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The *Cronobacter* genus: ubiquity and diversity

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Abstract

Members of the *Cronobacter* genus (formerly *Enterobacter sakazakii*) have become associated with neonatal infections and in particular contaminated reconstituted infant formula. However, this is only one perspective of the organism as the majority of infections are in the adult population, and the organism has been isolated from the enteral feeding tubes of neonates on non-formula diets. In recent years methods of detection from food and environmental sources have improved, though accurate identification has been problematic. The need for robust identification is essential in order to implement recent Codex Alimentarius Commission and related microbiological criteria for powdered infant formula (intended target age 0–6 months). Genomic analysis of emergent pathogens is of considerable advantage in both improving detection methods, and understanding the evolution of virulence. One ecosystem for *Cronobacter* is on plant material, which may enable the organism to resist desiccation, adhere to surfaces, and resist some antimicrobial agents. These traits may also confer survival mechanisms of relevance in food manufacturing and also virulence mechanisms.

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Introduction

Food safety is important to everyone, and especially the highly vulnerable members of our society. This article concerns the bacterial genus *Cronobacter*, which can cause severe illness in the highly vulnerable neonates, infants and the elderly. In recent years this group of organisms has gone from curiosity to notoriety and this article aims to deliver a balanced viewpoint on their importance and an overview of our knowledge.

Cronobacter is a genus within the *Enterobacteriaceae* family and is closely related to the *Enterobacter* and *Citrobacter* genera. It has come to prominence due to its association with severe neonatal infections [necrotizing enterocolitis (NEC), septicaemia and meningitis] that can be fatal. As neonates are frequently fed reconstituted powdered infant formula (PIF), which is not a sterile product, this potential vector has been the focus of attention for reducing infection risk to neonates as the number of exposure routes is limited. It should be noted, however, that such neonatal infections are rare, and not all have been associated with reconstituted formula ingestion. In fact (a) breast milk is also not sterile, (b) *Cronobacter malonaticus* LMG 23826^{T} (type strain) was isolated from a breast abscess, (c) *Cronobacter* has been isolated from breast milk, (d) breast milk from mastic mothers is used to feed neonates, (e) the organism has been isolated from enteral feeding tubes from neonates on non-formula diets and (f) the majority of *Cronobacter* infections, albeit less severe, are in the adult population.

To date the raised awareness of the organism has focussed on infant infections and resulted in changes in the microbiological criteria for PIF and reconstitution procedures. In other words, there have been required changes on two sides of the same coin; manufacturing practices and hygienic preparation practices. Such requirements need regulatory enforcement and support, but must be based on robust reliable information. Consequently there have been three FAO/WHO risk assessment meetings on the microbiological safety of PIF (FAO/WHO, 2004, 2006, 2008). Those

identified as being at high risk of *Cronobacter* infection are neonates (especially low-birth weight) for whom their source of nutrition will be limited to breast milk, fortified breast milk or breast milk replacement. Hence hygienic preparation of feed is essential due to their immature immune system and lack of competing intestinal flora. Key advice from these FAO/WHO risk assessments was that PIF should be reconstituted with water $> 70\,^{\circ}C$, minimize any storage time by not preparing in advance and if storage for short periods is necessary then the temperature should be $< 5\,^{\circ}C$. The high water temperature will drastically reduce the number of vegetative bacteria present, and minimizing the storage period will reduce the multiplication of any surviving organisms. These recommendations have been well addressed by the WHO 'Guidelines for the safe preparation, storage and handling of powdered infant formula' which are available online and can be downloaded using the URL given in the Reference section (Willis & Robinson, 1988). The FAO/WHO (2004) expert committee recommended that research should be promoted to gain a better understanding of the taxonomy, ecology, virulence and other characteristics of *Cronobacter*. This article addresses many of these topics, using our recent findings on the genomic analysis of the organism and similarities with closely related organisms as well as issues of detection and consumer protection.

Taxonomy and identification schemes

Taxonomy of *Cronobacter*

Initially the organism was regarded as a pigmented variety of *Enterobacter cloacae*. In 1974, Brenner showed that the pigmented strains had $< 50\%$ homology with non-pigmented strains and it was suggested that they should comprise a new species. *Enterobacter sakazakii* was later distinguished from *E. cloacae* based on DNA–DNA hybridization, pigment production, biotype assignment and antimicrobial resistance (Farmer, 1980; Izard *et al.*, 1983), the species name being derived from the Japanese bacteriologist Riichi Sakazaki. DNA–DNA hybridization values were 41% and 54% for *Citrobacter freundii* and *E. cloacae*, respectively, which were used as representatives of the *Citrobacter* and *Enterobacter* genera (Farmer, 1980). The results warranted the recognition of a separate species and, as they were phenotypically closer to *E. cloacae*, they were kept in the *Enterobacter* genus. Additional phenotypic analysis led to the description of 15 *E. sakazakii* biogroups, with biotype 1 being the most common (Farmer, 1980). At this point, there was no clear evidence of the generic assignment of *E. sakazakii* to the *Enterobacter* genus, however.

Since the 1980s, bacterial systematics has increasingly used DNA sequencing for its analysis and for determining relatedness. Analysis of both partial 16S rDNA and hsp60 gene sequencing by Iversen and Forsythe in 2004 showed that *E. sakazakii* isolates formed at least four distinct clusters which probably represented different closely related species (Iversen *et al.*, 2004). The 15 different biogroups fitted into the four clusters, and a 16th biogroup was added in subsequent work (Iversen *et al.*, 2006). However, full taxonomic revision required considerable further analysis for substantiation. The *Cronobacter* genus was defined first in 2007 and revised in 2008 (Iversen *et al.*, 2007, 2008). Differentiation between the newly defined *Cronobacter* species is primarily based on genotypic (DNA-based) analysis and is largely supported by biochemical traits (Table 1) (Iversen *et al.*, 2006). With a few exceptions, the former biotypes and genomogroups correspond with the new species; as shown in Table 1. The genus *Cronobacter* is currently composed of *Cronobacter sakazakii*, *C. malonaticus*, *Cronobacter turicensis*, *Cronobacter muytjensii* and *Cronobacter dublinensis* (Joseph *et al.*, 2011). Distinguishing between the two species *C. sakazakii* and *C. malonaticus* has been problematic and there are two primary reasons for this. Firstly, the use of biotype profile to designate the species was not totally robust as a few of the biotype index strains were themselves assigned the wrong species (Baldwin *et al.*, 2009). Secondly, there are seven copies of the rDNA gene in *Cronobacter* and intrageneric differences can lead to uncertain and inconsistent base calls.

