10 min to lyse the cells after which it was left to cool. The debris was pelleted by centrifugation at 10 000 *g* for 5 min and 200 µL of the supernatant was used as DNA template.

Sequencing

The identities of 28 presumptive *Cronobacter* isolates and 16 non-*Cronobacter* isolates were determined with sequence data based on the 16S rRNA gene. The PCR amplification of a 1.5 kilobase (kb) fragment of the 16S rRNA gene was performed using the primers F8 (5'- CAC GGA TCC AGA CTT TGA TYM TGG CTC AG -3') and R1512 (5'- GTG AAG CTT ACG GYT AGC TTG TTA CGA CTT -3') (Felske *et al*., 1997). The PCR reaction mixture (50 µL total volume) contained 1 µL (5 U) *Taq* DNA polymerase (Super-Therm, supplied by Southern Cross Biotechnologies, Cape Town, South Africa), 5 µL 10 X buffer (Super-Therm), 3 µL (1.5 mM) $MgCl_2$ (Super-Therm), 2 µL (400 nM) of each primer, 2 µL (400 nM) dNTPs (AB gene, supplied by Southern Cross Biotechnologies), 2 µL 99 % (v/v) DMSO (Merck) and 2 µL DNA template. Thermal cycling parameters (Eppendorf mastercycler personal, Eppendorf, Germany) were as follows: initial denaturation at 92 °C

for 3 min; 35 cycles of denaturation at 92 °C for 30 s, annealing at 54 °C for 30 s, and elongation at 68 °C for 60 s; and final elongation at 72 °C for 7 min (Felske *et al*., 1997).

Sequencing of the PCR products was performed using the ABI 3100 Genetic Analyser (Applied Biosystems, Foster City, USA). The closest recognised relatives of the isolates were determined by comparing the generated sequences to sequences in GenBank, using the BLASTn search option (Altschul *et al*., 1997).

Amplification of the *rpo*B gene

Primer design

The *rpo*B gene of the five *Cronobacter* type strains, *Cr. sakazakii* DSM 4485^T, *Cr. malonaticus* DSM 18702^T, *Cr. turicensis* DSM 18703^T, *Cr. dublinensis* DSM 18705^T and *Cr. muytjensii* ATCC 51329^T were used to design the primers. The *rpo*B sequences of these type strains were obtained from GenBank and aligned using Multalin (http://multalin.toulouse.inra.fr/multalin/). The conserved regions in this gene were evaluated for suitability to be used as primers, including the affinity for dimer and hairpin formation with Oligocal (http://www.basic.northwestern.edu/biotools/oligocalc.html).

PCR assay

Amplification of the *rpo*B fragment was performed using the newly designed primers CroF2 (5'- TCT CTG GGC GAT CTG GATA -3') and CroR (5'- TGC GCG CTG ATA AGC CGCT -3'). The PCR reaction mixture (50 µL total volume) contained 0.5 µL (2.5 U) *Taq* polymerase (Super-Therm), 1 µL 10 X buffer (Super-Therm), 2.5 µL (1.25 mM) $MgCl_2$ (Super-Therm), 1 µL (200 nM) of each primer, 1 µL (200 nM) dNTPs (AB gene) and 2.5 µL DNA template. Thermal cycling parameters (Eppendorf mastercycler) were as follows: initial denaturation at 92 °C for 3 min; 50 cycles of denaturation at 92 °C for 30 s, annealing at 61.5 °C for 30 s and elongation at 68 °C for 45 s; and final elongation at 72 °C for 7 min.

PCR products were separated with electrophoresis at 90 V for 50 min on a 1.5 % (m/v) agarose gel (Merck) containing 0.02 µl.ml^{-1} ethidium bromide in 0.5 X TBE electrophoresis buffer ((5.4 g.l^{-1} Tris (Fluka), 2.7 g.l^{-1} boric acid (Merck), 2.0 ml.l^{-1} 0.5 M EDTA (Merck)). The amplicons were visualised under UV light (Vilber Lourmat, Marne La Vallee, France) and a 100 bp ladder (Fermentas, supplied by Inqaba, Pretoria, South Africa) was used as a size indicator.

RFLP

The type strains, *Cr. sakazakii* DSM 4485^T, *Cr. malonaticus* DSM 18702^T, *Cr. turicensis* 18703^T, *Cr. dublinensis* DSM 18705^T and *Cr. muytjensii* ATCC 51329^T were used in preliminary PCR-RFLP analysis in which each restriction endonuclease was tested. *Enterobacter hormaechei* (strain 38) was used as a negative control as 4 of the 16 non-*Cronobacter* strains were identified as *E. hormaechei*. *Enterobacter helveticus* DSM 18963^T was used as an additional negative control as this species has been shown to be closely related to *Cronobacter* (El-Sharoud *et al*., 2009). The aligned *rpo*B gene sequences of the *Cronobacter* type strains were used to select restriction endonucleases for the PCR-RFLP analysis. Based on the variable regions in this gene, restriction endonucleases *Mbo*I (GATC·), *Csp*6I (G·TAC) and *Hin*P1I (G·CGC) (Fast Digest®, Fermentas) were selected.

PCR products were digested with each endonuclease separately and with a combination of *Csp*6I and *Hin*P1I. When investigating the restriction endonucleases as individual treatments, 10 µL PCR product was added to 1 µL endonuclease and 2 µL Fast Digest Universal Buffer® (Fermentas) in a 30 µL reaction volume. The reaction mixture of two endonucleases included 1 µL of each endonuclease, 2 µL Fast Digest Universal Buffer® (Fermentas) and 10 µL PCR product in a 30 µL reaction volume. Digestion took place at 37 °C for 10 min followed by electrophoresis at 100 V for 110 min. The fragments were separated on a 5 % (m/v) agarose gel containing 0.02 µl.ml^{-1} ethidium bromide in 1 X TBE electrophoresis buffer. The PCR-RFLP profiles were visualised under UV light (Vilber Lourmat) and an Ultra-Low Range Ladder (Fermentas) was used as a size indicator.

Results and discussion

Bacterial isolates

After 24 h incubation all the *Cronobacter* strains produced blue-green colonies on the Chromocult® agar (Merck), whereas the other related Enterobacteriaceae strains produced white colonies (Table 1, Fig. 1). The colonies of the *E. helveticus* strain DSM 18963^T, however, turned green after three days incubation. Strains of *E. helveticus, Enterobacter pulveris* and *Enterobacter turicensis* are reportedly responsible for the majority of false positive results on chromogenic agar containing the chromogen 5-bromo-4-chloro-3-indolyl-α-D-glycopyranoside (Stephan *et al.,* 2007). These false positive results can be attributed to different transport mechanisms of the chromogen which is induced by sugars

Table 1 Biochemical and sequence analysis results of *Cronobacter* and non-target Enterobacteriaceae strains

