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REVIEW ARTICLE

Cronobacter species (formerly known as *Enterobacter sakazakii*) in powdered infant formula: a review of our current understanding of the biology of this bacterium

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Summary

Cronobacter species (formerly known as *Enterobacter sakazakii)* are opportunistic pathogens that can cause necrotizing enterocolitis, bacteraemia and meningitis, predominantly in neonates. Infection in these vulnerable infants has been linked to the consumption of contaminated powdered infant formula (PIF). Considerable research has been undertaken on this organism in the past number of years which has enhanced our understanding of this neonatal pathogen leading to improvements in its control within the PIF production environment. The taxonomy of the organism resulted in the recognition of a new genus, *Cronobacter, which* consists of seven species. This paper presents an up-to-date review of our current knowledge of *Cronobacter* species. Taxonomy, genome sequencing, current detection protocols and epidemiology are all discussed. In addition, consideration is given to the control of this organism in the manufacturing environment, as a first step towards reducing the occurrence of this pathogen in PIF.

Introduction

Cronobacter species (formerly known as *Enterobacter sakazakii*) are Gram-negative rod-shaped, motile pathogenic bacteria of the family Enterobacteriaceae. These organisms are regarded as opportunistic pathogens linked with life-threatening infections predominantly in neonates (infants <4 weeks of age) (Bar-Oz *et al.* 2001; Gurtler *et al.* 2005, Anonymous 2006a,b; Mullane *et al.* 2007a). Clinical syndromes of *Cronobacter* infection include necrotizing enterocolitis (NEC), bacteraemia and meningitis, with case fatality rates ranging between 40 and 80% being reported (Bowen and Braden 2006; Friedemann 2009). Infections in older infants and among immuno-compromised adults, mainly the elderly, have also been noted (Bowen and Braden 2006; Gosney *et al.* 2006; See *et al.* 2007). The bacterium has been isolated from a range of food sources including dairy-based foods, dried meats, water, rice and others (Baumgartner *et al.* 2009; Chap *et al.* 2009; Healy *et al.* 2010). Surveillance studies detected *Cronobacter* in a variety of different environments including households, livestock facilities, food manufacturing operations, in particular PIF production facilities (Bar-Oz *et al.* 2001; Kandhai *et al.* 2004; Mullane *et al.* 2007b; Kilonzo-Nthenge *et al.* 2008). Contaminated powdered infant formula (PIF) has been epidemiologically linked with many of the infections reported (Bowen and Braden 2006). Controlling the microbiological load in infant food products and understanding the optimal growth conditions and epidemiology would contribute positively towards a reduction in the health risk to vulnerable individuals.

Classification of *Cronobacter*

Cronobacter species were originally referred to as yellow-pigmented *Enterobacter cloacae*, later being reclassified as a new species, *E. sakazakii* in 1980 (Farmer *et al.* 1980).

Using partial 16S ribosomal DNA (rDNA) and *hsp60* sequencing, Iversen *et al.* (2004a) divided 126 *Cronobacter* isolates into four clusters, suggesting that the genus may require re-classification. Later and following further extensive polyphasic analysis, Iversen *et al.* (2007a, 2008) proposed the reclassification of these bacteria into a new genus called *Cronobacter*. Originally, six species (*C. sakazakii, C. malonaticus, C. turicensis, C. muytjensii, C. dublinensis* and *C. genomospecies* 1) were defined and comprised of the 16 biogroups described in Table 1. A new species (*C. condimenti*) was identified recently by Joseph *et al.* (2011), and in addition, *C. universalis* now replaces the original *C. genomospecies* 1.

This re-classification by Iversen *et al.* (2007a, 2008) was subsequently supported by both optical mapping and genome sequencing data which confirmed the revision of the taxonomy (Kotewicz and Tall 2009; Kucerova *et al.* 2010). Although *C. sakazakii* and *C. malonaticus* were found to be closely related and difficult to distinguish by 16S rDNA sequence analysis, a seven loci (*atpD, fusA, glnS, gltB, gyrB, infB, ppsA*) multilocus sequence typing (MLST) scheme was developed to discriminate between these two species (Baldwin *et al.* 2009). Furthermore, recent findings reported by Joseph and Forsythe (2011) identified a highly stable sequence type (denoted as ST4) within *Cronobacter sakazakii* and which was responsible for a large proportion of severe neonatal infections, especially neonatal meningitis.

Two of the species genomes were subsequently published (as discussed below), and currently, a collaborative effort is underway to complete the genome sequences of a further 15 isolates of six of the seven *Cronobacter* species adding substantially to our knowledge of the core genome of this group of bacteria and highlighting particular species-specific features of interest.

Table 1 Distribution of biogroups among the genus *Cronobacter*

Cronobacter species	Biogroups
Cronobacter sakazakii sp. nov.	1, 2–4, 7, 8, 11, & 13
Cronobacter malonaticus sp. nov.	5, 9, &14
Cronobacter turicensis sp. nov.	16
Cronobacter muytjensii sp. nov.	15
Cronobacter condimenti sp. nov.	1
Cronobacter universalis sp. nov.	Separate genomospecies
Cronobacter dublinensis sp. nov.	
Cronobacter dublinensis subsp. *dublinensis* subsp. nov.	12
Cronobacter dublinensis subsp. *lausannensis* subsp. nov.	10
Cronobacter dublinensis subsp. *lactaridi* subsp. nov.	6

Another method of classification used is O-antigen typing and studies describing the nature of the O-antigen associated with *Cronobacter* species have been reported (Mullane *et al.* 2008a; Jarvis *et al.* 2011). The O-antigen is a component of the lipopolysaccharide (LPS) structure located on the outer surface of Gram-negative bacteria and is responsible for serological diversity. Mullane *et al.* (2008a) initially developed a molecular serotyping method, based on long-range amplification of the *rfb*-encoding locus (in Gram-negative enteric bacteria located between *galF-gnd*) followed by *MboII* digestion (Fig. 1). Using this approach, a PCR-RFLP profile was generated which can be compared across several isolates. Based on this approach, the first two O-serotypes were characterized and denoted as O:1 and O:2. More recently, another five additional O-antigens were serologically identified by Sun *et al.* (2011) and these correlated with the previously reported PCR-RFLP profiles. Jarvis *et al.* (2011) extended the original molecular-characterization scheme to include other *Cronobacter* species and defined new molecular O-serotype gene clusters. Two of these O-serotype gene clusters were shared among *C. sakazakii* and *C. muytjensii*, as well as *C. malonaticus* and *C. turicensis* strains (Tall, unpublished data). The structural composition of several O-serotypes has now been described (MacLean *et al.*

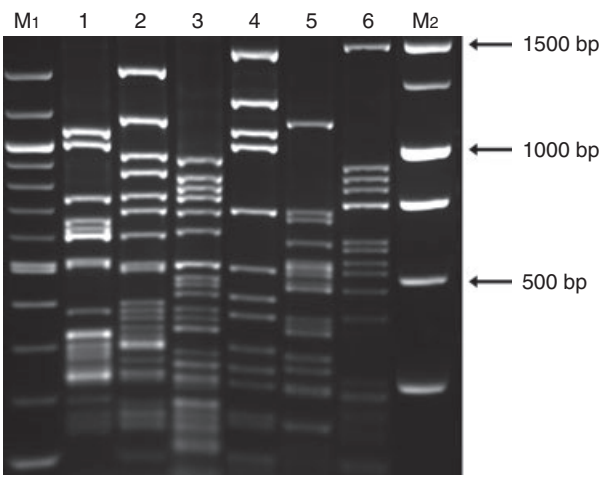

Figure 1 Restriction fragment length profiles of amplified *rfb*-encoding loci of *Cronobacter* following *MboII* digestion. A selection of some of the serotypes previously classified. Lane 1, ATCC®BAA 894, *Cronobacter sakazakii* O1; Lane 2, E830, *C. sakazakii* O2; Lane 3, E615, *Cronobacter malonaticus* O1; Lane 4, E618, *C. malonaticus* O2; Lane 5, E464, *Cronobacter dublinensis* O6; Lane 6, E797, *Cronobacter genomospecies* 1 O9; Lane M_1, 100 bp DNA ladder (New England Biolabs, Hertfordshire, England) and Lane M_2, 1 kb DNA ladder (New England Biolabs).

2009; Czerwicka *et al.* 2010; Maclean *et al.* 2011; Arbatsky *et al.* 2010, 2011; Shashkov *et al.* 2011).

Genomes of the genus *Cronobacter*

The first sequenced *Cronobacter* genome, *C. sakazakii* ATCC®BAA-894 was published by Kucerova *et al.* (2010). It revealed a single chromosome of 4·4 Mb (57% GC) along with two plasmids, denoted as pESA2 and pESA3 (31-kb, 51% GC and 131-kb, 56% GC, respectively). The source of this isolate was reported to be contaminated PIF used in a neonatal intensive care unit and which gave rise to an outbreak in 2001 in Tennessee, USA. Using comparative genomic hybridization (CGH) techniques, representative isolates including *C. malonaticus*, *C. turicensis*, *C. muytjensii and C. dublinensis* were further investigated, and those genes considered to be part of the core species genome along with other markers unique to *C. sakazakii* were identified (Kucerova *et al.* 2010).

Cronobacter sakazakii ATCC®BAA-894 contained approximately 4392 genes as part of its core genome. However, using the CGH approach, 4382 unique, annotated genes from both the chromosome and plasmids were noted, and only 54·9% of genes were common to all *C. sakazakii* with 43·3% being common across all *Cronobacter* species. Interestingly, 21 genes were found to be unique in five of the *C. sakazakii* tested, and these encoded proteins were involved in pilus assembly, a phosphotransferase system (PTS), an acid transporter, *N*-acetylneuraminate lyase and a toxin/antitoxin system.