Table 1 *Cronobacter* species groupings, updated from Baldwin *et al.* (2009) and Joseph *et al.* (2011)

Cronobacter species	16S cluster	Biotypes	MLST sequence types
C. sakazakii	1	1, 2–4, 7, 8, 11, 13	1, 2–4, 8, 9, 12–18, 20–23, 31, 40, 41, 45, 47, 50, 52
C. malonaticus	1	5, 9, 14	7, 10, 11, 25, 26, 29, 53
C. turicensis	2	16	5, 19, 24, 32, 35, 37
C. muytjensii	3	15	28, 33, 34, 44, 49
C. dublinensis	4	6, 10, 12	27, 36, 38, 39, 42, 43, 46
Cronobacter genomospecies 1	4	16	48, 51, 54

Since members of the *Cronobacter* genus were formerly known as the single species *E. sakazakii*, this name was used in publications before mid-2007. Subsequently it is uncertain which specific *Cronobacter* species were referred to in many pre-2007 publications. The majority of isolated strains are usually *C. sakazakii*, and it is probable that this has been the species of major study to date. For our part, we have tried to give cross-references for strains in our own publications to assist readers, and are available on request. Accurate bacterial taxonomy is essential for regulatory control because the detection methods must be based on a thorough understanding of the diversity of the target organism. A number of early methods were based on small numbers of poorly characterized, even misidentified, strains and therefore are not necessarily reliable for their stated purpose.

Identification schemes

The Codex Alimentarius Commission (CAC) (2008) microbiological criteria are applied to PIF with the intended age < 6 months. Hence a number of methods for the recovery of desiccation-stressed *Cronobacter* cells from this group of products have been developed. As the organism has only been reported at low numbers ($< 1\,\mathrm{CFU\,g^{-1}}$), a large volume of material needs to be tested. Subsequently the CAC requirement is to test thirty 10 g quantities, and therefore presence/absence testing of PIF is applied rather than direct enumeration. Initial *Cronobacter* detection methods were reminiscent of the stages for *Salmonella* isolation from milk powders. In brief, the steps were pre-enrichment (225 mL water or BPW +25 g formula), enrichment (EE broth), plate on to VRBG agar, pick off five colonies to TSA and identify phenotypically any yellow-pigmented colonies. It is now recognized that there are a number of limitations with this method. There is no initial selection for *Cronobacter*, instead any *Enterobacteriaceae* could be enriched in EE broth and grow on VRBG agar leading to possible overgrowth of *Cronobacter*. Furthermore, not all *Cronobacter* strains are pigmented and so could be overlooked on TSA plates. Finally phenotype databases did not adequately cover the genus resulting in conflicting results between commercial kits. These days improved methods use chromogenic agars, updated phenotyping databases, along with DNA-based identification and fingerprinting techniques. *Cronobacter* has a notable resistance to osmotic stresses, which may be linked to its ecology, and this trait has been used in the design of improved enrichment broths; modified lauryl sulphate broth containing 0.5 M NaCl and *Cronobacter* screening broth with 10% sucrose. The use of chromogenic agar (primarily based on the α-glucosidase reaction) to differentiate *Cronobacter* from other *Enterobacteriaceae* present on the plate was a major improvement. The α-glucosidase activity as a test differentiating the then *E. sakazakii* from *E. cloacae* had been reported in the early days by Harry Muytjens (Muytjens, van der Ros-van de Repe & Van Druten, 1984). As well as testing PIF, environmental samples are taken from the production environment as well as from ingredients (especially starches and other plant-derived material). In addition, production facilities and processes are already designed to control enteric pathogens, especially *Salmonella*.

Commercial companies producing phenotyping kits have been updating their databases due to the taxonomic revision, for example, the former *E. sakazakii* Preceptrol™ strain ATCC® 51329 has been reclassified as *C. muytjensii* and should not be confused with *C. sakazakii*. The specificity of some formerly *E. sakazakii* DNA-based PCR probes to the diverse *Cronobacter* genus needs to be re-evaluated, and new species-specific *Cronobacter* probes have been developed.

16S rDNA sequences have been traditionally used to determine phylogenetic relationships between organisms including *Enterobacteriaceae*. However, 16S rDNA sequencing is of limited use for very closely related organisms because of minor differences in the rDNA sequence. 16S rRNA gene sequencing can distinguish between the *Cronobacter* species as shown in Figure 1. Earlier difficulties in distinguishing between *C. sakazakii* and *C. malonaticus* was possibly due to polymorphic nucleotide sites and depended on the operator interpretation of the sequencing chromatograms for those loci. Additionally there is difficulty using biotyping to define the *Cronobacter* species as some strains defined as particular biotype index strains were misassigned their species. 16S rDNA sequence analysis has been applied to early strains of *E. sakazakii* and other mis-identifications include:

(1) Fatal case of neonatal sepsis infection in neonatal intensive care unit (NICU) by *E. sakazakii*, re-identified as *E. cloacae* (Caubilla-Barron *et al.*, 2007).
(2) NICU outbreak of *E. sakazakii*, re-identified as *Enterobacter hormaechei* (Townsend, Hurrell & Forsythe, 2008).
(3) Quinolone-resistant *E. sakazakii* strain, re-identified as *E. hormaechei* (Poirel *et al.*, 2007).
(4) Oligo-polysaccharide structure for *E. sakazakii*, strain re-identified as *Enterobacter ludwiggi* (Szafranek *et al.*, 2005).

Molecular typing methods

Although it is generally possible to differentiate *Cronobacter* species by biochemical profiling, molecular methods are