Strain Number	Strain	Source	Chromocult®	Malonate broth	16S rRNA[a] (% sequence similarity)[b]
1	*Cr. dublinensis* DSM 18705[T]	Type strain	P	P	*Cr. dublinensis*(97)
2	*Cr. turicensis* DSM 18703[T]	Type strain	P	P	*Cr. turicensis* (97)
3	*Cr. malonaticus* DSM 18702[T]	Type strain	P	P	*Cr. malonaticus* (98)
4	*Cr. muytjensii* ATCC 51329[T]	Type strain	P	P	*Cr. muytjensii* (97)
5	*Cr. sakazakii* DSM 4485[T]	Type strain	P	N	*Cr. sakazakii* (97)
6	E14	IFM environment	P	P	*Cr. sakazakii* (99)
7	E36	IFM environment	P	N	*Cr. sakazakii* (99)
8	E68	IFM environment	P	N	*Cr. sakazakii* (99)
9	Scoop	IFM environment	P	N	*Cr. sakazakii* (99)
10	797 (52-54) 5	IFM	P	P	*Cr. sakazakii* (99)
11	E39 chrom	IFM environment	P	N	*Cr. sakazakii* (99)
12	E71	IFM environment	P	N	*Cr. sakazakii* (99)
13	Esak 1039	Wine effluent	P	N	*Cr. sakazakii* (99)
14	B1	Beetroot	P	N	*Cr. sakazakii* (99)
15	B2	Beetroot	P	N	*Cr. sakazakii* (99)
16	B3	Beetroot	P	N	*Cr. sakazakii* (99)
17	B4	Beetroot	P	N	*Cr. sakazakii* (99)
18	C2	Carrot	P	N	*Cr. sakazakii* (99)
19	C3	Carrot	P	N	*Cr. sakazakii* (99)
20	C4	Carrot	P	N	*Cr. sakazakii* (99)
21	C5	Carrot	P	N	*Cr. sakazakii* (99)
22	CI	Industrial isolate (Canada)	P	N	*Cr. sakazakii* (99)
23	M3	Mince meat	P	N	*Cr. sakazakii* (99)
24	Mon	Montreal hospital	P	N	*Cr. sakazakii* (99)
25	P02	Potato	P	N	*Cr. sakazakii* (99)
26	P03	Potato	P	N	*Cr. sakazakii* (99)

Table 1 Continued

Strain number	Strain	Source	Chromocult®	Malonate broth	16S rRNA
27	PO4	Potato	P	N	*Cr. sakazakii* (99)
28	P07	Potato	P	N	*Cr. sakazakii* (99)
29	P08	Potato	P	N	*Cr. sakazakii* (99)
30	P1	PIF	P	N	*Cr. sakazakii* (99)
31	PM1	Powdered milk	P	N	*Cr. sakazakii* (99)
32	PM2	Powdered milk	P	N	*Cr. sakazakii* (99)
33	S1	Stellenbosch	P	N	*Cr. sakazakii* (99)
34	E2	IFM environment	N	N	*E. cloacae* (99)
35	18963	Type strain	N	N	*E. helveticus* (98)
36	R2	Raw milk	N	N	*E. hormaechei* (99)
37	872 C4	IFM	N	N	*E. hormaechei* (99)
38	P05	Potato	N	N	*E. hormaechei* (99)
39	R1	Raw milk	N	N	*E. hormaechei* (99)
40	E48	IFM environment	N	N	*Enterobacter* sp. (99)
41	E65	IFM environment	N	N	*Enterobacter* sp. (99).
42	SA1	Industrial isolate (SA)	N	N	*Enterobacter* sp. (99)
43	868 (2)	IFM	N	N	*Enterobacter* sp. (98)
44	C1	Carrot	N	N	*Enterobacter* sp. (99)
45	M1	Mince Meat	N	N	*Enterobacter* sp. (99)
46	W1	Whey	N	N	*Enterobacter* sp. (99)
47	883 A1	IFM	N	N	*Pantoea ananatis* (99)
48	877 C4		N	N	*Acinetobacter* (97)
49	763 P4 (3)	IFM	N	N	*Pseudomonas fulva* (98)

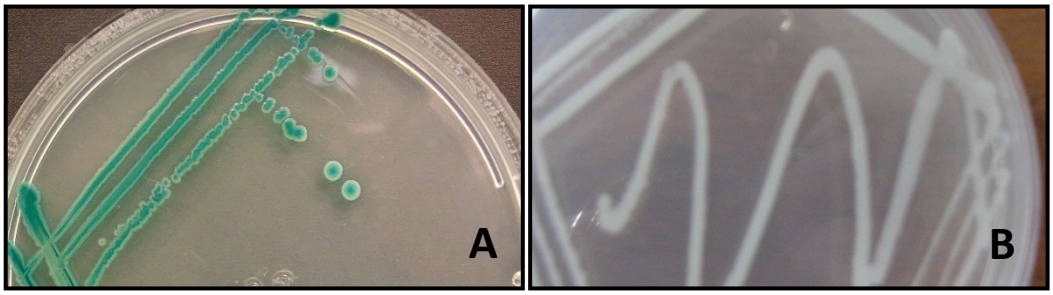


Figure 1 Chromocult® agar A: *Cr. muytjensii* ATCC 51329^{T}, B: *Enterobacter* sp. (strain 42).

such as maltose (Druggan & Iversen, 2009). The production of green colonies by non-*Cronobacter* strains on Druggan-Forsythe-Iversen (DFI) agar have also been reported after prolonged incubation (>30 h). Additionally, adding maltose to DFI resulted in entirely green colonies for strains of *Citrobacter koseri, Enterobacter aerogenes* and *Klebsiella pneumonia* (Iversen *et al*., 2004b). For these reasons, it is paramount that protocols are strictly followed to prevent false positive results.

Malonate production was used as one of the biochemical characteristics in the reclassification of the *Cronobacter* species and is used for biochemical differentiation between *Cr. sakazakii* and *Cr. malonaticus* (Iversen *et al*., 2006; 2007; Terragno *et al*., 2009). Positive results for the utilisation of malonate were obtained for all the *Cronobacter* type strains, except *Cr. sakazakii* DSM 4485^T (Table 1). Although *Cr. dublinensis* is known to be variable for malonate utilisation, it is generally the two subspecies *Cr. dublinensis* subsp*. lactaridi* and *Cr. dublinensis* subsp*. lausanensis* that do not utilise malonate (Iversen *et al*., 2006; 2007). As the type strain of *Cr. dublinensis* (*Cr. dublinensis* subsp. *dublinensis* DSM 18705^T) was used in this PCR-RFLP analysis, it was expected that this strain would test positive for malonate utilisation. The majority of *Cronobacter* strains isolated from South African products tested negative for malonate utilisation and, therefore, most likely belong to *Cr. sakazakii* and also as this species is the *Cronobacter* predominant species isolated from food products. Two strains, 6 and 10 did utilise malonate indicating that these strains may be one of the other *Cronobacter* species (Fig. 2). There is, however, a very small number (less than 5 %) of *Cr. sakazakii* strains that have produced positive results for malonate utilisation (Iversen *et al*., 2007). All the non-target Enterobacteriaceae strains tested negative for malonate production.