The genome of *C. turicensis* z3032 was published in early 2011 (Stephan *et al.* 2011) in an attempt to further determine virulence factors and mechanisms of pathogenicity in this bacterium. Following the deaths of two newborn infants in 2005, the latter isolate was cultured from the blood of one child with meningitis (Mange *et al.* 2006). In this sequenced isolate, the genome was 4·4 Mb (57% GC) in size and contained three plasmids of sizes approx 138-kb pCTU1 (56%), 22-kb pCTU2 (49%) and 54-kb pCTU3 (50% GC). Two hundred and twenty-three genes were annotated as virulence- and disease-related encoding open reading frames (ORFs); however, 9·27% (413 of 4455) encoded proteins were of unknown function. Because these latter ORFs lacked similarity to sequences already in the current databases, these could potentially have important pathogenic functions.

Franco *et al.* (2011a) reported the *in silico* analysis of two plasmids, one from *C. sakazakii* ATCC®BAA-894, pESA3 and the other from *Cronobacter turicensis* z3032, pCTU1, each of which contained a RepFIB replicon. Both plasmids possessed two iron acquisition systems (*eitCBAD* and *iucABCD/iutA*) essential for survival and successful pathogenesis. Ninety-seven per cent of 229 strains, representing seven of the eight *Cronobacter* species, possessed a RepFIB plasmid. The presence of a *Cronobacter* plasminogen activator-encoding gene (*cpa*) [encoded on pESA3], a type 6 secretion system (T6SS) [also encoded on pESA3] and a filamentous haemagglutinin/adhesin (FHA) gene locus (located on pCTU1) suggested the existence of unique virulence determinants in these species. The *cpa*-encoding gene encodes an outer membrane protease implicated in serum resistance, a feature that would facilitate *Cronobacter* species in crossing the blood–brain barrier and causing meningitis (Franco *et al.* 2011b). The T6SS acts to translocate putative effector proteins aiding in bacterial pathogenesis, while FHA pCTU1 contains *fhaB*, *fhaC* genes (which encode proteins with similarly identity to a transported and transporter protein as part of a two-partner secretion system) and five associate putative adhesins. Further studies are now in progress to extend our understanding of the functional roles of these plasmid-encoded loci.

Virulence characteristics of *Cronobacter*

Information from epidemiological studies along with *in vitro* mammalian tissue culture assays has shown that *Cronobacter* isolates demonstrate a variable virulence phenotype (Caubilla-Barron *et al.* 2007; Townsend *et al.* 2007, 2008). Only isolates of *C. sakazakii*, *C. malonaticus* and *C. turicensis* have been linked with neonatal infections (Healy *et al.* 2010; Kucerova *et al.* 2010).

Currently, few clues as to the mechanisms involved are known, though, data from genome sequencing efforts highlighted several potential markers that may be helpful candidates for future studies (Kucerova *et al.* 2010; Stephan *et al.* 2011).

The first putative *Cronobacter* virulence factors to be described were enterotoxin-like compounds produced by four of 18 isolates studied (Pagotto *et al.* 2003). Using conventional tissue culture-based assays, *Cronobacter* is known to attach to intestinal cell lines *in vitro* and survive within macrophages for periods of time (Townsend *et al.* 2008). Franco *et al.* (2011b) recently demonstrated resistance to complement-mediated killing of *C. sakazakii,* and this was associated with the presence of *cpa* which is contained on the pESA3 plasmid.

The outer membrane protein A, encoded by the *ompA* gene, is probably the best characterized virulence marker. This was originally reported by Nair and Venkitanarayanan (2006) and shown to be required for binding of the bacterium to human brain microvascular endothelial cells (BMEC) (Nair *et al.* 2009). More recently, Kim and Loessner (2008) reported that the disruption of tight junctions significantly enhanced adherence of *C. sakazakii* to Caco2 cells in culture and that the same marker was

required for basolateral invasion (Kim *et al.* 2010a,b). The *ompA*-encoding gene is thought to be present in all *Cronobacter* strains tested, and this marker has also been linked with invasive *Escherichia coli*, which causes neonatal meningitis (Prasadarao *et al.* 1996; Kim 2000).

At the epithelial cell surface, *C. sakazakii* infection results in damage of these cells, following the recruitment of greater numbers of dentritic cells, compared with macrophages and neutrophils (Emami *et al.* 2011). Using a NEC mouse model, these effects were shown to be mediated through OmpA and involved inducible NO synthase (iNOS).

From an analysis of the annotated genes in *C. sakazakii* ATCC®BAA-894, Kucerova *et al.* (2010) highlighted several markers including, *ibeA, ibeB, yijP* and *ompA*, which were previously identified in other organisms associated with invasion of BMEC (Prasadarao *et al.* 1996; Huang *et al.* 1999, 2001; Wang *et al.* 1999). Interestingly, *ibeB* (a gene synonymous with *cusC*), which belongs to a cluster of genes encoding a copper and silver resistance cation efflux system, facilitates the invasion of BMEC cells (Franke *et al.* 2003); although this gene was found in the reference strain *C. sakazakii* ATCC®BAA-894, the genes *ibeA* and *yijP* produced no matches. When assessed, it was found that the complete cation efflux operon (*cusA*, *cusB* and *cusC*) and its regulatory gene *cusR* were present in isolates associated with neonatal infections (including *C. sakazakii*, *C. turicensis* and *C. malonaticus*) and absent in the other tested strains (Kucerova *et al.* 2010).

Detection protocols

Conventional bacteriological culture

The first detection method developed for *Cronobacter* species was described by Muytjens *et al.* (1988). Based on this protocol, the US Food and Drug Administration (US FDA) recommended a method to isolate and enumerate *E. sakazakii* from powdered infant formula in 2002. In 2006, the International Organization for Standardization (ISO 2009) and the International Dairy Federation developed a technical standard protocol for the detection of *Cronobacter* species from milk-based powdered formula known as ISO/TS 22964 (Anonymous 2006a,b) (described in Table 2). More recently, the US–FDA method was revised to combine both a PCR assay and two newly developed chromogenic agars for detection (Chen *et al.* 2009; Chen 2011).

Pre-enrichment of the PIF samples to be tested is a requirement in these three protocols, and the time duration varies from a maximum overnight period (ranging from 18 to 24 h) to a minimum time period of 6 h, followed by selective enrichment and subsequent isolation using selective agars/media. Typical colonies are confirmed using a selective agar and/or a suitable real-time PCR assay, with the final identification based on either biochemical/molecular characterization. In the revised US–FDA protocol, there is one enrichment step which is then followed by a molecular method used for quick confirmation. This approach eliminates two days from

Table 2 Detection protocols for *Cronobacter* in PIF

Procedure	FDA (Original)	ISO/TS 22964	FDA (revised)
Pre-enrichment	Make 1 : 10 (w/v) of sample in distilled water, incubated overnight at 36°C	Make 1 : 10 (w/v) of sample in BPW, incubated at 37°C for 18 ± 2 h	Make 1 : 10 (w/v) of sample in BPW, incubated at 36°C for 6 h
Selective enrichment	Transfer 10 ml pre-enrichment to 90 ml EE broth, incubated overnight at 36°C	Transfer 100 μl pre-enrichment to 10 ml mLST/vancomycin medium, incubated at 44°C for 24 ± 2 h	
Selection/ isolation	Make an isolation streak and spread plate from each EE broth onto VRBG agar, incubated overnight at 36°C	Streak from the cultured mLST/ vancomycin medium one loopful on the chromogenic agar in Petri dishes, incubated at 44°C for 24 ± 2 h	Centrifuge 40 ml samples, 3000 ***g***, 10 min and resuspend pellet in 200 μl PBS; Spread 100 μl onto chromogenic media, incubated overnight at 36°C
Confirmation	Pick five presumptive positive colonies and streak onto TSA, incubated overnight at 25°C	Select five typical colonies and streak on TSA agar, incubated at 25°C for 48 ± 4 h	Pick two typical colonies from each chromogenic media confirmed with real-time PCR, API 20E, Rapid ID 32 E
Identification	Yellow colonies are confirmed with the API 20E test kit	Select one yellow colony from each TSA plate for biochemical characterization	
Detection time (days)	5	6	3

the detection procedure compared with the original protocol.

Selective media for *Cronobacter*, including Leuschner–Bew agar (Leuscher and Bew 2004), Druggan-Fosythe-Iversen agar (Iversen *et al.* 2004b), Oh-Kang agar (Oh and Kang 2004), ESPM agar (Restaino *et al.* 2006) and HiCrome™ *Cronobacter* spp. agar (Sigma-Aldrich, Switzerland), have been developed, based on the α-glucosidase enzyme marker (Iversen *et al.* 2004b) identified by Muytjens *et al.* (1984) and β-cellobiosidase activity (Restaino *et al.* 2006) which is present in all *Cronobacter* strains. Moreover, violet red bile agar (VRBA), MacConkey agar and desoxycholate agar, which are selective for Gram-negative bacteria, are also available for the isolation of *Cronobacter* (Druggan and Iversen 2009; Forsythe 2009) from foods.

However, despite the availability of selective agar media, some were shown to insufficiently support the growth of all *Cronobacter* strains (Iversen and Forsythe 2007) and other related species, such as *Enterobacter helveticus*, *Enterobacter pulveris* and *Enterobacter turicensis*, which are often found in the same ecological niches. Therefore, improvements in the design of the selective media for the isolation and identification of *Cronobacter* were required.