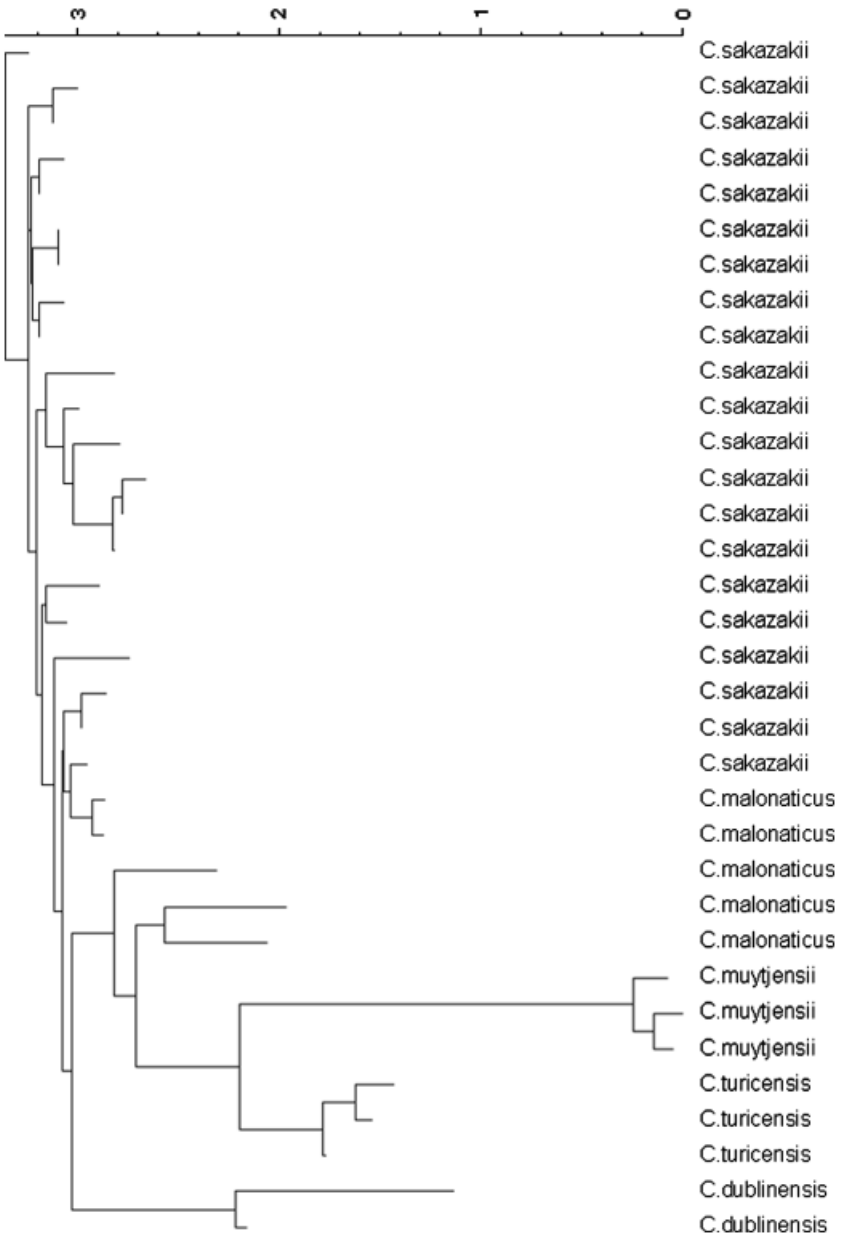

Figure 1 16S rDNA gene neighbour joining phylogenetic tree of *Cronobacter* genus.

increasingly used as a more rapid and reliable tool to study bacterial genomic diversity and to track sources of infection. As the organism is ubiquitous, typing schemes are required both for epidemiological and environmental investigation. As given above, initially 15 biogroups of *Cronobacter* were defined with biogroup 1 being the most common. These divisions, however, are not specific enough for epidemiological investigations. Instead, initial procedures used plasmid profiling, chromosomal restriction endonuclease analysis and multilocus enzyme electrophoresis (Clark *et al.*, 1990; Nazarowec-White & Farber, 1999). This was followed by the application of random amplified polymorphic DNA ribotyping, pulsed-field gel electrophoresis (PFGE) and multiple-locus variable number tandem repeat analysis (Mullane *et al.*, 2008). To date, PFGE with two restriction enzymes (*Xba*1 and *Spe*1) is the most common method (Caubilla-Barron *et al.*, 2007). The technique is widely used and can be used for transnational investigations, as per PulseNet, because the gel results can be electronically analysed (http://www.cdc.gov/pulsenet/). PFGE is considered the gold standard for genetic typing and is recommended to be used in surveillance and investigations of sources of outbreaks.

Typing *Cronobacter* to understand its diversity has led to the development of a multilocus sequence typing (MLST) scheme which is available online (http://www.pubMLST.org/cronobacter) (Baldwin *et al.*, 2009). The sequencing of protein coding genes is a useful, more discriminatory alternative to partial 16S rDNA sequencing (ca. 528 nucleotide length), especially as unlinked sequences from multiple protein-coding genes are used. The *Cronobacter* MLST analysis is based on seven housekeeping genes; ATP synthase β chain (*atp*D), elongation factor G (*fus*A), glutaminyl-tRNA synthetase (*gln*S), glutamate synthase large subunit (*glt*B), DNA gyrase subunit B (*gyr*B), translation initiation factor IF-2 (*inf*B) and phosphoenolpyruvate synthase A (*pps*A). The seven sequenced alleles can be concatenated together to give $>$ 3000 nucleotides for phylogenetic analysis (Figure 2). This is six times the length of the commonly used partial 16S rDNA sequences, and has the additional advantage of considerably greater number of variable loci. The initial publication was focussed on *C. sakazakii* and *C. malonaticus* due to the reported difficulties in distinguishing between them (Baldwin *et al.*, 2009). The seven allele phylogenetic tree (Figure 2) is comparable to the 16S tree (Figure 1). The MLST analysis has revealed a remarkably strong clonal nature in *Cronobacter*. Of particular note, is that this study showed that sequence types (ST) existed which were primarily associated with infant formula (ST3), another both infant formula and clinical isolations (ST4), and another which was primarily composed of clinical isolates (ST8). The strains analysed were widely geographically, temporally and source distributed, some of which could be traced over a 50-year period. These clones may reflect different ecologies of the organism. ST8 indicates that there may be a source of *Cronobacter* which is not PIF associated. Therefore to focus on PIF analysis following a *Cronobacter* outbreak on a NICU may lead to oversight of the true source of the infection. As given above, the MLST scheme is accessible online and has been extended to cover all *Cronobacter* species. The scheme will be of considerable use in the future for choosing representative *Cronobacter* strains when undertaking further studies.