16S rRNA gene sequencing

The sequencing results of the 16S rRNA gene and isolation sources of the target and non-target strains are shown in Table 1. All the *E. sakazakii* isolates from South Africa were identified as *Cr. sakazakii* based on the 16S rRNA gene sequences, despite the diverse isolation sources. In a recent study by Miled-Bennour *et al*. (2010) 150 *E. sakazakii* isolates obtained from food products, clinical and environmental samples were reclassified. The majority of these isolates were identified as *Cr. sakazakii* (82.5 %), with 8 % of the strains designated as *Cr. malonaticus*, 5 % as *Cr. muytjensii*, 3 % as *Cr. dublinensis* and 1.5 % as *Cr. turicensis*. The absence of the other *Cronobacter* species in the South African

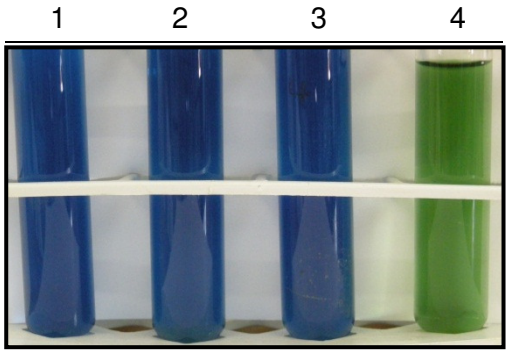


Figure 2 Malonate broth Tube 1: *Cr. malonaticus* DSM 18702^T, 2: *Cr. malonaticus* (strain 6), 3: *Cr. malonaticus* (strain 10), 4: *Cr. sakazakii* DSM 4485^T.

isolates may, therefore, be contributed to the smaller number of isolates evaluated in this study and the fact that *Cr. sakazakii* is the dominant species in terms of isolation frequency. The majority of the 16 non-target strains were identified as *Enterobacter* spp. Strains 36 to 39 that were isolated from milk products and fresh produce were identified as *E. hormaechei*. Another three of the non-target strains isolated from IFM were identified as *Pantoea ananatis*, *Acinetobacter* sp. and *Pseudomonas fulva*.

PCR-RFLP

The aligned sequences of the *Cronobacter* type strains indicated several conserved regions within the *rpo*B gene (Fig. 3). The blocks highlighted in Fig. 3 indicate the conserved regions that were used for the design of the primers to ensure amplification of a 660 bp fragment of all five *Cronobacter* species. The oligonucleotide used as forward primer CroF2 is 19 bp long and has a 53 % GC content. The reverse primer, CroR, was also 19 bp long with a GC % content of 63 %. Both primers had no affinity for secondary structure formation such as dimers and hairpins. Amplification of the partial region of the *rpo*B gene between basepairs 328247 and 328907 in the genome was successful for all the target and non-target strains (Fig. 4). An unusually large number of cycles (50 cycles) during PCR amplification provided sufficient amplicons for digestion with the restriction endonucleases. The fragments in the profiles ranged from 20 to 300 bp. Fragments smaller than 50 bp were not always clearly visible and, therefore, not included in defining profiles.

Single restriction endonuclease digestion

Digestion with *Mbo*I resulted in unique profiles for only *Cr. dublinensis*, *Cr. muytjensii* and *Cr. turicensis* as identical profiles were obtained for *Cr. sakazakii* and *Cr. malonaticus* (Fig. 5). Unlike the theoretical profiles based on the sequences available in GenBank, *Cr. muytjensii* had an identical profile to *E. hormaechei*. The PCR-RFLP assay with *Mbo*I was, therefore, unsuitable for differentiation between the *Cronobacter* species, as well as differentiating between *Cronobacter* and Enterobacteriaceae strains.

Restriction digestion of the type strains and two negative controls with *Csp*61I resulted in unique profiles for *Cr. sakazakii* and *E. helveticus* (Fig. 6). *Cronobacter muytjensii* and *Cr. dublinensis* had identical profiles as predicted by the theoretical profiles. These two profiles were, however, also similar to the profile of *Cr. malonaticus* due to the

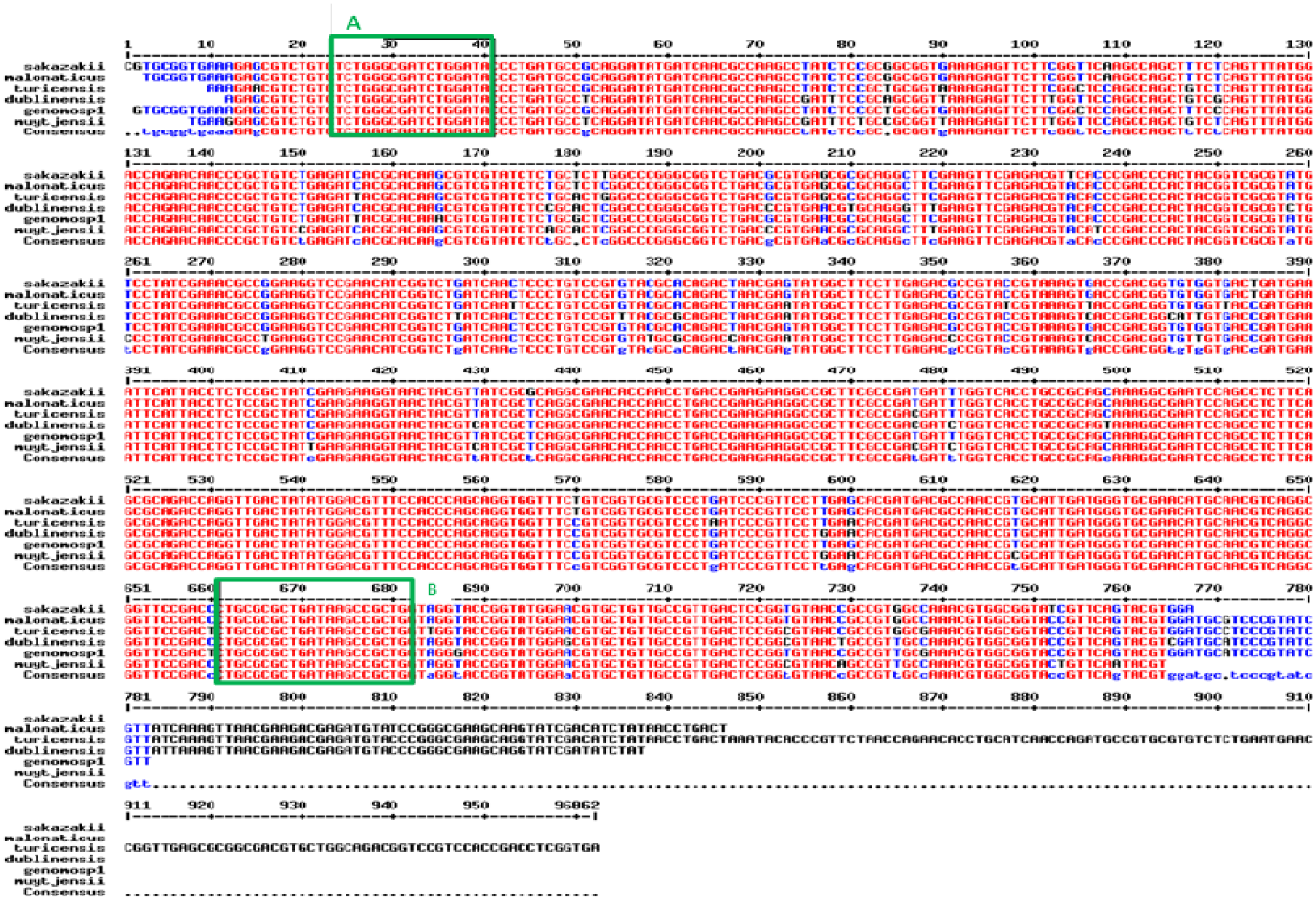


Figure 3 Alignment of *rpo*B gene sequences of five *Cronobacter* strains (Block A indicates the forward primer (Crof2) and B the reverse primer (CroR)).