O'Brien *et al.* (2009) described the design of a one-step pre-enrichment and enrichment protocol using a chromogenic medium. In this detection strategy, the specific broth developed [denoted as *Cronobacter* enrichment broth (CEB)] facilitated a shortened two-day culture method for the detection of *Cronobacter* species in PIF. Mullane *et al.* (2006) utilized a cationic-magnetic-bead capture technique to improve the sensitivity of detection for *Cronobacter* from PIF.

The accuracy and reliability of commercially available identification kits for *Cronobacter* have been questioned, with reports of false-negative and false-positive identification (Restaino *et al.* 2006; Iversen and Forsythe 2007). However, Gen III is currently the only commercially available identification kit with the original six species included (Healy 2010).

Immuno-based detection protocols

Immuno-based assays are convenient for detection methods that can be applied to detect specific bacteria. Assays using monoclonal antibodies are widely used in research as rapid detection tools. These approaches can improve the sensitivity and specificity for detection. Commercial kits based on enzyme-linked immunosorbent assay (ELISA) technology have been developed, and the VITEK® immunodiagnostic assay system (denoted as VIDAS®, bioMerieux Vitek Inc., Hazelwood, MO, USA) has been used as a rapid detection platform for *Salmonella*, *E. coli* O157:H7, *Listeria* species, *Campylobacter jejuni* and *Staphylococcus* species enterotoxins. The VIDAS® *Salmonella* method has been validated and certified by the Association of Official Analytical Chemists (AOAC) as an approved method of analysis in foods. This protocol was also approved by other regulatory organisations including Health Canada, the European Microbiological Method Assessment Scheme (EMMAS) and German Normalization Institute (DIN). Research on a VIDAS®-based *Cronobacter* method is currently in progress; early results suggest that the method is fast, reliable and sensitive for the detection of *Cronobacter* in a range of test matrices (Q.Q. Yan, unpublished data).

Molecular-based detection protocols

Molecular detection techniques have always been regarded as useful tools to extend our understanding of the epidemiology of an organism. Usually, these assays are designed to target unique genes present in the pathogen of interest. Many of the more recent assay formats are based on real-time PCR and several have been designed for the specific detection of *Cronobacter* (Malorny and Wagner 2005; Seo and Brackett 2005; Drudy *et al.* 2006;

Table 3 Gene targets useful for the detection of *Cronobacter* and related species

	Gene targets	Reference
Genus loci		
ribosomal DNA (rDNA)	16S rRNA	Iversen *et al.* (2004a)
	23S rRNA	Derzelle *et al.* (2007)
	$tRNA_{Glu}$	Hassan *et al.* (2007)
	FISH	Iversen *et al.* (2007a)
		Almeida *et al.* (2009)
1,6 α-glucosidase	*gluA*	Iversen *et al.* (2007b)
MMS operon	*dnaG*	Seo and Brackett (2005)
		Drudy *et al.* (2006)
Zinc-containing metalloprotease	*zpx*	Jaradat *et al.* (2009)
Outer membrane protein A	*ompA*	Nair and Venkitanarayanan (2006)
Species loci		
rfb (O-antigen)	*wehC* [CsakO:1] & *wehI* [CsakO:2]	Mullane *et al.* (2008a)
	wzx [CsakO:3; CturO:1;Cmuy O:1; and Cmal O:1 and O:2]	Jarvis *et al.* 2011)
β-subunit of RNA polymerase	*rpoB*	Stoop *et al.* (2009)
Other gene targets		
RNaseP		
infB (initiation factor)		

Nair and Venkitanarayanan 2006; Kothary *et al.* 2007; Zhou *et al.* 2008). Targets used (Table 3) include the 16S rRNA gene, 16S -23S rDNA intergenic region, the *dnaG* gene, the *ompA* gene, the 1,6 α-glucosidase-encoding gene (*gluA*) and a zinc-containing methalloprotease (*zpx*). More recently, a PCR-based method developed by Stoop *et al.* (2009) was extended by A. Lehner, C. Fricker-Feer and R. Stephan (unpublished data) to include the new species described. The latter protocol facilitated the detection of all seven known species within the *Cronobacter* genus using a mismatch-PCR-based approach. The applications of these molecular-based protocols can support the traditional culture-based approaches (Fig. 2). Interestingly, using the latter protocol, *C. malonaticus* and *C. sakazakii* could not be differentiated using this approach, and therefore, a second PCR was required to accurately identify each of these species. In 2011, Yan *et al.* (2011) published a PCR- and array-based biomarker verification study for the detection and identification of *Cronobacter* spp. Here, these authors propose to elucidate virulence markers which may be helpful as biomarkers for differentiating *Cronobacter* spp. and *Salmonella* spp. from other food-borne pathogens. While these putative markers were identified, further validation experiments are currently being conducted.

Molecular subtyping has long been regarded as a useful approach that can be applied to elucidate the nature of those bacteria colonizing a particular ecological niche. Mullane *et al.* (2007b) applied pulsed-field gel electrophoresis (PFGE) to characterize and track *Cronobacter* species in a PIF processing facility (Fig. 3). The study provided a basis for the development of efficient intervention measures contributing to the reduction of *Cronobacter* in the PIF manufacturing environment. A similar approach using the second generation subtyping method, multiple-locus variable-number tandem-repeat analysis (MLVA), was subsequently applied to subtype a collection of geno- and phenotypically diverse *Cronobacter* isolates (Mullane

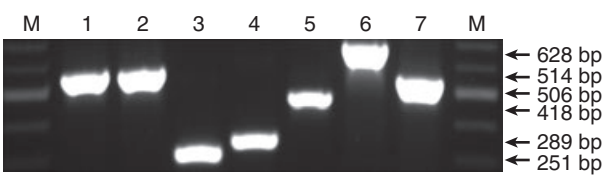

Figure 2 A 1·0% agarose gel showing the amplification of *rpoB* amplicons used to identify six of the genus species (Stoop *et al.* 2009). Lane 1, *Cronobacter sakazakii* SP291; Lane 2, *Cronobacter malonaticus* E766; Lane 3, *C. malonaticus* E766 (following a confirmatory second PCR); Lane 4, *Cronobacter muytjensii* ATCC®51329; Lane 5, *Cronobacter dublinensis* CFS237; Lane 6, *Cronobacter turicensis* E626; Lane 7, *Cronobacter* genomospecies 1 E797 and Lane M, 100 bp DNA ladder.

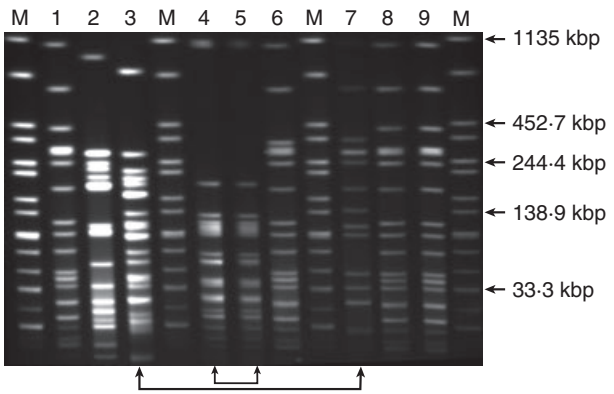

Figure 3 Pulsed-field gel electrophoresis (PFGE) profiles used to characterize and track *Cronobacter* species in a PIF processing facility. Lane 1, factory sample 1; Lane 2, factory sample 2; Lane 3, factory sample 3; Lane 4, factory sample 4; Lane 5, factory sample 5; Lane 6, factory sample 6; Lane 7, factory sample 7; Lane 8, factory sample 8; Lane 9, factory sample 9 and Lane M, *Salmonella* Braenderup, molecular marker, genomic DNA digested with *XbaI*. The arrow heads at the foot of the image show that in lanes 3 and 7, these isolates have the same PFGE profile and would be considered to be indistinguishable. Similarly, isolates contained in lanes 4 and 5 have another indistinguishable profile cluster.

et al. 2008b). However, a standardized PFGE protocol is close to completion and has already been validated by PulseNet, a network of national and regional laboratory networks dedicated to tracking food-borne infections.

A significant reduction in both time and cost associated with genome sequencing has made molecular detection methods increasingly accessable. Multilocus sequence analysis (MLSA) based on *recN*, *rpoA* and *thdF* genes was used in describing the genomic similarity of *Cronobacter* genes by Kuhnert *et al.* (2009). El-Sharoud *et al.* (2009) applied *recN* gene sequence analysis to isolate *Cronobacter* strains recovered from dried milk and related products. A similar MLST typing scheme using the following seven housekeeping genes*: atpD, fusA, glnS, gltB, gyrB, infB, ppsA* (3036 nt concatenated length) has been developed by Baldwin *et al.* (2009), and a database containing defined sequence types covering all *Cronobacter* spp. is currently being maintained at Oxford University. The database and MLST analytics can be accessed at www.pubMLST.org/cronobacter. However, the scheme has not been applied for use in any ongoing epidemiologic investigation.

Whole-genome sequencing efforts can be expected to facilitate the correct identification of the bacterial species being studied; it can also provide detailed information regarding the unique geno- and phenotypic features. Moreover, these approaches can be used for comparative purposes to rapidly and simultaneously investigate the presence/absence of all annotated genes or coding

sequences or/and polymorphisms that may contribute to a specific morphology or physiology. Microarray-based comparative genomic indexing analysis of *Cronobacter* genus was originally performed by Healy *et al.* (2009), and this study identified species-specific genes that could be evaluated as candidate markers for inclusion in a molecular-based detection protocol. More recently, next generation sequencing has been used for the comprehensive analysis of eight *Cronobacter* genomes to better understand the pathogenicity and evolution of the genus alongside the characterization of virulence genes (Ji 2011). While this approach might reveal species-specific genomic information, in essence, this is a method that will provide much more detail necessary for subtyping an organism.