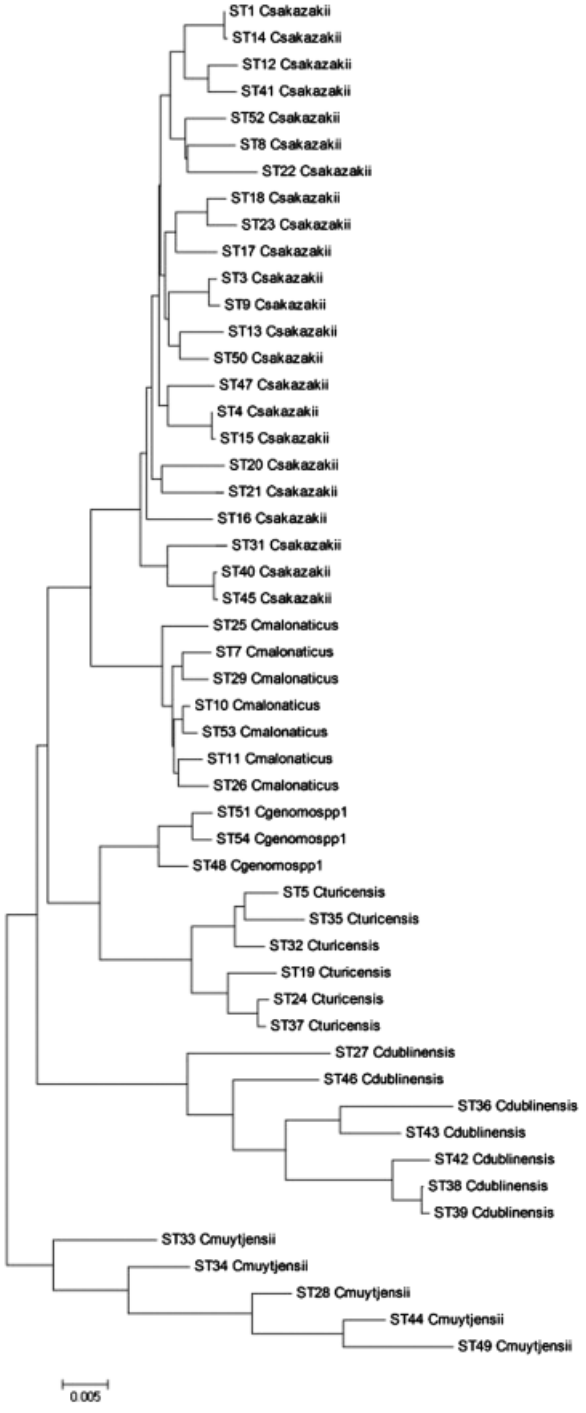

Figure 2 Mulitlocus sequence typing (7 loci, 3036 nt) gene neighbour joining phylogenetic tree of *Cronobacter* genus.

Ecology and physiological aspects

Plant-associated traits and sources

Iversen and Forsythe (2003) hypothesized that the *Cronobacter* species might be of plant origin due to physiological features such as the production of a polysaccharide capsule, production of a yellow pigment and its desiccation resistance. These traits may enable the organism to stick to plant leaves, protect against oxygen radicals from sunlight exposure and survive dry periods including autumn. About 80% of *Cronobacter* strains produce a non-diffusible, yellow pigment on Tryptone Soya Agar at 25 °C. Pigment production is temperature dependent, and even fewer strains

produce it at 37 °C. As given above, the organism probably colonizes plant material and the yellow carotenoid-based pigmentation may protect it from sunlight-generated oxygen radicals.

A productive source of *Cronobacter* strains are fresh or dried herbs and spices with ~30% incidence. In fact an early patent for a food thickener was material extracted from *E. sakazakii* isolated from Chinese tea (Scheepe-Leberkuhne & Wagner, 1986; Harris & Oriel, 1989). In order to provide evidence for the plant origin of *Cronobacter*, Schmid *et al.* (2009) investigated biochemical traits associated with plant microorganisms in nine strains representing the then recognized five *Cronobacter* species. All strains were able to solubilize mineral phosphate, produce indole acetic acid and produce siderophores. The strains were also able to endophytically colonize tomato and maize roots. The authors concluded that plants may be the natural habitat of *Cronobacter* spp. and that the rhizosphere might act as a reservoir of the bacterium. The plant association of *Cronobacter* may account for physiological traits such as surviving spray drying and prolonged periods in dry materials (i.e. starches), and presence in ingredients that are added to PIF without additional heat treatment (FAO/WHO, 2004, 2006). Unlike most other *Enterobacteriaceae*, the organism can persist in PIF for 2 years (Caubilla-Barron *et al.*, 2007). It is notable that, when the *E. sakazakii* species was defined, it included a strain that had been isolated from dried milk in 1960. Therefore, possibly *Cronobacter* has been present in dried milk products for many decades. For a fuller review of desiccation survival mechanisms please see Osaili and Forsythe (2009).

Cronobacter have the ability to survive osmotic stress and desiccation (Riedel & Lehner 2007; Osaili & Forsythe, 2009). They are able to take up osmoprotectants including trehalose [via phosphotrasferase system (PTS)], glycine, betaine, proline, spermidine and putrescine using ABC transporters.

Because the organism is probably plant associated it is not surprising that the organism can be isolated from a wide range of environments, including water, soil, herbs and spices, and a variety of processed foods and fresh produce (Friedemann, 2007). The resistance to plant essential oils may be linked to efflux pumps which contribute to the organism's resistance to osmotic pressure, and can be of use in the design of selective media.

As given above, MLST has revealed the organism may have a more complex ecology with non-plant ecosystems. The bacterium has been isolated from the hospital environment and clinical samples; cerebrospinal fluid, blood, bone marrow, sputum, urine, inflamed appendix, neonatal enteral feeding tubes and conjunctivae. Asymptomatic human carriage (intestines and throat) have also been reported. The bacterium has been isolated from factories producing milk powder, household vacuum cleaning bags and also from household utensils used for the reconstitution of PIF (Muytjens, Roelofs-Willemse & Jaspar, 1988; Bar-Oz *et al.*, 2001; Block *et al.*, 2002; Kandhai *et al.*, 2004).

PIF, follow-up formula and weaning foods

Cronobacter was first associated with contaminated PIF by Muytjens, Roelofs-Willemse and Jaspar (1988) when it was isolated from prepared formula and reconstitution equipment. They reported 52.2% ($n = 141$) of PIF samples from 35 countries contained *Enterobacteriaceae*, with 14% containing *Cronobacter* spp. (Muytjens, Roelofs-Willemse & Jaspar, 1988). A more recent international survey for *Cronobacter* and related organisms in PIF, follow-up formula and infant foods was conducted by eight laboratories in seven countries in response to the call for data in preparation for the FAO/WHO (2008) risk assessment. In total, 290 products were analysed using a standardized procedure. *Cronobacter* was isolated from 3% ($n = 91$) follow up formulae and 12% ($n = 199$) infant foods and drinks (Chap *et al.*, 2009). The few reported quantitative studies do not show any samples with *Cronobacter* at levels > 1 cell g^{-1} PIF. In fact < 1 cell in 100 g may be more representative and explains why large sample volumes (30×10 g) are required for testing. Hence, the need to consider opportunities for extrinsic bacterial contamination and multiplication during formula preparation. Because of the ubiquitous presence of *Cronobacter* and its resistance to dry conditions, contamination of food products including PIF is difficult to avoid. *Cronobacter* does posses the gene encoding the universal stress protein UspA, which is also found in other closely related *Enterobacteriaceae* (*Escherichia coli*, *E. cloacae*, *Citrobacter koseri* and *Pantoea* spp.). In *E. coli* the protein is induced following both heat and osmotic shock. Hence it may be important in the survival of *Cronobacter* during manufacturing processes and the cross-induction of other protection mechanisms.