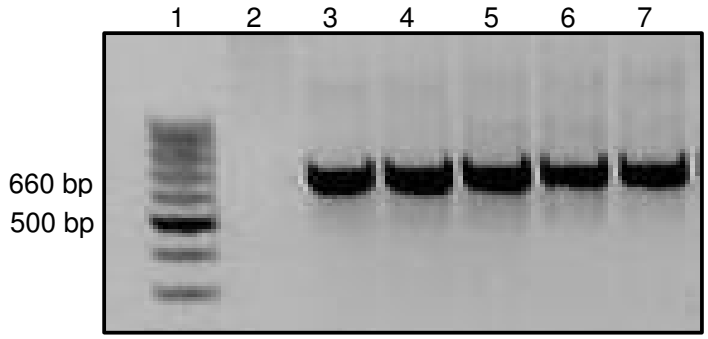


Figure 4 Amplification of a *rpo*B gene fragment of *Cronobacter* spp. Lane 1: 100 bp ladder, 2: negative control containing no DNA template, 3: *Cr. sakazakii* DSM 4485^T, 4: *Cr. malonaticus* DSM 18702^T, 5: *Cr. turicensis* DSM 18703^T, 6: *Cr. muytjensii* ATCC 51329^T, 7: *Cr. dublinensis* DSM 18705^T.

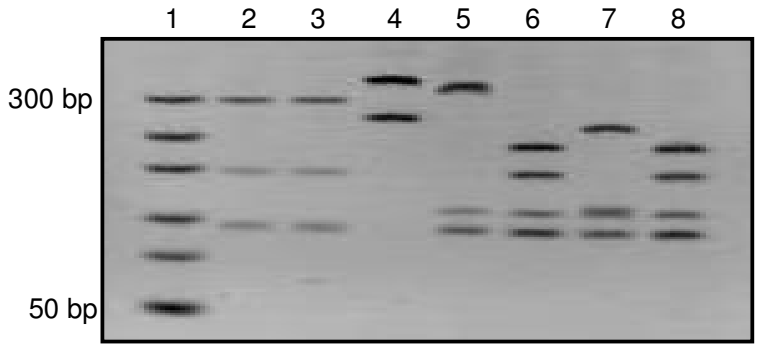


Figure 5 PCR-RFLP of the *rpo*B gene fragment digested with *Mbo*I. Lane 1: Ultra low range ladder, 2: *Cr. sakazakii* DSM 4485^T, 3: *Cr. malonaticus* DSM 18702^T, 4: *Cr. turicensis* DSM 18703^T, 5: *Cr. dublinensis* DSM 18705^T, 6: *Cr. muytjensii* ATCC 51329^T 7: *Enterobacter helveticus* 18963^T, 8: *Enterobacter hormaechei* (strain 38).

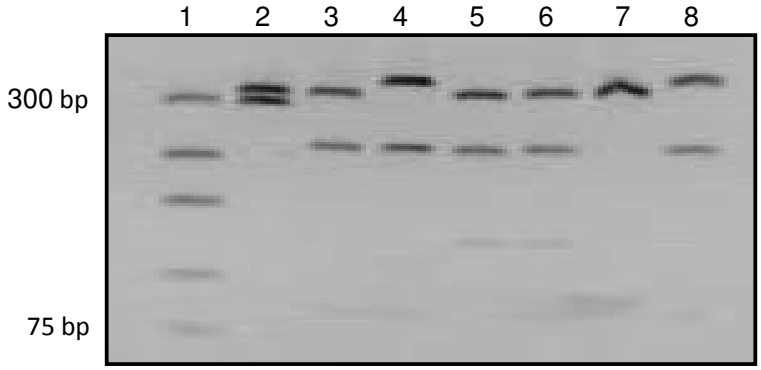


Figure 6 RFLP of the *rpo*B gene fragment digested with *Csp*6I. Lane 1: Ultra low range ladder, 2: *Cr. sakazakii* DSM 4485^T, 3: *Cr. malonaticus* DSM 18702^T, 4: *Cr. turicensis* DSM 18703^T, 5: *Cr. dublinensis* DSM 18705^T, 6: *Cr. muytjensii* ATCC 51329^T 7: *Enterobacter helveticus* 18963^T, 8: *Enterobacter hormaechei* (strain 38).

fragments smaller than 50 bp which were not clearly visible on the gel in the profile of *Cr. malonaticus*. The profile of *E. hormaechei* obtained with PCR-RFLP analysis was identical to the profile of *Cr. turicensis*. The profile of *E. helveticus* was as expected since the restriction endonuclease cut the *rpo*B gene fragment only once resulting in two fragments of 330 bp each. The similar profiles of *Cr. dublinensis*, *Cr. muytjensii* and *Cr. malonaticus,* as well as *Cr turicensis* and *E. hormaechei* obtained with *Csp*61I rendered this restriction endonuclease unsuitable for the aims of this study.

Restriction analysis of the type strains with *Hin*P1I resulted in identical profiles for *Cr. dublinensis* and *Cr. malonaticus* (Fig. 7). *Cronobacter turicensis* had a profile which contained two smaller fragments (45 and 43 bp) which were not clearly visible. Consequently, the profile of *Cr. turicensis* was very similar to those of *Cr. malonaticus* and *Cr. dublinensis*. Since unique profiles were obtained for only *Cr. sakazakii*, *Cr. muytjensii, E. helveticus* and *E. hormaechei,* digestion with *Hin*P1I did not meet the aims of this study. Therefore, PCR-RFLP used in combination with each of these restriction endonucleases failed to simultaneously distinguish between all the *Cronobacter* species, as well as between *Cronobacter* and other Enterobacteriaceae strains.

Multiple restriction digestion

A multiple restriction digestion was used as none of the single restriction digestions simultaneously differentiated between the *Cronobacter* spp. and between the *Cronobacter* spp. and other Enterobacteriaceae species. Based on the theoretical profiles, it was determined that a combination of *Hin*P1I and *Mbo*I would have resulted in too many fragments that were smaller than 50 bp. Furthermore, a combination of *Csp*6I and *Mbo*I would have resulted in similar profiles for *E. hormaechei* and *Cr. muytjensii*. Therefore, restriction endonucleases *Csp*6I and *Hin*P1I were selected for combined digestion of the *rpo*B gene. This combination resulted in unique profiles for each of the *Cronobacter* species as predicted by the theoretical profiles (Figs. 8 and 9). All the profiles for the *Cronobacter* species had four fragments of varying sizes. Digestion of *Cr. sakazakii* with *Csp*6I and *Hin*P1I resulted in fragments of 190, 140, 87 and 78 bp, whereas *Cr. malonaticus* showed a profile with fragments of 190, 170, 140 and 83 bp. *Cronobacter turicensis* had

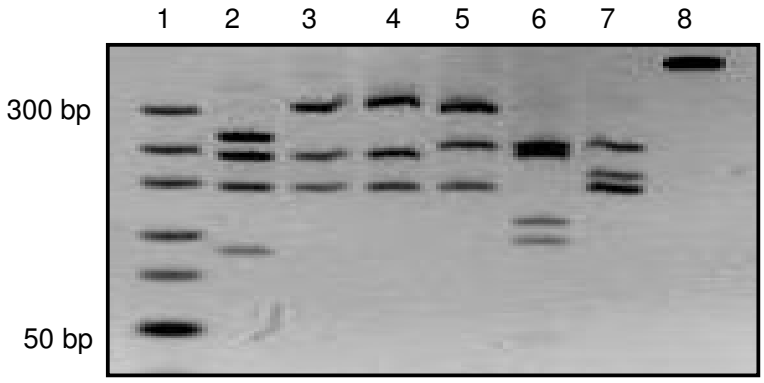


Figure 7 RFLP of the *rpo*B gene fragment digested with *Hin*P1I. Lane 1: Ultra low range ladder, 2: *Cr. sakazakii* DSM 4485^T, 3: *Cr. malonaticus* DSM 18702^T, 4: *Cr. turicensis* DSM 18703^T, 5: *Cr. dublinensis* DSM 18705^T, 6: *Cr. muytjensii* ATCC 51329^T 7: *Enterobacter helveticus* 18963^T, 8: *Enterobacter hormaechei* (strain 38).