Epidemiology of *Cronobacter* species

Cronobacter infections are rare and are often underreported, especially in developing and less-developed countries (Estuningsih and Sani 2008; Friedemann 2009). Thus, the epidemiology of *Cronobacter* species is incomplete and poorly described. Bowen and Braden (2006) first attempted to describe the epidemiology related to these infections. These authors analysed the clinical case notes of 46 invasive infant *E. sakazakii* infections between 1961 and 2005. These included 12 infants presenting with bacteraemia, 33 with meningitis, and 1 with a urinary tract infection. Infants presenting with bacteraemia had higher birth weights (2454 g), and a gestational age of 37 weeks, with infections occurring at a younger age (6 days), compared with those infants presenting with meningitis. Meningitis was reported to have a high mortality rate (42%) and many of the survivors (more than 74%) suffer chronic neurological and developmental complications (Reij and Zwietering 2008). More recently, Friedemann (2009) analysed 120–150 neonatal *Cronobacter*-confirmed infections based on data published between 2000 and 2008. The overall lethality of the 67 invasive infections noted was 26·9%. Lethality of *Cronobacter* meningitis, bacteraemia and necrotizing enterocolitis (NEC) was calculated to be 41·9% ($P < 0·0001$), <10% and 19·0% ($P < 0·05$), respectively. Interestingly, this study identified two key risk factors, a longer gestational age at birth and parentage not from Europe, as significant factors for a higher reporting probability of neonatal *Cronobacter* meningitis based on a logistic regression models.

Many infections in newly born babies are transmitted from mother to child through the mother's birth canal, which has been suspected as a source of *Cronobacter* infections (Townsend and Forsythe 2008; Kandhai 2010). In most cases, both the route of exposure and the incubation period are generally unclear. Two early described outbreaks demonstrated a clear relationship between *Cronobacter* isolates from infected patients and the isolates cultured from unopened cans of PIF of the same batch consumed by these same patients (Clark *et al.* 1990; Block *et al.* 2002). Although powdered infant formula is regarded as an important source of this pathogen, environmental or extrinsic sources of contamination should not be excluded (Noriega *et al.* 1990). It has been reported that plant material may be the natural habitat for *Cronobacter* species (Schmid *et al.* 2009). Moreover, reports on *Cronobacter* species infections in immune-compromised adults (Gosney *et al.* 2006; See *et al.* 2007) may indicate other potential sources of contamination, such as the home environment or transient carriage states present in adult care takers, among others (Kandhai 2010; unpublished data and personal communication with Anna Bowen, CDC). It was estimated that the annual incidence rate among the low birth weight infants (i.e. weight <2500 g; children <12 months of age) was 8·7 per 100 000 infants in the United States of America (FAO/WHO 2006). Similarly, another study estimated an incidence rate of 9·4 per 100 000 among very low birth weight infants (i.e. weight < 1500 g) (Stoll *et al.* 2004).

Additionally, the prevalence of *Cronobacter* species infections in adults is increased in the elderly who have experienced strokes that have affected their abilities to swallow (dysphagia) and may therefore require reconstituted powdered protein supplements as part of their daily diet (Gosney *et al.* 2006; FAO/WHO 2008). This is a problem that is likely to become more common because of the ageing of the world's population, and as trends for consumption of synthetic, dehydrated formulas for such patients increase.

Control in manufacturing environment

Describing the epidemiology of *Cronobacter* in a PIF production environment can be regarded as a useful first step in an attempt to reduce the bacterial load and control dissemination. Mullane *et al.* (2008c) investigated *Cronobacter* species in a powdered milk protein manufacturing facility and highlighted the importance attached to the correct installation and maintenance of air filters to reduce the dissemination of *Cronobacter* and other biological hazards in the food production setting. Furthermore, these strategies facilitate the distinction between transient colonizing bacteria and those that can persist for long periods of time (Osaili and Forsythe 2009). These latter organisms could be regarded as having adapted to the production environment. Data from on-going surveillance studies identified a *Cronobacter sakazakii* isolate that demonstrated a remarkable phenotype. This isolate adapted and acquired a tolerance to temperatures of 60°C for

periods of time. The bacterium also remained in a viable state when in a desiccated state for several weeks (Cooney, unpublished data). This finding clearly supports the need to continuously monitor *Cronobacter* species in the production environment and also to identify those isolates that persist. An understanding the molecular mechanisms associated with such characteristics may be helpful as a means of eliminating them.

Walsh *et al.* (2011) provided further insights into the variability among strains in terms of their survival characteristics. Environmental strains appeared to survive better in dry ingredients, when tested in inulin and lecithin over a 338-day period. Also, clinical strains appeared to be more thermotolerant compared with their environmental counterparts. This resistance may be linked to a phenotype involving the production of extracellular polysaccharide (EPS). Walsh *et al.* (2011) proposed, based on these data, that the ability to produce EPS reduces thermotolerance. Based on these observations, clinical strains may have patho-adaptation resulting in a less desiccation-resistant phenotype.

Gajdosova *et al.* (2011) characterized an 18-kbp region from a collection of *Cronobacter* isolates and compared this locus to members belonging to other genera, such as *Enterobacter*, *Citrobacter* and *Escherichia,* where its presence was positively correlated with increased thermotolerance. The contribution of the 22 open reading frames annotated within this region to the thermotolerance phenotype remains unclear and requires further detailed experimental investigation.

Farmer *et al.* (1980) observed that many of the isolates that were studied originally had two different colony types referred to as types A and B. Type A colonies were described as 'either dry or mucoid, crenated (notched or scalloped edges), and rubbery when touched with a loop'. Type B colonies were described as possessing 'a typical smooth colony appearance, easily removed with a loop'. Based on similar descriptions of colonies of *Salmonella* (Anriany *et al.* 2006), we currently related these *Cronobacter* colony descriptions to those reported by Zogaj *et al.* (2003) as colonies expressing the rugose phenotype. Various studies using other enteric organisms such as *Salmonella* (Anriany *et al.* 2006), *Vibrio cholerae* (Ali *et al.* 2002) and *Grimontia hollisae* (formerly *Vibrio hollisae)* (Curtis *et al.* 2007) have shown that strains expressing the rugose phenotype impart: (i) a resistance to desiccation and antimicrobial agents such as hypochlorite; (ii) an increased ability to form biofilms; and (iii) the reversible rugose to smooth colony phase variation that was originally described by Farmer *et al.* (1980), respectively. It has been reported that *Cronobacter* species possesses a *bcsABZC* operon (Grimm *et al.* 2008) which is required for cellulose expression. Grimm *et al.* (2008) showed that a strain of *E. sakazakii*, ES5, which was used in development of a bacterial artificial chromosome (BAC) library, possesses the genetic machinery for cellulose biosynthesis. These studies also suggested that the overexpression of an exopolysaccharide composed of cellulose may play a role in rugosity, but the involvement and expression of other bacterial exopolysaccharides in rugosity should not be discounted. Understanding how these phenotypes evolve at a molecular level may facilitate the development of strategies to eliminate the persistent population of *Cronobacter.* Several such strategies have been suggested recently. These include the use of biocides and natural antibacterial compounds, such as essential oils and polyphenols, all of which are effective against *Cronobacter* (Brul 2004; Manach *et al.* 2004; Kim *et al.* 2010a,b).

Biocides to control *Cronobacter*

In attempts to ensure food safety and improve hygiene measures, the use of biocides and chemical-based disinfection protocols to control the microbial ecology of the production environment is in widespread use throughout the modern food industry. Several studies evaluated the ability of *Cronobacter* to survive treatment with common biocides used for this purpose. A study by Condell *et al.* (2012) evaluated the efficacy of eight commercially available biocide formulations against a collection of 90 *Cronobacter* species cultured from various origins: food, water, clinical and environmental. This study determined that each biocide formulation was completely effective in killing all *Cronobacter* strains tested, when cultured in a planktonic state, at the working concentration recommended by the manufacturer (Fig. 4). Mean minimum inhibitory concentration (MIC) values determined for these biocides ranged from 0·2 to 50% of the recommended working concentration. However, when the biocide formulations were re-analysed for their efficacy against surface-dried bacterial cells and *Cronobacter* contained in a biofilm, they all displayed a reduced killing effect. Five of the biocides were ineffective at killing

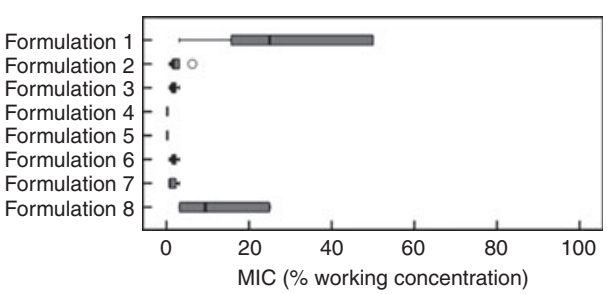

Figure 4 Minimum inhibitory concentration of planktonic *Cronobacter* measured against eight food industry biocide formulations.

Cronobacter contained in a biofilm at twice the reported working concentration and three were unable to eradicate surface-dried *Cronobacter* after an hour of contact time. These results further emphasized a critical need to understand the role of stress responses in *Cronobacter* species.

In a similar approach, Kim *et al.* (2007) reported that different disinfectants varied in their lethality to *Cronobacter* and showed reduced activity against *Cronobacter* in a biofilm, or when dried on stainless steel.