Capsule and biofilm formation

Cronobacter do not have the *pgaABCD* locus which in *E. coli* promotes its binding to abiotic surfaces and encodes for β-1,6-*N*-acetyl-D-glucosamine. Instead, the organism often produces a heteropolysaccharide capsule composed of glucuronic acid, D-glucose, D-galactose, D-fucose and D-mannose. Strains from NICU outbreaks produce so much

capsular material that on milk agar plates the colonies drip onto the lid of inverted Petri dishes (Caubilla-Barron *et al.*, 2007). This material has been patented for use as a thickening agent in foods (Scheepe-Leberkuhne & Wagner, 1986; Harris & Oriel, 1989). The capsular material, induced under nitrogen-limited conditions, could facilitate the organism's attachment to plant surfaces. Combined with a tolerance to desiccation, this gives the organism an armoury to colonize plant material and maybe survive harsh, environmental conditions. These traits may also contribute to the organism's presence in starches used in the manufacture of infant formula and persistence during the manufacturing process. The organism attaches to surfaces, forming biofilms that are resistant to cleaning and disinfectant agents, and the organism has been isolated as part of the mixed flora biofilm in enteral feeding tubes of neonates not fed PIF (Hurrell *et al.*, 2009b).

Cronobacter is able to adhere to silicon, latex and polycarbonate and to a lesser extent to stainless steel (Iversen & Forsythe, 2003). Furthermore, *Cronobacter* has been reported to attach and form biofilm on glass and polyvinyl chloride (Lehner *et al.*, 2005). All of these materials are commonly used for infant-feeding and food preparation equipment and, if contaminated, may increase the risk of infection. Beuchat *et al.* (2009) reported that the ability of *Cronobacter* to form a biofilm is affected by the composition of the media, and that it is enhanced by infant formula components. The infant formula composition can also increase *Cronobacter* resistance to disinfectants, as shown by Beuchat and colleagues who examined the effect of thirteen disinfectants commonly used in infant formula preparation areas (Kim *et al.*, 2007). Populations of *Cronobacter* cells suspended in water (ca. $7 \log \mathrm{CFU\,mL^{-1}}$) decreased to undetectable levels ($< 0.3 \log \mathrm{CFU\,mL^{-1}}$) within 1–5 min of treatment with disinfectants, whereas numbers of cells in reconstituted PIF diminished by only $0.02–3.69 \log \mathrm{CFU\,mL^{-1}}$ after treatment for 10 min. Furthermore, cells attached to stainless steel were less resistant to disinfectants. It is clear that the ability of *Cronobacter* to attach to surfaces, form biofilms, and resist dry stress conditions contribute to the risk of *Cronobacter* ingestion. Moreover, the composition of PIF has a strong protective effect on the survival of *Cronobacter*.

Cronobacter appear to have the carbon storage regulatory (Csr) system as evident by the presence of *Csr*A in both *C. sakazakii* and *C. turicensis* genomes. Although its regulatory role in *Cronobacter* is unknown at present, its role in *E. coli* has been well established. CsrA is an RNA-binding protein that binds to the untranslated leader sequences of target mRNAs and alters their translation and stability. It represses stationary phase processes, including glycogen synthesis and catabolism, gluconeogenesis and biofilm formation. It also activates glycolysis, motility and biofilm dispersal. Repression of biofilm formation by *Csr*A involves the synthesis and catabolism of intracellular glycogen. Therefore biofilm formation in *Cronobacter* is probably linked to central carbon metabolism.

Finally, high levels of heat-stable lipopolysaccharide (endotoxin) in infant formula may enhance the translocation of *Cronobacter* across both the intestines and the blood–brain barrier, and therefore increase the risk of bacteraemia in neonates (Townsend *et al.*, 2007). Levels of endotoxin vary 500-fold in PIF. The chemical structure of oligopolysaccharide from three *Cronobacter* species; *C. sakazakii* BAA-894, 767, *C. malonaticus* and *C. muytjensii* have been derived (MacLean *et al.*, 2009a, b; MacLean, Vinogradov *et al.*, 2009; Czerwicka *et al.*, 2010). The material is branched in *C. sakazakii* and linear in *C. muytjensii*. Whether the surface structure is important in virulence remains to be determined but may serve as a basis for serotyping and other characterization methods.

Temperature response

Cronobacter can grow over a wide temperature range. Because of interest in the organism and infant infections, growth and death rates have been determined in reconstituted infant formula. At room temperature (21 °C), *Cronobacter* had a doubling time of 40–94 min. The lowest permissible growth temperature is near refrigeration (~5 °C) and therefore the organism may grow following prolonged storage or poor temperature control. The maximum growth temperature (44–47 °C) is strain dependent, and the *C. sakazakii* type strain (ATCC 29544^{T}) is reported not to grow above 42 °C (Nazarowec-White & Farber, 1999) the temperature used for the ISO/TS 22964 isolation method . Recent studies show that *C. turicensis* grows at < 5 °C which is lower than other *Cronobacter* species and has a lower maximum temperature (Caubilla-Barron *et al.*, 2009).

Decimal reduction times and *z*-values vary considerably between strains, i.e. D_{55} 2–49 min, *z*-values 2–14 °C (Caubilla-Barron *et al.*, 2009). Early studies inferred the organism was very thermotolerant. However, subsequent work clarified that the organism was less thermotolerant than *Listeria monocytogenes* (Nazarowec-White & Farber, 1999). Nevertheless, the organism can survive spray drying albeit with a considerable reduction in viability, and the surviving cells may be severely damaged (Caubilla-Barron *et al.*, 2009). The

organism's tolerance to drying has been well noted, and it can survive for 2 years desiccated in infant formula and then rapidly grow on reconstitution (Barron & Forsythe, 2007).

The first and second FAO/WHO meetings (2004 and 2006) reviewed the organisms associated with neonatal infections, those found in PIF, and also those that had been epidemiologically linked (FAO/WHO, 2004, 2006). Subsequently, *Salmonella* and *Cronobacter* were designated Category A (clear evidence of causality), and other named *Enterobacteriaceae* and *Acinetobacter* were in Category B (causality plausible, but not yet demonstrated). In order to reduce the number of intrinsic bacteria and limit bacterial growth, the FAO/WHO (2004, 2006) expert committees proposed that PIF be reconstituted at temperatures no cooler than 70 °C, and that it is used immediately rather than stored (FAO/WHO, 2006, 2008). As stated earlier, a common feature in a number of outbreaks has been a lack of adequate hygienic preparation and temperature control of the reconstituted infant formula. A second outcome from the FAO/WHO meetings was the production of an online risk model; http://www.mramodels.org/ESAK/default.aspx. The model allows the user to compare the level of risk between different levels of contamination and reconstitution practices. The model was based on growth and death kinetic data for a limited number of *Cronobacter* strains. We recently extended the Risk Model to cover all organisms in Categories A and B; *Cronobacter* species, *Salmonella*, other named *Enterobacteriaceae* and *Acinetobacter* spp. It can be accessed from the UK FSA web site at http://www.foodbase.org.uk/results.php?f_category_id=&f_report_id=395. The data were generated using casein- and whey-based formulas as the type of formula affects bacterial lag times, growth and death rates.