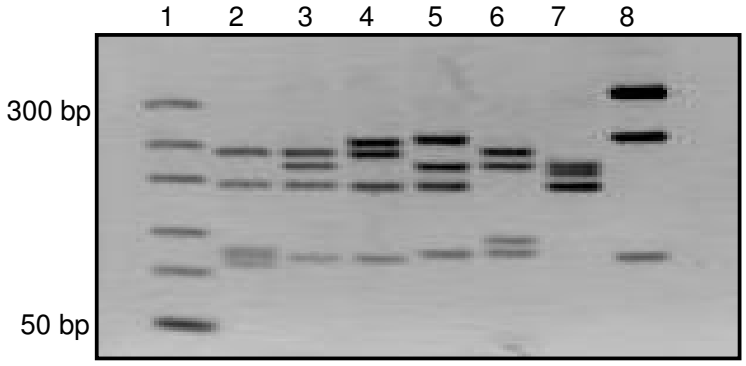
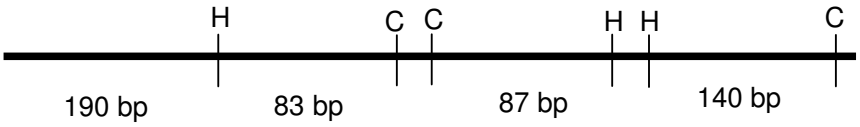
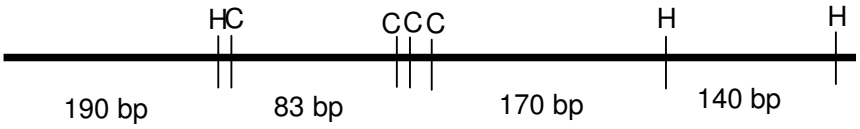


Figure 8 RFLP of the *rpo*B gene fragment digested with a combination of *Csp*6I and *Hin*P1I. Lane 1: Ultra low range ladder, 2: *Cr. sakazakii* DSM 4485^T, 3: *Cr. malonaticus* DSM 18702^T, 4: *Cr. turicensis* DSM 18703^T, 5: *Cr. dublinensis* DSM 18705^T, 6: *Cr. muytjensii* ATCC 51329^T 7: *Enterobacter helveticus* 18963^T, 8: *Enterobacter hormaechei* (strain 38).

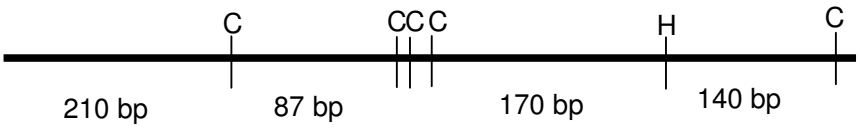
***Cr. sakazakii* DSM 4485^T**



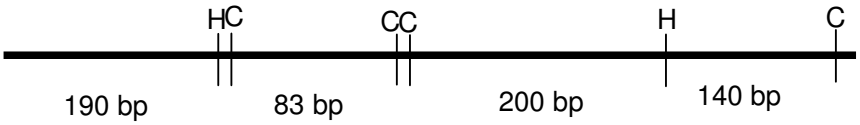
***Cr. malonaticus* DSM 18702^T**



***Cr. dublinensis* DSM 18705^T**



***Cr. turicensis* DSM 18703^T**



***Cr. muytjensii* ATCC 51329^T**

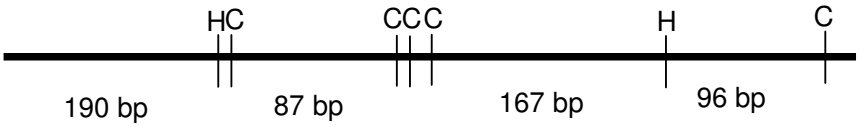


Figure 9 Restriction endonuclease maps of *Csp*6I and *Hin*P1I, indicating how the 660 bp fragment of the *rpo*B gene was digested for each of the five *Cronobacter* species (only fragments larger than 50 bp are indicated).

H = *Hin*P1I

C = *Csp*6I

slightly bigger fragments of 200, 190, 140 and 83, *Cr. dublinensis* had a profile with fragments of 210, 170, 140 and 87 bp in size and *Cr. muytjensii* had fragments of 190, 167, 96 and 87 bp. The two negative control strains also had unique profiles as *E. helveticus* showed three fragments of 170, 160 and 140 bp and *E. hormaechei* (strain 38) had fragments of 365, 211 and 84 bp in size.

PCR-RFLP of South African strains

In total 28 *Cr. sakazakii* strains isolated from South Africa were subjected to the PCR-RFLP assay using the *Csp*6I and *Hin*P1I endonuclease combination. All but two of the strains had identical profiles to the *Cr. sakazakii* type strain. Strains 6 and 10 had profiles identical to that of the *Cr. malonaticus* type strain (Fig. 10). These two strains also tested positive for the utilisation of malonate which is a distinctive characteristic of *Cr. malonaticus* (Fig. 2). Finally, strains 6 and 10 were identified as *Cr. malonaticus* based on the sequencing of the *rpo*B gene. These results proved the two strains (6 and 10) isolated from IFM to be *Cr. malonaticus* and not *Cr. sakazakii*.

The level of similarity between *Cr. sakazakii* and *Cr. malonaticus* is very high and sequence analysis based on 16S rRNA is not sufficient to distinguish between these two species (Iversen *et al*., 2007; 2008; Kuhnert *et al*., 2009). Miled-Bennour *et al*. (2010) also reported controversial results regarding these two species in that biochemical analysis of 05CHPL02 identified the strain as *Cr. sakazakii* and 05CPL53 as *Cr. malonaticus*. However, ribotyping results placed the *Cr. sakazakii* strain closer to the non-*sakazakii* strains and the *Cr. malonaticus* strain was grouped with the *Cr. sakazakii* strains (Miled-Bennour *et al*., 2010).

The 15 non-*Cronobacter* stains used in this study were also evaluated with the PCR-RFLP assay using the restriction endonucleases *Csp*6I and *Hin*P1I. A clear distinction could be made between the *Cronobacter* strains and the related Enterobacteriaceae strains (Fig. 10). The negative control, *E. hormaechei* (strain 38), had a profile containing three fragments including a fragment bigger than 300 bp (Fig. 8). In total, 11 of the non-*Cronobacter* strains (36 – 40, 42, 44 – 48) exhibited this particular profile (Figs. 10 and 11). These strains included four *E. hormaechei* strains, five *Enterobacter* spp. strains, an *Acinetobacter* sp. and a *Pantoea ananatis* strain. The two remaining *Enterobacter* sp. strains (strains 41 and 43) had profiles similar to that of the *Pseudomonas fulva* strain (strain 49) (Fig. 11).