Both of these studies indicated that biofilm or surface-dried-associated phenotypes may contribute to the persistence of *Cronobacter* in the production environment. Cleaning regimes should consider this possibility, incorporating control strategies to prevent the development of biofilm and surface-dried communities in the food processing environment.

Other studies evaluated the use of natural biocides as food additives as an alternative natural means to control *Cronobacter*. Al-Holy *et al.* (2010) studied the effect of using natural biocides as food additives for the control of *Cronobacter*. In their study, lactic acid (LA), copper sulfate and monolaurin were used to inactivate *Cronobacter* species. Data showed that the use of a synergistic interactive combination of LA and copper sulfate could be beneficial to control *Cronobacter* in PIF industry.

Although biocides play an important role, their widespread use is not without its risks. The current scientific literature continues to report on the links between the over-use of biocides in the clinical and domestic environments and the subsequent selection of bacterial isolates displaying an increased tolerance to these agents concomitant with the emergence of cross-resistance to clinically important antibiotics. Condell *et al.* (unpublished data) evaluated the ability of *Cronobacter* to develop a tolerance to commercial food-grade biocide formulations and to active biocidal compounds contained in these products. Sublethal exposure of *Cronobacter* failed to increase their tolerance to any of the formulations tested or the actives contained therein. Nonetheless, the potential for selection and dissemination of pathogens, such as *Cronobacter* in the food chain, which have become biocide tolerant, is an area that should be considered and monitored in the designing and implementation of cleaning regimes.

Essential oils

Essential oils, aromatic-based liquids derived from plants, have been shown to inhibit some food-borne pathogen (Brul 2004). These oils may demonstrate potential for use in the food industry, particularly as consumer demands for pathogen-free food along with the minimal use of artificial preservatives continues to increase (Brul 2004). The capacity for the use of essential oils in the control of *Cronobacter* has been evaluated, with work mainly focusing on 'trans'-cinnamaldehyde (TC), a component of bark extract from the cinnamon plant (Amalaradjou and Venkitanarayanan 2011a).

Studies examined the inhibition of *Cronobacter* on abiotic surfaces (Amalaradjou and Venkitanarayanan 2011b) as well as evaluating its use in reducing the tolerance of *Cronobacter* to environmental stresses (Amalaradjou and Venkitanarayanan 2011c). These studies determined that TC was an effective agent in the inhibition and inactivation of *Cronobacter* biofilms, in the reduction of *Cronobacter* tolerance to desiccation, acid and osmotic stresses and in enhancing the killing effect of heat treatments. Indeed, this compound was shown to down-regulate genes involved in biofilm formation (Amalaradjou and Venkitanarayanan 2011b) in addition to many important stress regulators within the genus, such as *rpoS*, *phoP/-phoQ* and *ompR* (Amalaradjou and Venkitanarayanan 2011c). Further work by Amalaradjou and Venkitanarayanan examined the effect of TC on *Cronobacter* by analysing alterations in the proteome following exposure to TC. This work determined that TC disrupts *Cronobacter* metabolism, down-regulating proteins involved in amino acid metabolism as well as the F_0F_1 ATPase, disrupting the production of ATP. TC was also found to inhibit proteins involved in active transport across the membrane, flagellar biosynthesis, many genes associated with bacterial survival and defence such as catalase, superoxide dismutase and metaloprotease, as well as OmpA, a known *Cronobacter* virulence factor involved in adherence and invasion. The ability of TC to disrupt proteins associated with motility and survival in the host indicated that it may have a compromising effect on *Cronobacter* virulence (Amalaradjou and Venkitanarayanan 2011a). These data suggest that TC may be a promising agent useful in the control of *Cronobacter*. However, further work is required to identify and verify an appropriate application protocol for essential oils in the control of food-borne pathogens.

Polyphenols

Polyphenols are compounds that are abundant in nature. They are produced by plants and animals and often found in food and soil. Plants produce polyphenols as a defence mechanism to protect against infections, thus many plant polyphenols elaborate an antibacterial activity (Manach *et al.* 2004). Polyphenols have been evaluated as a potential food additive for the control of *Cronobacter*. A study by Kim *et al.* (2010a,b) concluded that red muscadine juice, a rich source of polyphenols, displayed strong antimicrobial action against *Cronobacter,* with tannic acid showing the greatest effect. This study suggested that the red muscadine juice had the potential for use in

baby food as an inhibitor of *Cronobacter* (Kim *et al.* 2010a,b).

Prebiotics

Prebiotics have been emerging over recent years as a beneficial food ingredient. These are known to stimulate the growth of beneficial bacteria in the intestine and have been recently reported to inhibit bacterial adherence to host cell surfaces *in vitro* (Gibson *et al.* 2005). Prebiotics, in particular polydextrose (PDX) and galactooligosaccharides (GOS), have been evaluated for use in the prevention of infection by *Cronobacter*. A study by Quintero *et al.* (2011) determined that GOS and a PDX/GOS combination had an inhibitory effect on the adherence of *Cronobacter* to intestinal-derived cells in tissue culture experiments. This work indicated that these prebiotics are inhibitory during the initial step in the establishment of a *Cronobacter* infection and therefore show some potential in the prevention of *Cronobacter*-related illness (Quintero *et al.* 2011). Furthermore, prebiotics have the added advantage of being food grade agents, and further work in this area could be important in the development of prebiotics as a natural, noninvasive and safe method for the control of *Cronobacter* infection.

Risk analysis

The implementation of microbiological criteria is one of the control measures that should be employed to reduce the risk of *Cronobacter* infection associated with PIF. There are several types of sampling plans that can be employed for the microbiological testing of PIF. The most common approach is *attribute testing*, which can be used to determine the *presence/absence* of *Cronobacter*.

A workshop on *Cronobacter* was convened in 2004, jointly by the United Nations (UN)-Food and Agriculture Organization (FAO) and World Health Organization (WHO) in response to a request for scientific advice from the Codex Alimentarius Committee on Food Hygiene (EC 2005), to provide information or guidance to PIF manufacturing companies and parents in regard to *Cronobacter* infections (FAO/WHO 2004). For any single lot of PIF, the level of contamination and the within-lot variability will determine the likelihood that a sample will be positive for *Cronobacter* and thus accepted or rejected. Three parameters can be used to characterize PIF production: the mean log concentration (MLC) (CFU g^{-1}) of *Cronobacter* across all PIF lots, the between-lot standard deviation (σ_b) across all PIF lots and the within-lot standard deviation (σ_w) for individual PIF lots. Therefore, related process controls should be developed according to these parameters.

In recent years, Hazard Analysis and Critical Control Point (HACCP) systems designed for prevision and management of contamination has been widely accepted and applied. HACCP programmes are mandatory in the food industry, but infant formula establishments are not required at this stage to have quality system standards such as ISO 9000 in place. Nevertheless, all infant formula industry-related manufactures are required to have in place effective good manufacturing practice (GMP) and related quality control procedures, which help these food factories to monitor the product line, manage the production quality and further improve products and processes.

Future considerations

Future attention to improve the control *of Cronobacter* should focus on five aspects as follows (FAO/WHO 2004):

1. For manufacturing and factories:
   - i Implementing an effective environmental monitoring programme, such as GMP and HACCP, to control the microbiological hazards from the raw materials, during the entire processing chain, until the final products, so as to minimize the entry of *Cronobacter* into the PIF environment and avoid the growth/persistence of this pathogen in PIF products.
   - ii Improving PIF product labels and communicating with consumers to create awareness of the correct method to be used for reconstituting PIF products.
   - iii Collaborating with researchers and governments to provide assistance on solving *Cronobacter*-related issues.
2. For governments and intergovernmental bodies:
   - i Setting a standard regulation and/or legislation directive for *Cronobacter* to guide food manufacturing towards improved control in the quality of their PIF products and further reduce the risk of *Cronobacter* infection.
   - ii Educating healthcare professionals to provide high-quality training to parents and professional caregivers to ensure PIF is prepared, handled and stored properly.
3. For hospitals:
   - i Using commercial sterile liquid formula or formula, which has undergone an effective point of use decontamination procedure and which is to be given to high-risk infants.
   - ii Educating parents in relation to the proper way of raising children being fed with PIF.
   - iii Assisting developing countries in establishing effective measures to minimum risk on *Cronobacter* infection.

4. For researchers and public health officials:
   i Developing a better understanding of the ecology, virulence and other characteristics of *Cronobacter* as a means of developing effective ways to reduce contamination in reconstituted PIF.
   ii Investigating and reporting of sources and vehicles and establishing laboratory-based domestic and international networks such as an integrated food safety system (IFSS), a mandate put forth by of the United States Food Safety and Modernization Act of 2011 (US-FSMA) which will house the Pathogen-Annotated Tracking Resource Network system (PATRN) (Tall 2010) as a prototype of an IFSS.
   iii Developing effective and rapid *Cronobacter* detection protocols for the PIF industry.
4. For consumers:
   i Being aware of the healthcare information-related infants.
   ii Obtaining scientifically grounded assistance from professionals, such as caregivers, doctors, PIF researchers.

References

Al-Holy, M.A., Castro, L.F. and Al-Qadiri, H.M. (2010) Inactivation of *Cronobacter* spp. (Enterobacter sakazakii) in infant formula using lactic acid, copper sulfate and monolaurin. *Lett Appl Microbiol* **50**, 246–251.

Ali, A., Rashid, M.H. and Karaolis, D.K. (2002) High-frequency rugose exopolysaccharide production by *Vibrio cholerae*. *Appl Environ Microbiol* **68**, 5773–5778.