As referred to above, the WHO guidelines for hygienic preparation of PIF are aimed at reducing the number of bacteria in the reconstituted product using hot water and limiting the time available for any survivors to multiply. However, a wider perspective is that neonates are frequently fed via enteral feeding tubes. These tubes are in place for prolonged periods (even several days) to reduce distress to the neonate by the gagging reaction. However, *Cronobacter*, and other opportunistic pathogens can attach and colonize these tubes which are at 37 °C, and at regular intervals receive fresh feed (Hurrell *et al.*, 2009a, b). This scenario is applicable to all neonates with nasogastric tubes, and not only those on reconstituted PIF. In fact *Cronobacter* and other *Enterobacteriaceae* have been isolated from such tubes in intensive care units from neonates receiving breast milk and various other feeding regimes at levels up to 10^7 CFU tube^{-1} (Hurrell *et al.*, 2009a, b). Therefore hygienic practices and avoidance of temperature abuse are vitally important regardless of the type of feed.

Cronobacter virulence

Adult and neonate infections

It may be a surprise to some readers but *Cronobacter* infections are not unique to neonates. In fact they occur in all age groups, with the greater incidence in the more immuno-compromised very young and elderly. A major difference between the age groups is the severity of infection in neonates. Infections caused by *Cronobacter* in adults comprise a wide range of symptoms from conjunctivitis, biliary sepsis, urosepsis and appendicitis to wound infection and pneumonia. Infections in neonates include infant meningitis and NEC (Gurtler, Kornacki & Beuchat, 2005). Adult patients at increased risk include those treated previously with antibiotics, immuno-compromised and elderly patients, those with medical implants or with acute, chronic, or serious illnesses (Pitout *et al.*, 1997; Sanders & Sanders, 1997).

According to the FAO/WHO (2008) around the world there have been 120 documented *Cronobacter* cases and at least 27 deaths (FAO/WHO, 2008). This is undoubtedly an underestimate. In the United States, the reported *Cronobacter* infection incidence rate is 1 per 100 000 infants. This incidence rate increases to 9.4 per 100 000 in infants of very low birth weight, i.e. < 1.5 kg. Symptoms in neonates include NEC, septicaemia and meningitis. The former is non-invasive (and is multifactorial), whereas in septicaemia and meningitis the organism has attached and invaded presumably through the intestinal epithelial layer. NEC is a common gastrointestinal illness in neonates and can be caused by a variety of bacterial pathogens. It is characterized by ischaemia, bacterial colonization of the intestinal tract and increased levels of proteins in the gastrointestinal lumen. The incidence of NEC is 2–5% of premature infants and 13% in those weighing < 1.5 kg at birth. It is 10 times more common in infants fed formula compared with those fed breast milk (Lucas & Cole, 1990). NEC has a high mortality rate; 15–25% of cases (Henry & Moss, 2009).

Cronobacter has been implicated as a causative agent of NEC, but its role in the pathogenesis of the disease is somewhat unclear. There are reports of *Cronobacter* isolation from babies who developed NEC (van Acker *et al.*, 2001; Caubilla-Barron *et al.*, 2007). This suggests that there is an association between *Cronobacter* occurrence and NEC,

although until recently, the organism has not been conclusively proven to cause the disease.

Infant meningitis can be caused by a variety of bacterial pathogens, including *Cronobacter* and its close relatives *E. cloacae* and *C. koseri*. *Cronobacter*-related meningitis is characterized by a mortality rate of 40–80% and generally a very poor clinical outcome. The bacterium causes cystic changes, abscesses, fluid collection, brain infarctions, hydrocephalus, necrosis of brain tissue and liquefaction of white cerebral matter. Patients surviving *Cronobacter*-related meningitis often suffer from severe neurological sequelae, such as hydrocephalus, quadriplegia and retarded neural development (Lai, 2001). The infection usually arises between the fourth and fifth day after birth and it can be fatal within hours to days following the first clinical signs (Muytjens *et al.*, 1983). Compared with patients suffering from *Cronobacter*-induced enterocolitis, infants in whom meningitis developed tend to have normal gestational age and birth weight (Bowen & Braden, 2006).

Sources of infection

While the source of contamination in *Cronobacter*-related outbreaks has not always been confirmed, breast milk substitutes (one group of PIF products) have been epidemiologically or microbiologically established as the source of infection in a number of cases (Muytjens *et al.*, 1983; Biering *et al.*, 1989; Simmons *et al.*, 1989; Clark *et al.*, 1990; Muytjens & Kollee, 1990; van Acker *et al.*, 2001; Weir, 2002; Iversen & Forsythe, 2003). A strong link between the presence of *Cronobacter* in formula feeding and an outbreak of *Cronobacter* infection was established by Center for Disease Control and Prevention in 2002 following the outbreak in a NICU in Tennessee in 2001. In this outbreak, one neonate died from *Cronobacter*-induced meningitis and further 10 cases of *Cronobacter* colonization were found on the same unit. Later investigation revealed that the formula fed to the infant in Tennessee was in fact a formula that was not intended for neonates.

Infections have been directly linked to reconstituted PIF, which may have been contaminated intrinsically or during preparation and administration. A common feature in some of these outbreaks is the opportunity for temperature abuse of the prepared feed, which would permit bacterial growth. In reported outbreaks in France and United States, the neonates were fed using perfusion devices whereby the reconstituted PIF is slowly pumped at ambient temperature into the neonate through an enteral feeding tube (Himelright *et al.*, 2002; Caubilla-Barron *et al.*, 2007). Using this procedure there is the possibility of bacterial multiplication in the syringe leading to the ingestion of large numbers of *Cronobacter* by the neonate. The neonate has an immature immune system and a low intestinal microflora density. Consequently, if a large number of *Cronobacter* cells were ingested they would not be outcompeted by the resident intestinal flora. Following invasion of the intestinal cells, the lack of a developed immune system could make the neonate more prone to systemic infection. No infectious dose has been determined for neonates. Animal studies by Pagotto *et al.* (2003) and Richardson, Lambert and Smith (2009) have used large numbers of *Cronobacter* cells ($\sim 10^8$) for infection studies. Whether this number is reflective of that necessary for neonates is uncertain, but it does contrast with the number of cells reported in contaminated PIF (< 1 CFU g^{-1}), and may therefore indicate the role of temperature abuse in enabling bacterial multiplication.

It is pertinent to note that the bacterium is isolated from the tracheae and has been recovered from the feeding tubes of neonates fed breast milk and ready-to-feed formula, not infant formula (Hurrell *et al.*, 2009b). Therefore wider sources of the organism during an outbreak need to be investigated, not just the use of PIF. Infants can be colonized by more than one strain of *Cronobacter*, and therefore multiple isolates need to be characterized by PFGE in epidemiological investigations (Caubilla-Barron *et al.*, 2007).