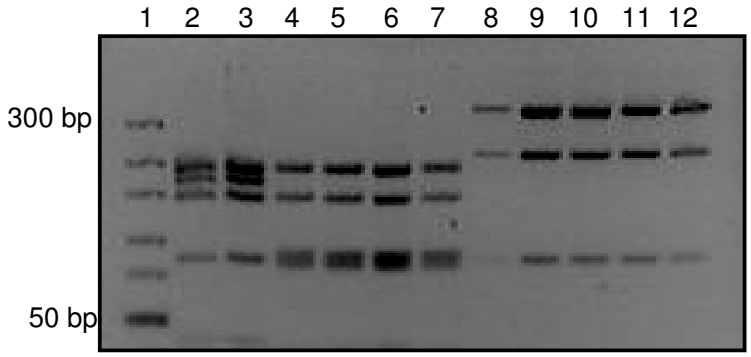


Figure 10 PCR-RFLP of the *rpo*B gene fragment of *Cronobacter* spp. digested with a combination of *Csp*6I and *Hin*P1I. Lane 1: Ultra low range ladder, 2: *Cr. malonaticus* (strain 6), 3: *Cr. malonaticus* (strain 10), 4: *Cr. sakazakii* (strain 9), 5: *Cr. sakazakii* (strain 8), 6: *Cr. sakazakii* (strain 11), 7: *Cr. sakazakii* (strain 32), 8: *E. hormaechei* (strain 37), 9: *E. hormaechei* (strain 36), 10: *E. hormaechei* (strain 39), 11 *Enterobacter* sp. (strain 40), 12: *Enterobacter* sp. (strain 45).

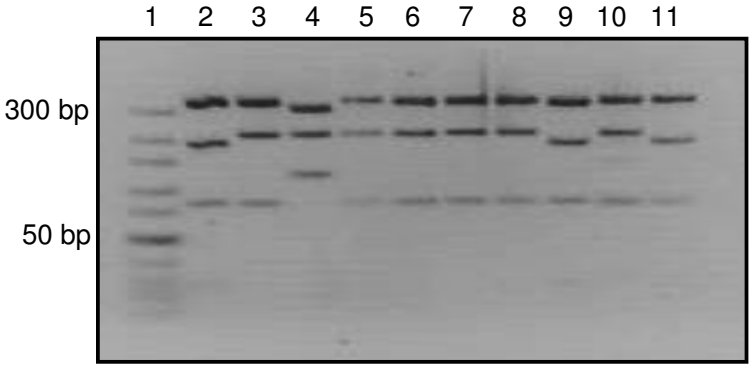


Figure 11 PCR-RFLP of the *rpo*B gene fragment of Enterobacteriaceae digested with a combination of *Csp*6I and *Hin*P1I. Lane 1: Ultra low range ladder, 2: *Pseudomonas fulva* (strain 49), 3: *Acinetobacter* sp. (strain 48), 4: *Enterobacter cloacae* (strain 34), 5: *Enterobacter hormaechei* (strain 38), 6: *Pantoea ananatis* (strain 47), 7: *Enterobacter* sp. (strain 46), 8: *Enterobacter* sp. (strain 44), 9: *Enterobacter* sp. (strain 43), 10: *Enterobacter* sp. (strain 42), 11: *Enterobacter* sp. (strain 41).

Lastly the E. cloacae strain had an unique profile with fragments of 300, 210 and 140 bp (Fig. 11). Although there were differences between the non-*Cronobacter* strains, there was not enough evidence to support any conclusions. This particular gene and endonuclease combination is not sufficient to distinguish between non-*Cronobacter* strains as some of these species had similar profiles.

Conclusions

The formation of risk management strategies is essential to produce food products that are safe for consumption. The recent reclassification of *Enterobacter sakazakii* to *Cronobacter* simplified the characterisation of these pathogens and, therefore, assisted in the formation of effective risk management strategies. The definition of the five species has, however, led to the challenge of designing rapid and reliable typing methods that can differentiate between these species. The ability to differentiate between the *Cronobacter* species is particularly important since there are indications that these species do not display the same virulence traits. This study described the development of a novel PCR-RFLP typing method for the *Cronobacter* species that can be used as a reliable alternative to sequencing based on 16S rRNA gene sequences. The PCR-RFLP assay is based on the *rpo*B gene and used in combination with restriction endonucleases *Csp*6I and *Hin*P1I. The results from this study indicated that the *rpo*B gene of the *Cronobacter* spp. is divergent enough to be used in typing methods. This assay can also be used to differentiate between *Cronobacter* and other Enterobacteriaceae strains, however, the PCR-RFLP assay was not optimised for species other than *Cronobacter*.

References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, Z., Miller, W. & Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acid Research*, **25**, 3389-3402.

Arroyo, C., Condon, S. & Pagan, R. (2009). Thermobacteriological characterization of *Enterobacter sakazakii*. *International Journal of Food Microbiology,* **136** (1), 110-118.

Druggan, P. & Iversen, C. (2009). Culture media for the isolation of *Cronobacter* spp. *International Journal of Food Microbiology,* **136** (2), 169-178.

El-Sharoud, W., O'Brien, S., Negredo, C., Iversen, C., Fanning, S. & Healy, B. (2009). Characterization of *Cronobacter* recovered from dried milk and related products. *BMC Microbiology,* **9**, 9.

Farmer, J.J., Asbury, M.A., Hickman, F.W., Brenner, D.J. & the Enterobacteriaceae Study Group (1980). *Enterobacter sakazakii*, new species of Enterobacteriaceae isolated from clinical specimens. *International Journal of Systematic Bacteriology*, **30**, 569-584.

Felske, A., Rheims, H., Wolterink, A., Stackebrandt, E. & Akkermans, A.D.L. (1997). Ribosome analysis reveals prominent activity of an uncultured member of the class *Acinetobacteria* in grassland soils. *Microbiology*, **143**, 2983-2989.

Food and Agriculture Organization / World Health Organization (FAO/WHO) (2004). *Enterobacter sakazakii* and other microorganisms in powdered infant formula: Meeting Report, MRA series 6. Geneva, Switzerland. [www document]. URL http://www.who.int/foodsafety/micro/meetings/feb2004/en/. 6 March 2009.

Food and Agriculture Organization / World Health Organization (FAO/WHO) (2008). *Enterobacter sakazakii* (*Cronobacter* spp.) in powdered follow-up formulae: Meeting Report, MRA series 15. Rome. [www document]. URL http://www.who.int/foodsafety/micro/meetings/2008/en/. 11 May 2009.

Healy, B., Huynh, S., Mullane, N., O'Brien, S., Iversen, C., Lehner, A., Stephan, R., Parker, C.T. & Fanning, S. (2009). Microarray-based comparative genomic indexing of the *Cronobacter* genus (*Enterobacter sakazakii*). *International Journal of Food Microbiology,* **136** (2), 159-164.

Iversen, C., Druggan, P. & Forsythe, S. (2004a). A selective differential medium for *Enterobacter sakazakii*, a preliminary study. *International Journal of Food Microbiology,* **96** (2), 133-139.

Iversen, C., Waddington, M., On, S.L.W. & Forsythe, S. (2004b). Identification and phylogeny of *Enterobacter sakazakii* relative to *Enterobacter* and *Citrobacter* species. *Journal of Clinical Microbiology,* **42** (11), 5368-5370.

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

Iversen, C., Lehner, A., Mullane, N., Bidlas, E., Cleenwerck, I., Marugg, J., Fanning, S., Stephan, R. & Joosten, H. (2007). The taxonomy of *Enterobacter sakazakii*: proposal of a new genus *Cronobacter* gen. nov. and descriptions of *Cronobacter*

sakazakii comb. nov. *Cronobacter sakazakii* subsp. *sakazakii*, comb. nov., *Cronobacter sakazakii* subsp. *malonaticus* subsp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov., *Cronobacter* genomospecies 1. *BMC Evolutionary Biology,* **64** (7), 1471-2148.