Almeida, C., Azevedo, N.F., Iversen, C., Fanning, S., Keevil, C.W. and Vieira, M.J. (2009) Development and application of a novel peptide nucleic acid probe for the specific detection of *Cronobacter* genomospecies (*Enterobacter sakazakii*) in powdered infant formula. *Appl Environ Microbiol* **75**, 2925–2930.

Amalaradjou, M.A. and Venkitanarayanan, K. (2011a) Proteomic analysis of the mode of antibacterial action of trans – cinnamaldehyde against *Cronobacter sakazakii* 415. *Foodborne Pathog Dis* **8**, 1095–1102.

Amalaradjou, M.A. and Venkitanarayanan, K. (2011b) Effect of *trans* – cinnamaldehyde on inhibition and inactivation of *Cronobacter sakazakii* biofilm on abiotic surfaces. *J Food Prot* **74**, 200–208.

Amalaradjou, M.A. and Venkitanarayanan, K. (2011c) Effect of trans – cinnamaldehyde on reducing resistance to environmental stresses in *Cronobacter sakazakii*. *Foodborne Pathog Dis* **8**, 403–409.

Anonymous (2006a) *Milk and Milk Products – Detection of Enterobacter sakazakii*. Technical Specification ISO/TS 22964. ISO/TS 22964:2006(E) and IDF/RM 210:2006(E), 1st edn. Geneva: International Organization for Standardization.

Anonymous (2006b) *Enterobacter sakazakii* and *Salmonella* in powdered infant formula. Available at: http://www.fao.org/ag/agn/agns/jemra_riskassessment_enterobacter_en.asp. Second Risk Assessment Workshop. Joint FAO/WHO Workshop: Rome, Italy.

Anriany, Y., Ahu, S.N., Wessels, K.R., McCann, L.M. and Joseph, S.W. (2006) Alteration of the rugose phenotype in *waaG* and *ddhC* mutants of *Salmonella enterica* serovar Typhimurium DT104 is associated with inverse production of curli and cellulose. *Appl Environ Microbiol* **72**, 5002–5012.

Arbatsky, N.P., Wang, M., Shashkov, A.S., Chizhov, A.O., Feng, L., Knirel, Y.A. and Wang, L. (2010) Structure of the O-polysaccharide of *Cronobacter sakazakii* O2 with a randomly O-acetylated l-rhamnose residue. *Carbohydr Res* **345**, 2090–2094.

Arbatsky, N.P., Wang, M., Daeva, E.D., Shashkov, A.S., Feng, L., Knirel, Y.A. and Wang, L. (2011) Elucidation of the structure and characterization of the gene cluster of the O-antigen of *Cronobacter sakazakii* G2592, the reference strain of *C. sakazakii* O7 serotype. *Carbohydr Res* **346**, 1169–1172.

Baldwin, A., Loughlin, M., Caubilla–Barron, J., Kucerova, E., Manning, G., Dowson, C. and 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 Microbiol* **9**, 223.

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

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

Block, C., Peleg, O., Minster, N., Simhon, A., Arad, I. and Shapiro, M. (2002) Cluster of neonatal infections in Jerusalem due to unusual biochemical variant of *Enterobacter sakazakii*. *Eur J Clin Microbiol Infect Dis* **21**, 613–616.

Bowen, A.B. and Braden, C.R. (2006) Invasive *Enterobacter sakazakii* disease in infants. *Emerg Infect Dis* **12**, 1185–1189.

Brul, S. (2004) Essential oils: their antibacterial properties and potential applications in foods – a review. *Int J Food Microbiol* **94**, 223–253.

Caubilla-Barron, J., Hurrell, E., Townsend, S., Cheetham, P., Loc-Carrillo, C., Fayet, O., Prère, M.F. and 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. *J Clin Microbiol* **45**, 3979–3985.

Chap, J., Jackson, P., Siqueira, R., Gaspar, N., Quintas, C., Park, J., Osaili, T., Shaker, R. *et al.* (2009) International survey of *Cronobacter sakazakii* and other *Cronobacter* spp.

in follow up formulas and infant foods. *Int J Food Microbiol* **136**, 185–188.

Chen, Y. (2011) Development and validation of a revised FDA method for the detection of *Cronobacter* in powdered infant formula. *Department Seminar Series in University of Helsinki*, April 21 2011, Latokartanonkaari, Finland.

Chen, Y., Hammack, T.S., Song, K.Y. and Lampel, K.A. (2009) Evaluation of a revised U.S. Food and Drug Administration for the detection and isolation of Enterobacter sakazakii in powdered infant formula: precollaborative study. *J AOAC Int* **92**, 862–872.

Clark, N.C., Hill, B.C., O'Hara, C.M., Steingrimsson, O. and Cooksey, R.C. (1990) Epidemiologic typing of *Enterobacter sakazakii* in two neonatal nosocomial outbreaks. *Diagn Microbiol Infect Dis* **13**, 467–472.

Condell, O., Iversen, C., Cooney, S., Power, K.A., Walsh, C., Burgess, C. and Fanning, S. (2012) Efficacy of biocides used in the modern food industry to control *Salmonella*–links between biocide tolerance and resistance to clinically relevant antimicrobial compounds. *Appl Environ Microbiol*, in press, doi: 10.1128/AEM.07534-11.

Curtis, S.K., Kothary, M.H., Blodgett, R.J., Raybourne, R.B., Ziobro, G.C. and Tall, B.D. (2007) Rugosity in *Grimontia hollisae*. *Appl Environ Microbiol* **73**, 1215–1224.

Czerwicka, M., Forsythe, S.J., Bychowska, A., Dziadziuszko, H., Kunikowska, D., Stepnowski, P. and Kaczynski, Z. (2010) Chemical structure of the O-polysaccharide isolated from *Cronobacter sakazakii* 767. *Carbohydr Res* **345**, 908–913.

Derzelle, S., Dilasser, F., Maladen, V., Soudrie, N., Leclercq, A., Lombard, B. and Lafarge, V. (2007) Comparison of three chromogenic media and evaluation of two molecular-based identification systems for the detection of *Enterobacter sakazakii* from environmental samples from infant formulae factories. *J Food Prot* **70**, 1678–1684.

Drudy, D., O'Rourke, M., Murphy, M., Mullane, N., O'Mahony, R., Kelly, L., Fischer, M., Sanjaq, S. *et al.* (2006) Characterization of a collection of *Enterobacter sakazakii* isolates from environmental and food sources. *Int J Food Microbiol* **110**, 127–134.

Druggan, P. and Iversen, C. (2009) Culture media for the isolation of *Cronobacter* spp. *Int J Food Microbiol* **136**, 169–178.

El-Sharoud, W.M., O'Brien, S., Negredo, C., Iversen, C., Fanning, S. and Healy, B. (2009) Characterization of *Cronobacter* recovered from dried milk and related products. *BMC Microbiol* **9**, 24.

Emami, C.N., Mittal, R., Wang, L., Ford, H.R. and Prasadarao, N.V. (2011) Recruitment of dendritic cells is responsible for intestinal epithelial damage in the pathogenesis of necrotizing enterocolitis by *Cronobacter sakazakii*. *J Immunol* **186**, 7067–7079.

Estuningsih, S. and Sani, N.A. (2008) Powdered infant formula in developing and other countries – issues and prospects. In *Enterobacter sakazakii In Enterobacter sakazakii (Emerging Issues in Food Safety)* ed. Farber, J.M. and Forsythe, S.J. pp. 221–234. Washington, DC: ASM Press.

European Commission (EC) (2005) Commission Regulation (EC) No. 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Available at: http://www.fsai.ie/uploadedFiles/Reg2073_2005%281%29.pdf.

FAO/WHO (2004) Workshop on *Enterobacter sakazakii* and other microorganism in powdered infant formula. FAO/WHO, Geneva.

FAO/WHO (2006) *Enterobacter sakazakii* and *Salmonella* in powdered infant formula: meeting report. Microbiological Risk Assessment Series No.10, Rome, Italy.

FAO/WHO (2008) *Enterobacter sakazakii* (*Cronobacter* spp.) in powdered following formula: meeting report. Microbiological Risk Assessment Series No.15, Rome, Italy.

Farmer, J.J. III, Hickmann, A.M. and Brenner, D.J. (1980) *Enterobacter sakazakii*: a new species of 'Enterobacteriaceae' isolated from clinical specimens. *Int J Syst Evol Microbiol* **30**, 569–584.

Forsythe, S. (2009) *Cronobacter* species. *Culture* **31**, 1.

Franco, A.A., Hu, L., Grim, C.J., Gopinath, G., Sathyamoorthy, V., Jarvis, K.G., Lee, C., Sadowski, J. *et al.* (2011a) Characterization of putative virulence genes on the related RepFIB plasmids harbored by *Cronobacter* spp. *Appl Environ Microbiol* **77**, 3255–3267.

Franco, A.A., Kothary, M.H., Gopinath, G., Jarvis, K.G., Grim, C.J., Hu, L., Datta, A.R., McCardell, B.A. *et al.* (2011b) Cpa, the outer membrane protease of *Cronobacter sakazakii*, activates plasminogen and mediates resistance to serum bactericidal activity. *Infect Immun* **79**, 1578–1587.

Franke, S., Grass, G., Rensing, C. and Nies, D.H. (2003) Molecular analysis of the copper-transporting efflux system CusCFBA of *Escherichia coli*. *J Bacteriol* **185**, 3804–3812.

Friedemann, M. (2009) Epidemiology of invasive neonatal *Cronobacter* (*Enterobacter sakazakii*) infections. *Eur J Clin Microbiol Infect Dis* **28**, 1297–1304.