Bowen and Braden (2006) have reported that there are a number of neonatal cases, which have no links with the ingestion of infant formula. Therefore in epidemiological investigations multiple sources should be sampled. Breast milk can contain the bacterium, and the *C. malonaticus* type strain (LMG 23826^T) was isolated from a breast abscess. In some countries breast milk from mothers with mastitis is still fed to the neonate. The organism has also been isolated from hospital air, human intestines and throats. So control of microbiological content of PIF will not necessarily totally remove the risk of neonate infection by this bacterium.

Virulence factors

All *Cronobacter* species have been associated with clinical infections in infants or adults and are considered potentially pathogenic. To date, isolates from infected neonates have been limited to only three species; *C. sakazakii*, *C. malonaticus* and *C. turicensis* (Kucerova *et al.*, 2010). These species can invade human intestinal cells, replicate in macrophages and invade the blood–brain barrier. It is known that *Cronobacter* strains and species vary in their virulence (Caubilla-Barron *et al.*, 2007). *In vitro* studies have shown

that bacterial attachment and invasion of mammalian intestinal cells, macrophage survival and serum resistance is comparable with *E. cloacae* and *C. freundii*, but less than that for *Salmonella* Typhimurium (Townsend *et al.*, 2007). Strains from *C. sakazakii* and *C. malonaticus* showed higher invasion of Caco-2 (human cell line) than other *Cronobacter* species. Similarly *C. sakazakii* and *C. malonaticus* survive and replicate in macrophages inside phagosomes, whereas *C. muytjensii* die and *C. dublinensis* is serum sensitive. Virulence also varies within the *C. sakazakii* species. This was determined from epidemiological studies of an NICU outbreak in France where the clinical outcome of three *C. sakazakii* pulsetypes varied with only one pulsetype causing the three deaths (Caubilla-Barron *et al.*, 2007). Additionally this variation in virulence is supported by mammalian tissue culture studies (Pagotto *et al.*, 2003; Townsend *et al.*, 2007; Townsend, Hurrell & Forsythe, 2008) and appears to have been confirmed by recent MLST studies (Baldwin *et al.*, 2009). OmpA is produced by *Cronobacter* and has been used as an identification trait (Mohan Nair & Venkitanarayanan, 2006). This protein has been extensively studied in *E. coli* K1 as contributes to the organism's serum resistance, adhesion to host cells and invasion of brain microvascular endothelial cells. It is logical to predict that it also has a role in *Cronobacter* penetrating the blood–brain barrier. However, the mechanism leading to the destruction of the brain cells is unknown and could, in part, be a host response. *Cronobacter* may invade the tissues using pathogenic secretory factors (elastases, glycopeptides, endotoxins, collagenases and proteases) which increase the permeability of the blood–brain barrier and allow the organism to gain access to the nutrient-rich cerebral matter (Iversen & Forsythe, 2003). Only a limited number of animal studies have been undertaken on *C. sakazakii*, principally by Pagotto *et al.* (2003) and Richardson, Lambert and Smith (2009) but these have confirmed the variation within the species.

In *Cronobacter* meningitis there is gross destruction of the brain, leading sadly to either death (40–80% of cases) or severe neurological damage. This pathogenesis is different to that caused by both *Neisseria meningitidis* and neonatal meningitic *E. coli*. Some reports suggest a similarity between the tropism of *Cronobacter* and the closely related organism *C. koseri* for invasion and infection of the central nervous system (Willis & Robinson, 1988; Burdette & Santos, 2000). Kline noted that brain abscesses caused by *Cronobacter* and *C. koseri* were morphologically similar and may be due to similar virulence mechanisms (Kline, 1988). Although the production of an enterotoxin by some *Cronobacter* strains described by Pagotto *et al.* (2003) is widely acknowledged, the genes encoding the putative toxin have yet to be identified. The *C. sakazakii* type strain ATCC 29544^T showed no enterotoxin production in their study, which confirms that there are considerable differences in virulence among *Cronobacter* strains and that some strains may be non-pathogenic. Kothary *et al.* (2007) characterized a zinc metalloprotease, zpx, which was unique to 135 *Cronobacter* strains tested, which could allow the bacterium to penetrate the blood–brain barrier and cause meningitis. The protein is found in all *Cronobacter* species (Kucerova *et al.*, 2010), although there is some sequence variation (Kothary *et al.*, 2007). Although *C. muytjensii* has not been associated with neonatal infections, one strain (ATCC 51329^T, source unknown) has been used in animal studies to demonstrate its potential to infect neonates (Mittal *et al.*, 2009).

Townsend *et al.* (2007) showed that *Cronobacter* can attach to intestinal Caco2 cells and survive in macrophages, but the invasion mechanism remains unknown. Kucerova *et al.* (2010) referred to a prophage encoding a protein homologous to the Eae adhesion protein. This, however, only encodes for a small portion of the protein and probably has no physiological relevance. Kim and Loessner (2008) suggested that the invasion of *Cronobacter* to Caco2 cells may be receptor-mediated, as the bacterial invasion showed characteristics of saturation kinetics. The authors also concluded that bacterial *de novo* protein synthesis was required for invasion. In the same study, pretreatment of Caco2 cells with an actin polymerization inhibitor resulted in decreased invasiveness of *L. monocytogenes* and *Salmonella* Typhimurium, but enhanced the invasiveness of *Cronobacter*. The authors hypothesized that this enhancement was due to the disruption of tight junction, a membrane-associated structure that acts as a barrier against the molecular exchange between epithelial cells. This was confirmed when the disruption of the tight junction by EGTA significantly increased the invasive properties of *Cronobacter*. They also speculated that frequent lipopolysaccharide contamination of PIF that is known to disrupt tight junctions might contribute to the invasiveness of *Cronobacter* (Kim & Loessner, 2008).

Townsend, Hurrell and Forsythe (2008) studied seven *C. sakazakii* strains associated with the largest reported NICU outbreak with the most reported deaths to date. All strains were able to attach and invade intestinal cells Caco2 more than *E coli* K12 and *Salmonella* Enteritidis. Two strains (767 and 701), both associated with fatal cases of meningitis and NEC, showed the highest invasion rates. These two strains were also able to replicate within macrophages, while all other strains survived inside macrophages for at least 48 h (Townsend, Hurrell & Forsythe, 2008).

Antibiotic susceptibility

When an infection by *Cronobacter* occurs, it is essential to provide rapid antibiotic treatment. Although the bacterium tends to be more sensitive to most antibiotics compared with other *Enterobacteriaceae*, its increasing resistance to some antibiotics has proven problematic. *Cronobacter*-related infections have been traditionally treated with ampicillin combined with gentamicin or chloramphenicol (Lai, 2001). Unfortunately, the organism has developed resistance to ampicillin (Muytjens *et al.*, 1983; Lai, 2001) and gentamicin use is limited as it fails to reach sufficient concentrations in the cerebral spinal fluid (Iversen & Forsythe, 2003). In 1980, all strains tested by Farmer (1980) were susceptible to ampicillin. In 2001, Lai described five cases of *Cronobacter* infection in which one or more of the isolates were resistant to ampicillin and most cephalosporins of first and second generation. Kim *et al.* (2008) reported frequent resistance of *Cronobacter* food isolates to ampicillin and cephalotin. For this reason, the shift to carbapenems or third generation cephalosporins with an aminoglycoside or trimethoprim with sulfamethoxazole was proposed. This treatment improved the outcome of *Cronobacter* meningitis, but may also have caused the increase in resistance to these antimicrobials (Lai, 2001).