Iversen, C., Mullane, N., McCardell, B., Tall, B.D., Lehner, A., Fanning, S., Stephan, R. & Joosten, H. (2008). *Cronobacter* gen. nov., a new genus to accommodate the biogroups of *Enterobacter sakazakii*, and proposal of *Cronobacter sakazakii* gen. nov., comb. nov., *Cronobacter malonaticus* sp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov., *Cronobacter* genomospecies 1, and of three subspecies, *Cronobacter dublinensis* subsp. *dublinensis* subsp. nov., *Cronobacter dublinensis* subsp. *lausannensis* subsp. nov. and *Cronobacter dublinensis* subsp. *lactaridi* subsp. nov. *International Journal of Systematic and Evolutionary Microbiology,* **58** (6), 1442-1447.

Kucerova, E., Clifton, S.W., Xia, X., Long, F., Porwollik, S., Fulton, L., Fronick, C., Minx, P., Kyung, K.,Warren, W., Fulton, R., Feng, D., Wollam, A., Shah, N., Bhonagiri, V., Nash, W.E., Hallsworth-Pepin, K., Wilson, R.K., McClelland, M. & Forsythe, S.J. (2010). Genome sequence of *Cronobacter sakazakii* BAA-894 and comparative genomic hybridisation analysis with other *Cronobacter* species. *PLoS ONE*. **5** (3) e9556. Doi: 10.1371/ journal.pone0009556.

Kuhnert, P., Korczak, B.M., Stephan, R., Joosten, H. & Iversen, C. (2009). Phylogeny and prediction of genetic similarity of *Cronobacter* and related taxa by multilocus sequence analysis (MLSA). *International Journal of Food Microbiology,* **136** (2), 152-158.

Kuzina, L.V., Peloquin, J.J., Vacek, D.C. & Miller, T.A. (2001). Isolation and identification of bacteria associated with adult laboratory Mexican fruit flies, *Anastrepha ludens*. *Current Microbiology*, **42**, 290-294.

Lai, K.K. (2001). *Enterobacter sakazakii* infections among neonates, infants, children, and adults: case reports and a review of the literature. *Medicine Baltimore*, **80**, 113–122.

MacLean, L.L., Pagotto, F., Farber, J.M. & Perry, M.B. (2009). The structure of the O-antigen in the endotoxin of the emerging food pathogen *Cronobacter* (*Enterobacter*) *muytjensii* strain 3270. *Carbohydrate Research,* **344** (5), 667-671.

Miled-Bennour, R., Ells, C., Pagotto, F.J., Farber, J.M., Kerouanton, A., Meheut, T., Colin, P., Joosten, H., Leclercq, A. & Besse, N.G. (2010). Genotypic and phenotypic

characterisation of a collection of *Cronobacter* (*Enterobacter sakazakii*) isolates. *International Journal of Food Microbiol*ogy, **139,** 116–125.

Mollet, C., Drancourt, M. & Raoult, D. (1997). *rpoB* sequence analysis as a novel basis for bacterial identification. *Molecular Microbiology*. **25** (5), 1005-1011.

Nazarowec-White, M. & Farber, J.M. (1997). *Enterobacter sakazakii*: a review. *International Journal of Food Microbiology,* **34** (2), 103-113.

Pagotto, F.J., Nazarowec-White, M., Bidawid, S. & Farber, J.M. (2003). *Enterobacter sakazakii*: infectivity and enterotoxin production in vitro and in vivo. *Journal of Food Protection,* **66** (3), 370-375.

Stephan,R., Van Trappen, S., Cleenwerck, I., Vancanneyt, M., De Vos, P. & Lehner, A. (2007). *Enterobacter turicensis* sp. nov. and *Enterobacter helveticus* sp. nov., isolated from fruit powder. *International Journal of Systematic and Evolutionary Microbiology*, **57**, 820–826.

Stoop, B., Lehner, A., Iversen, C., Fanning, S. & Stephan, R. (2009). Development and evaluation of rpoB based PCR systems to differentiate the six proposed species within the genus *Cronobacter*. *International Journal of Food Microbiology,* **136** (2), 165-168.

Terragno, R., Salve, A., Pichel, M., Epszteyn, S., Brengi, S. & Binsztein, N. (2009). Characterization and subtyping of *Cronobacter* spp. from imported powdered infant formulae in Argentina. *International Journal of Food Microbiology,* **136** (2), 193-197.

Van Acker, J., Smet, F., Muyldermans, G., Bougatef, A., Naessens, A. & Lauwers, S. (2001). Outbreak of necrotizing enterocolitis associated with *Enterobacter sakazakii* in powdered milk formula. *Journal of Clinical Microbiology,* **39** (1), 293-297.

Van Elsas, J. D.; Mäntynen, V.; Wolters, A.C. (1997). Soil DNA extraction and assessment of the fate of *Mycobacterium chlorophenolicum* strain PCP-1 in different soil by 16S ribosomal RNA gene sequence based most-probable-number PCR and immunofluorescence. *Biol Fertil Soils*. **24**,188-195.

Wang, S. & Levin, R.E. (2006). Discrimination of viable *Vibrio vulnificus* cells from dead cells in real-time PCR. *Journal of Microbiological Methods*, **64**, 1-8.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

The genus *Cronobacter* (previously known as *Enterobacter sakazakii*) can cause foodborne infections such as neonatal meningitis, septicaemia and necrotising enterocolitis (Nazarowec-White & Farber, 1997; Van Acker *et al*., 2001). These pathogens have been associated with disease outbreaks, as well as infections and are a health concern for immuno-compromised individuals, especially premature infants (FAO/WHO, 2004). The number of *Cronobacter* related infections in South Africa (SA) is unknown although these pathogens have been isolated from environmental sources and food produced in SA. Given the risk posed by these pathogens a comprehensive understanding of *Cronobacter* is necessary.

This study describes the evaluation of 24 isolates previously identified as *E. sakazakii.* The isolates originated from IFM processing facilities and final products, as well as fresh produce from SA (Cawthorn *et al*., 2008; Mofokeng *et al*., 2010). All the strains isolated from SA were reclassified as *Cr. sakazakii* despite the wide variety of isolation sources. Since *Cr. sakazakii* is always the dominant species in terms of isolation frequency (Iversen *et al*., 2007; 2008; Baldwin *et al*., 2009; Kuhnert *et al*., 2009) the absence of the other *Cronobacter* species can be attributed to the small number of isolates used in this study. It should, however, not be concluded that the other *Cronobacter* species do not occur in SA as they could be identified when more isolates are evaluated. Furthermore, the phylogram based on the 16S rRNA gene showed that the *Cr. sakazakii* strains were divided into two significantly different clusters, separated by a 93 % bootstrap value.

The phylogram based on the combined sequences of the *rpo*A and 16S rRNA genes showed distinct clusters for *Cr. dublinensis, Cr. muytjensii, Cr. turicensis* and *Cr. sakazakii*. *Cronobacter malonaticus* was not phylogenetically distinct from *Cr. sakazakii* since the type strain, *Cr. malonaticus* DSM 18702^{T} grouped in a cluster with *Cr. sakazakii* strains. The phylogenetic analysis in this study showed that these two species are very closely related and it is, therefore, not advisable to use the *rpo*A and 16S rRNA genes as a basis for differentiation between these two *Cronobacter* species. If these two genes are used in

future studies an additional test such as malonate utilisation should be included to distinguish between *Cr. sakazakii* and *Cr. malonaticus*.