Gajdosova, J., Benedikovicova, K., Kamodyova, N., Tothova, L., Kaclikova, E., Stuchlik, S., Turna, J. and Drahovska, H. (2011) Analysis of the DNA region mediating increased thermotolerance at 58°C in *Cronobacter* spp. and other enterobacterial strains. *Antonie Van Leeuwenhoek* **100**, 279–289.

Gibson, G.R., Mccartney, A.L. and Rastall, R.A. (2005) Prebiotics and resistance to gastrointestinal infections. *Br J Nutr* **93**, 31–34.

Gosney, M.A., Martin, M.V., Wright, A.E. and Gallagher, M. (2006) *Enterobacter sakazakii* in the mouths of stroke patients and its association with aspiration pneumonia. *Eur J Intern Med* **17**, 185–188.

Grimm, M., Stephan, R., Iversen, C., Manzardo, G.G., Rattei, T., Riedel, K., Ruepp, A., Frishman, D. *et al.* (2008) Cellulose as an extracellular matrix component present in *Enterobacter sakazakii* biofilms. *J Food Prot* **71**, 13–18.

Gurtler, J.B., Kornacki, J.L. and Beuchat, L.R. (2005) *Enterobacter sakazakii*: a coliform of increased concern to infant health. *Int J Food Microbiol* **104**, 1–34.

Hassan, A.A., Akineden, O., Kress, C., Estuningsih, S., Schneider, E. and Usleber, E. (2007) Characterization of the gene encoding the 16S rRNA of *Enterobacter sakazakii* and development of a species-specific PCR method. *Int J Food Microbiol* **116**, 214–220.

Healy, B. (2010) *Characterization of Cronobacter spp. (Enterobacter sakazakii)*. Dublin: University College Dublin.

Healy, B., Huynh, S., Mullane, N., O'Brien, S., Iversen, C., Lehner, A., Stephan, R., Parker, C.T. *et al.* (2009) Microarray-based comparative genomic indexing of the *Cronobacter* genus (*Enterobacter sakazakii*). *Int J Food Microbiol* **136**, 159–164.

Healy, B., Cooney, S., O'Brien, S., Iversen, C., Whyte, P., Nally, J., Callanan, J.J. and Fanning, S. (2010) *Cronobacter* (*Enterobacter sakazakii*): an opportunistic foodborne pathogen. *Foodborne Pathog Dis* **7**, 339–350.

Huang, S.H., Chen, Y.H., Fu, Q., Stins, M., Wang, Y. and Kim, K.S. (1999) Identification and characterization of an *Escherichia coli* invasion gene locus, *ibeB*, required for penetration of brain microvascular endothelial cells. *Infect Immun* **67**, 2103–2109.

Huang, S.H., Wan, Z.S., Chen, Y.H., Jong, A.Y. and Kim, K.S. (2001) Further characterization of *Escherichia coli* brain microvascular endothelial cell invasion gene *ibeA* by deletion, complementation, and protein expression. *J Infect Dis* **183**, 1071–1078.

International Organization for Standardization (2009) ISO 9000 Essentials. Available at: http://www.iso.org/iso/iso_catalogue/management_standards/iso_9000_iso_14000/iso_9000_essentials.htm.

Iversen, C. and Forsythe, S.J. (2007) Comparison of media for the isolation of *Enterobacter sakazakii*. *Appl Environ Microbiol* **73**, 48–52.

Iversen, C., Waddington, M., On, S.L. and Forsythe, S. (2004a) Identification and phylogeny of *Enterobacter sakazakii* relative to *Enterobacter* and *Citrobacter* Species. *J Clin Microbiol* **42**, 5368–5370.

Iversen, C., Druggan, P. and Forsythe, S. (2004b) A selective differential medium for *Enterobacter sakazakii*, a preliminary study. *Int J Food Microbiol* **96**, 133–139.

Iversen, C., Lehner, A., Mullane, N., Bidlas, E., Cleenwerck, I., Marugg, J., Fanning, S., Stephan, R. *et al.* (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. and *Cronobacter* genomospecies 1. *BMC Evol Biol* **7**, 64.

Iversen, C., Lehner, A., Mullane, N., Marugg, J., Fanning, S., Stephan, R. and Joosten, H. (2007b) Identification of "*Cronobacter*" spp. (Enterobacter sakazakii). *J Clin Microbiol* **11**, 3814–3816.

Iversen, C., Mullane, N., McCardell, B., Tall, B.D., Lehner, A., Fanning, S., Stephan, R. and 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. *Int J Syst Evol Microbiol* **58**, 1442–1447.

Jaradat, Z.W., Ababneh, Q.O., Saadoun, I.M., Samara, N.A. and Rashdan, A.M. (2009) Isolation of *Cronobacter* spp. (formerly Enterobacter sakazakii) from infant food, herbs and environmental samples and the subsequent identification and confirmation of the isolates using biochemical, chromogenic and molecular methods. *BMC Microbiol* **9**, 225–235.

Jarvis, K.G., Grim, C.J., Franco, A.A., Gopinath, G., Sathyamoorthy, V., Hu, L., Sadowski, J.A., Lee, C.S. *et al.* (2011) Molecular characterization of *Cronobacter* lipopolysaccharide O-antigen gene clusters and development of serotype-specific PCR assays. *Appl Environ Microbiol* **77**, 4017–4026.

Ji, Y.M. (2011) Next generation sequencing and comparative analysis of eight *Cronobacter* genomes. *The American Society for Microbiology (ASM) 2011*, May 21–24, New Orleans, LA, USA.

Joseph, S. and Forsythe, S.J. (2011) Predominance of *Cronobacter sakazakii* sequence type 4 in neonatal infections. *Emerg Infect Dis* **17**, 1713–1715.

Joseph, S., Cetinkaya, E., Drahovska, H., Levican, A., Figueras, M.J. and Forsythe, S.J. (2011) *Cronobacter condimenti* sp. 1 nov., isolated from spiced meat and *Cronobacter universalis* sp. nov., a novel species designation for *Cronobacter* sp. genomospecies 1, recovered from a leg infection,water, and food ingredients. *IJSEM*, in press, doi: 10.1099/ijs.0.032292–0.

Kandhai, M.C. (2010) *Detection, Occurrence, Growth and Inactivation of Cronobacter* spp. (*Enterobacter sakazakii*). Wageningen: Wageningen University.

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

Kilonzo-Nthenge, A., Chen, F.C. and Godwin, S.L. (2008) Occurrence of *Listeria* and *Enterobacteriaceae* in domestic refrigerators. *J Food Prot* **71**, 608–612.

Kim, K.S. (2000) *E. coli* invasion of brain microvascular endothelial cells as a pathogenetic basis of meningitis. *Subcell Biochem* **33**, 47–59.

Kim, K.P. and Loessner, M.J. (2008) *Enterobacter sakazakii* invasion in human intestinal Caco-2 cells requires the host cell cytoskeleton and is enhanced by disruption of tight junction. *Infect Immun* **76**, 562–570.

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. *Appl Environ Microbiol* **73**, 1256–1265.

Kim, K., Kim, K.P., Choi, J., Lim, J.A., Lee, J., Hwang, S. and Ryu, S. (2010a) Outer membrane proteins A (*OmpA*) and X (*OmpX*) are essential for basolateral invasion of *Cronobacter sakazakii*. *Appl Environ Microbiol* **76**, 5188–5198.

Kim, T.J., Weng, W.L., Silva, J.L., Jung, Y.S. and Marshall, D. (2010b) Identification of natural antimicrobial substances in red muscadine juice against *Cronobacter sakazakii*. *J Food Sci* **75**, 150–154.

Kotewicz, M.L. and Tall, B.D. (2009) Optical maps of subgroups of *Cronobacter* (formerly *Enterobacter sakazakii*) show large chromosomal regions of homology and differences among isolates. *1st International Conference on Cronobacter,* Poster Abstract 60, Dublin, Ireland.

Kothary, M.H., McCardell, B.A., Frazar, C.D., Deer, D. and Tall, B.D. (2007) Characterization of the zinc–containing metalloprotease encoded by *zpx* and development of a species–specific detection method for *Enterobacter sakazakii*. *Appl Environ Microbiol* **73**, 4142–4251.

Kucerova, E., Clifton, S.W., Xia, X.Q., Long, F., Porwollik, S., Fulton, L., Fronick, C., Minx, P. *et al.* (2010) Genome sequence of *Cronobacter sakazakii* BAA–894 and comparative genomic hybridization analysis with other *Cronobacter* species. *PLoS ONE* **5**, e9556.

Kuhnert, P., Korczak, B.M., Stephan, R., Joosten, H. and Iversen, C. (2009) Phylogeny and prediction of genetic similarity of Cronobacter and related taxa by multilocus sequence analysis (MLSA). *Int J Food Microbiol* **136**, 152–158.

Leuscher, R.G. and Bew, J. (2004) A medium for the presumptive detection of Enterobacter sakazakii in infant formula: interlaboratory study. *J AOAC Int* **87**, 604–613.

MacLean, L.L., Vinogradov, E., Pagotto, F., Farber, J.M. and Perry, M.B. (2009) Characterization of the O-antigen in the lipopolysaccharide of *Cronobacter* (*Enterobacter*) *malonaticus* 3267. *Biochem Cell Biol* **87**, 927–932.