The reclassification of the five *Cronobacter* species has led to the challenge of designing rapid and reliable typing methods that can differentiate between these species. The ability to differentiate between the *Cronobacter* species is particularly important since there are indications that these species do not display the same virulence traits. Recently, Kucerova *et al*. (2010) reported that only *Cr. sakazakii*, *Cr. malonaticus* and *Cr. turicensis* have been associated with neonatal infections. Since it is still unclear whether all of the species are virulent, the whole genus is currently classified as pathogenic (FAO/WHO, 2008). The few typing methods available for *Cronobacter* species such as sequencing based on the 16S rRNA gene and biochemical analysis are either time consuming or inaccurate and other molecular typing methods should be evaluated for the accurate differentiation between the species of *Cronobacter*.

This study describes the development of a novel PCR-RFLP assay based on the *rpo*B gene that facilitates differentiation between the five *Cronobacter* species. Single digestions with the restriction endonucleases, *Mbo*I, *Csp*6I and *Hin*P1I did not simultaneously differentiate between all the *Cronobacter* species and, therefore, the PCR-RFLP assay was combined with a multiple restriction digestion that included *Csp*6I and *Hin*P1I. With this optimised PCR-RFLP assay it was possible to differentiate between *Cronobacter* strains and other related Enterobacteriaceae. Additionally, two strains isolated from SA that were reclassified as *Cr. sakazakii* based on sequence data of the *rpo*A and 16S rRNA genes, were identified as *Cr. malonaticus* with the PCR-RFLP assay. Sequence data based on the *rpo*B gene of these two strains and a biochemical test of malonate utilisation confirmed the PCR-RFLP results. The newly developed PCR-RFLP assay may, therefore, serve as an accurate and rapid typing method for the genus *Cronobacter*.

Recommendations

Currently the most significant challenge regarding the *Cronobacter* genus is the determination of the virulence status of the five species. This study addresses the problem of differentiation between the species by evaluating *Cronobacter* strains isolated from SA. The data from this study showed that the *rpo*A and 16S rRNA genes were highly conserved and did not provide enough genetic variation to simultaneously differentiate

between the *Cronobacter* species. It is, therefore, advisable to use multiple genes when evaluating the phylogeny of this genus. Future phylogenetic studies should focus on the genetic variation of virulence genes such as *omp*A, *cus*A, *cus*B, *cus*C, *cus*F, *cus*R, *ibe*A and *yij*P. These genes are all associated with the invasion of brain microvascular endothelial cells and should aid in the understanding of the virulence characteristics of the *Cronobacter* genus (Kucerova *et al*., 2010). Furthermore, evaluation of clinical *Cronobacter* isolates from SA should also shed light on the virulence of these pathogens.

The inability to differentiate between all five *Cronobacter* species based on the *rpo*A and 16S rRNA genes led to the development of a PCR-RFLP typing method that can simultaneously differentiate between all the *Cronobacter* species. This PCR-RFLP method can be used in combination with a chromogenic agar such as Chromocult® to identify *Cronobacter* isolates. The outcome of this study increases the understanding of the phylogeny of these pathogens and aid in the development of risk management strategies for *Cronobacter* contamination.

References

Baldwin, A., Loughlin, M., Caubilla-Barron, J., Kucerova, E., Manning, G., Dowson, C. & Forsythe, S. (2009). Multilocus sequence typing of *Cronobacter sakazakii* and *Cronobacter malonaticus* reveals stable clonal structures with clinical significance which do not correlate with biotypes. *BMC Microbiology,* **9**, 223.

Cawthorn, D.M., Botha, S. & Witthuhn, R.C. (2008). Evaluation of different methods for the detection and identification of *Enterobacter sakazakii* isolated from South African infant formula milks and the processing environment. *International Journal of Food Microbiology,* **127** (1-2), 129-138.

Food and Agriculture Organization / World Health Organization (FAO/WHO) (2004). *Enterobacter sakazakii* and other microorganisms in powdered infant formula: Meeting Report, MRA series 6. Geneva, Switzerland. [www document]. URL http://www.who.int/foodsafety/micro/meetings/feb2004/en/. 6 March 2009.

Food and Agriculture Organization / World Health Organization (FAO/WHO) (2008). *Enterobacter sakazakii* (*Cronobacter* spp.) in powdered follow-up formulae: Meeting Report, MRA series 15. Rome. [www document]. URL http://www.who.int/foodsafety/micro/meetings/2008/en/. 11 May 2009.

Iversen, C., Lehner, A., Mullane, N., Bidlas, E., Cleenwerck, I., Marugg, J., Fanning, S., Stephan, R. & Joosten, H. (2007). The taxonomy of *Enterobacter sakazakii*: proposal of a new genus *Cronobacter* gen. nov. and descriptions of *Cronobacter sakazakii* comb. nov. *Cronobacter sakazakii* subsp. *sakazakii*, comb. nov., *Cronobacter sakazakii* subsp. *malonaticus* subsp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov., *Cronobacter* genomospecies 1. *BMC Evolutionary Biology,* **64** (7), 1471-2148.

Iversen, C., Mullane, N., McCardell, B., Tall, B.D., Lehner, A., Fanning, S., Stephan, R. & Joosten, H. (2008). *Cronobacter* gen. nov., a new genus to accommodate the biogroups of *Enterobacter sakazakii*, and proposal of *Cronobacter sakazakii* gen. nov., comb. nov., *Cronobacter malonaticus* sp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov., *Cronobacter* genomospecies 1, and of three subspecies, *Cronobacter dublinensis* subsp. *dublinensis* subsp. nov., *Cronobacter dublinensis* subsp. *lausannensis* subsp. nov. and *Cronobacter dublinensis* subsp. *lactardi* subsp. nov.. *International Journal of Systematic and Evolutionary Microbiology,* **58** (6), 1442-1447.

Kuhnert, P., Korczak, B.M., Stephan, R., Joosten, H. & Iversen, C. (2009). Phylogeny and prediction of genetic similarity of *Cronobacter* and related taxa by multilocus sequence analysis (MLSA). *International Journal of Food Microbiology,* **136** (2), 152-158.

Kucerova, E., Clifton, S.W., Xia, X., Long, F., Porwollik, S., Fulton, L., Fronick, C., Minx, P., Kyung, K.,Warren, W., Fulton, R., Feng, D., Wollam, A., Shah, N., Bhonagiri, V., Nash, W.E., Hallsworth-Pepin, K., Wilson, R.K., McClelland, M. & Forsythe, S.J. (2010). Genome sequence of *Cronobacter sakazakii* BAA-894 and comparative genomic hybridisation analysis with other *Cronobacter* species. *PLoS ONE*. **5** (3) e9556. Doi: 10.1371/ journal.pone0009556.

Mofokeng, L., Cawthorn, D. M., Witthuhn, C.R., Anelich, L. E. M. & Jooste, P. J. (2010). Characterization of *Cronobacter* species (*Enterobacter Sakazakii*) isolated from various South African food sources. *Journal of Food Safety*, 272, 1745-4565.

Nazarowec-White, M. & Farber, J.M. (1997). *Enterobacter sakazakii*: a review. *International Journal of Food Microbiology,* **34** (2), 103-113.

Van Acker, J., de Smet, F., Muyldermans, G., Bougatef, A., Naessens, A. & Lauwers, S.

(2001). Outbreak of necrotizing enterocolitis associated with *Enterobacter sakazakii* in powdered milk formula. *Journal of Clinical Microbiology*, **39**, 293-297.

Dennison & Morris 2002