Maclean, L.L., Vinogradov, E., Pagotto, F. and Perry, M.B. (2011) Characterization of the lipopolysaccharide O-antigen of *Cronobacter turicensis* HPB3287 as a polysaccharide containing a 5, 7-diacetamido-3, 5, 7, 9-tetradeoxy-d-glycero-d-galacto-non-2-ulosonic acid (legionaminic acid) residue. *Carbohydr Res* **346**, 2589–2594.

Malorny, B. and Wagner, M. (2005) Detection of *Enterobacter sakazakii* strains by real-time PCR. *J Food Prot* **68**, 1623–1627.

Manach, C., Scalbert, A., Morand, C., Rémésy, C. and Jiménez, L. (2004) Polyphenols: food sources and bioavailability. *Am J Clin Nutr* **79**, 727–747.

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

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

Mullane, N.R., Iversen, C., Healy, B., Walsh, C., Whyte, P., Wall, P.G., Quinn, T. and Fanning, S. (2007a) *Enterobacter sakazakii* an emerging bacterial pathogen with implications for infant health. *Minerva Pediatr* **59**, 137–148.

Mullane, N.R., Whyte, P., Wall, P.G., Quinn, T. and Fanning, S. (2007b) Application of pulsed–field gel electrophoresis to characterise and trace the prevalence of *Enterobacter sakazakii* in an infant formula processing facility. *Int J Food Microbiol* **116**, 73–81.

Mullane, N.R., O'Gaora, P., Nally, J.E., Iversen, C., Whyte, P., Wall, P.G. and Fanning, S. (2008a) Molecular analysis of the *Enterobacter sakazakii* O–antigen gene locus. *Appl Environ Microbiol* **12**, 3783–3794.

Mullane, N.R., Ryan, M., Iversen, C., Murphy, M., O'Gaora, P., Quinn, T., Whyte, P., Wall, P.G. *et al.* (2008b) Development of multiple–locus variable–number tandem–repeat analysis for the molecular subtyping of *Enterobacter sakazakii*. *Appl Environ Microbiol* **74**, 1223–1231.

Mullane, N.R., Healy, B., Meade, J., Whyte, P., Wall, P.G. and Fanning, S. (2008c) Dissemination of *Cronobacter* spp. (Enterobacter sakazakii) in a powdered milk protein manufacturing facility. *Appl Environ Microbiol* **74**, 5913–5917.

Muytjens, H.L., van der Ros-van de Repe, J. and van Druten, H.A. (1984) Enzymatic profiles of *Enterobacter sakazakii* and related species with special reference to the alpha glucosidase reaction and reproducibility of the test system. *J Clin Microbiol* **20**, 684–686.

Muytjens, H.L., Roelofs–Willemse, H. and Jaspar, G.H. (1988) Quality of powdered substitutes for breast milk with regard to members of the family *Enterobacteriaceae*. *J Clin Microbiol* **26**, 743–746.

Nair, M.K. and 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. *Appl Environ Microbiol* **72**, 2539–2546.

Nair, M.K., Venkitanarayanan, K., Silbart, L.K. and Kim, K.S. (2009) Outer membrane protein A (*OmpA*) of *Cronobacter sakazakii* binds fibronectin and contributes to invasion of human brain microvascular endothelial cells. *Foodborne Pathog Dis* **6**, 495–501.

Noriega, F.R., Kotloff, K.L., Martin, M.A. and Schwalbe, R.S. (1990) Nosocomial bacteremia caused by *Enterobacter sakazakii* and *Leuconostoc mesenteroides* resulting from extrinsic contamination of infant formula. *Pediatr Infect Dis J* **9**, 447–449.

O'Brien, S., Healy, B., Negredo, C., Fanning, S. and Iversen, C. (2009) Evaluation of a new one-step enrichment in conjunction with a chromogenic medium for the detection of *Cronobacter* spp. (Enterobacter sakazakii) in powdered infant formula. *J Food Prot* **72**, 1472–1475.

Oh, S.W. and Kang, D.H. (2004) Fluorogenic selective and differential medium for isolation of *Enterobacter sakazakii*. *Appl Environ Microbiol* **70**, 5692–5694.

Osaili, T. and Forsythe, S. (2009) Desiccation resistance and persistence of *Cronobacter* species in infant formula. *Int J Food Microbiol* **136**, 214–220.

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

Prasadarao, N.V., Wass, C.A., Weiser, J.N., Stins, M.F., Huang, S.H. and Kim, K.S. (1996) Outer membrane protein A of *Escherichia coli* contributes to invasion of brain microvascular endothelial cells. *Infect Immun* **64**, 146–153.

Quintero, M., Maldonado, M., Perez–Munoz, M., Jimenez, R., Fangman, T., Rupnow, J., Wittke, A., Russell, M. *et al.* (2011) Adherence inhibition of *Cronobacter sakazakii* to intestinal epithelial cells by prebiotic oligosaccharides. *Curr Microbiol* **62**, 1448–1454.

Reij, M.W. and Zwietering, M.H. (2008) Integrating concepts: a case study using *Enterobacter sakazakii* in infant formula. In: *Microbial Risk Analysis of Foods* ed. Schaffner, D.W. pp. 177–204. Washington, DC: ASM Press.

Restaino, L., Frampton, E.W., Lionberg, W.C. and Becker, R.J. (2006) A chromogenic plating medium for the isolation and identification of *Enterobacter sakazakii* from foods, food ingredients and environmental sources. *J Food Prot* **69**, 315–322.

Schmid, M., Iversen, C., Gontia, I., Stephan, R., Hofmann, A., Hartmann, A., Jha, B., Eberl, L. *et al.* (2009) Evidence for a plant-associated natural habitat for *Cronobacter* spp. *Res Microbiol* **160**, 608–614.

See, K.C., Than, H.A. and Tang, T. (2007) *Enterobacter sakazakii* bacteremia with multiple splenic abscesses in a 75-year-old woman: a case report. *Age Ageing* **36**, 595–596.

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

Shashkov, A.S., Arbatsky, N.P. and Knirel, Y.A. (2011) Structures and genetics of Kdo-containing O-antigens of Cronobacter sakazakii G2706 and G2704, the reference strains of serotypes O5 and O6. *Carbohydr Res* **346**, 1924–1929.

Stephan, R., Lehner, A., Tischler, P. and Rattei, T. (2011) Complete genome sequence of *Cronobacter turicensis* LMG 23827, a food-borne pathogen causing deaths in neonates. *J Bacteriol* **193**, 309–310.

Stoll, B.J., Hansen, N., Fanaroff, A.A. and Lemons, J.A. (2004) *Enterobacter sakazakii* is a rare cause of neonatal septicemia or meningitis in VLBW infants. *J Pediatr* **144**, 821–823.

Stoop, B., Lehner, A., Iversen, C., Fanning, S. and Stephan, R. (2009) Development and evaluation of *rpo*B-based PCR systems to differentiate the six proposed species within the genus *Cronobacter*. *Int J Food Microbiol* **136**, 165–168.

Sun, Y.M., Wang, M., Liu, H.B., Wang, J.J., He, X., Zeng, J., Guo, X., Li, K. *et al.* (2011) Development of an O-antigen serotyping scheme for *Cronobacter sakazakii*. *Appl Environ Microbiol* **77**, 2209–2214.

Tall, B.D. (2010) Development and application of a Pathogen Annotated Tracking Resource Network (PATRN) system to aid analysis of foodborne pathogens, abstract R-2447. Abstract. 110th General Meeting of the American Society for Microbiology. American Society for Microbiology, Washington, DC.

Townsend, S. and Forsythe, S. (2008) The neonatal intestinal microbial flora, immunity and infections. In *Enterobacter sakazakii* ed. Farber, M. and Forsythe, S. pp. 61–100. Washington, DC: ASM Press.

Townsend, S.M., Hurrell, E., Gonzalez-Gomez, I., Lowe, J., Frye, J.G., Forsythe, S. and Badger, J.L. (2007) *Enterobacter sakazakii* invades brain capillary endothelial cells, persists in human macrophages influencing cytokine secretion and induces severe brain pathology in the neonatal rat. *Microbiology* **153**, 3538–3547.

Townsend, S., Hurrell, E. and Forsythe, S. (2008) Virulence studies of *Enterobacter sakazakii* isolates associated with a neonatal intensive care unit outbreak. *BMC Microbiol* **8**, 64.

Walsh, D., Molloy, C., Iversen, C., Carroll, J., Cagney, C., Fanning, S. and Duffy, G. (2011) Survival characteristics of environmental and clinically derived strains of *Cronobacter sakazakii* in infant milk formula (IMF) and ingredients. *J Appl Microbiol* **110**, 697–703.

Wang, Y., Huang, S.H., Wass, C.A., Stins, M.F. and Kim, K.S. (1999) The gene locus *yijP* contributes to *Escherichia coli* K1 invasion of brain microvascular endothelial cells. *Infect Immun* **67**, 475–4756.

Yan, X., Gurtler, J., Fratamico, P.M., Hu, J., Gunther IV, N.W., Juneja, V.K. and Huang, L. (2011) Comprehensive approaches for molecular biomarker discovery for the detection and identification of *Cronobacter* spp. (*Enterobacter sakazakii*) and *Salmonella*. *Appl Environ Microbiol* **77**, 1833–1843.

Zhou, Y., Wu, Q., Xu, X., Yang, X., Ye, Y. and Zhang, J. (2008) Development of an immobilization and detection method of *Enterobacter sakazakii* from powdered infant formula. *Food Microbiol* **25**, 648–652.

Zogaj, X., Bokranz, W., Nimitz, M. and Romling, U. (2003) Production of cellulose and curli fimbriae by members of the family *Enterobacteriaceae* isolated from the human gastrointestinal tract. *Infect Immun* **71**, 415–4158.