Initial reports concerning the ability of *Cronobacter* to produce β-lactamases gave conflicting results. The presence of β-lactamases in *Cronobacter* was reported in a study by Pitout *et al.* (1997) when all tested strains were positive for Bush group 1 β-lactamase (cephalosporinase). In 2001, Lai reported increasing β-lactamase production among *Cronobacter* strains. Similarly, Block *et al.* (2002) reported that all *Cronobacter* isolates tested were β-lactamase positive. However, Stock and Wiedemann (2002) did not find any evidence of β-lactam production in the 35 *Cronobacter* strains tested. The discrepancy in the results might be due to the different selection of strains, the limited number of strains used, as well as differences in the experimental protocol. Also, some *Enterobacter* strains express β-lactamases at very low levels, which might have not been detectable by the methods used.

Genome studies

Genome description of *C. sakazakii* and *C. turicensis*

The genome of the *C. sakazakii* strain (BAA-894) from the formula associated with the neonate infection in Tennessee (Himelright *et al.*, 2002) has been sequenced and published (Kucerova *et al.*, 2010). This can be compared with the genome sequence of *C. turicensis* strain z3032, which was also associated with a neonatal infection and has been sequenced by the Technische Universitaet Muenchen, Germany. The sequences are available online (RefSeq numbers NC_009778 and NC_013282, respectively). Additionally, the proteome of the same *C. turicensis* strain has been published, which will considerably assist in our understanding the organism (Carranza *et al.*, 2009). The genome of *C. sakazakii* comprises a 4.4 Mbp chromosome and two plasmids (31 and 131 kbp). The *C. turicensis* z3032 genome is similar; chromosomal size 4.4 Mbp, three plasmids (20, 50 and 140 kbp). The %GC of both *Cronobacter* strains is 57–58%, which is greater than that of the closely related organism *E. cloacae*. Both *Cronobacter* strains have a large plasmid (131 and 140 kbp) with the same %GC ratio as the chromosome and a large number of genes, as well as smaller plasmids with a lower (51%) GC content. Despite the apparent similarities between the plasmids with respect to sizes and %GC content, caution should be exercised as plasmids do vary between species and in early work plasmid profiling was used for epidemiological purposes (Clark *et al.*, 1990). Three putative prophage genomes and three putative prophage fragments were identified in *C. sakazakii* BAA-894. These have been described in detail already (Kucerova *et al.*, 2010). *C. turicensis* z3032 genome also contains at least three putative prophages as identified by Prophinder (Lima-Mendez *et al.*, 2008). The presence of these phage regions is important as prophages can play an important role in evolution of bacteria by introducing novel genes of different biological functions and contribute to their virulence properties.

Comparative genomic hybridization studies of the *Cronobacter* species

Of the 4382 annotated genes, ~55% (2404) were present in all *C. sakazakii* strains, and 43% (1899) were present in all *Cronobacter* species (Kucerova *et al.*, 2010). Note that when genes defined as intermediate are included, the core gene set for *C. sakazakii* species constitutes 80.9% (3547) genes and core gene for *Cronobacter* genus includes 75.3% (3301) genes. The vast majority of these shared genes are predicted to encode cellular essential functions such as energy metabolism, biosynthesis, DNA, RNA and protein synthesis, cell division and membrane transport. The proportion of genes absent from test strains compared with *C. sakazakii* BAA-894 ranged from 10.3% (453) in *C. sakazakii* strain 20–17.1% (751) in *C. muytjensii* (Kucerova *et al.*, 2010).

Whole-genome clustering based on the comparative genomic hybridization data by Kucerova and colleagues revealed that *Cronobacter* strains formed two distinct

Table 2 Distribution of gene clusters across the *Cronobacter* genus

Gene loci	Description	*C. sakazakii* (*n* = 6)	*C. malonaticus*	*C. turicensis*	*C. muytjensii*	*C. dublinensis*
ESA_00257–ESA_00258	Toxin/antitoxin RelE/RelB	Yes	No	No	No	No
ESA_01116–ESA_01119	ABC-type multidrug efflux	Yes	No	No	No	No
ESA_01448–ESA_01450	Fatty acid desaturases	Yes	No	Yes	Yes	Yes
ESA_02125–ESA_02129	Ecotin (ESA_02129)	Yes	Yes	No[1]	No[1]	No[1]
ESA_02538–ESA_02542	Fimbriae	Yes	No	No	No	No
ESA_02544–ESA_02547	β-glucosides metabolism	Yes	Yes	No	Yes	Yes
ESA_02549–ESA_02553	Multidrug efflux system	Yes	Yes	Yes	No	Yes
ESA_02616–ESA_02618	Mannosyl-D-glycerate uptake	Yes	Yes	No	Yes	Yes
ESA_02795–ESA_02799	Fimbriae	Yes	No	No	No	No
ESA_03301–ESA_03320	Mannose metabolism	Yes	No	No	No	No
ESA_03609–ESA_03613	Mannose metabolism	Yes	No	No	No	No
ESA_04067–ESA_04073	Fimbriae	Yes	No	No	No	No
ESA_04101–ESA_04106	Cell wall biogenesis	Yes	No	Yes[2]	Yes[2]	Yes[2]

[1]The presence/absence status in *Cronobacter* species relates to the gene for ecotin.
[2]See main text for details about absence/presence of particular genes from this cluster.

phylogenetic clusters. All *C. sakazakii* strains formed one cluster, whereas *C. malonaticus*, *C. turicensis*, *C. dublinensis* and *C. malonaticus* formed a second, separate cluster. Within *C. sakazakii*, strains 701 and 767 were the most closely related and clustered together with strain 20. Previously, strains 701 and 767 were shown to belong to the same PFGE restriction digestion type (Caubilla-Barron *et al.*, 2007). Although the clinical details of the source of *C. sakazakii* strain 20 are unknown, the strain belongs to MLST 4 (as do 701 and 767). This is a stable clone of *C. sakazakii* isolated from both PIF and clinical sources (Baldwin *et al.*, 2009). *C. sakazakii* strain ATCC 29544^T (species type strain) formed a separate branch within the *C. sakazakii* cluster. The remaining *Cronobacter* species formed sub-clusters: *C. malonaticus* clustered with *C. turicensis* and *C. dublinensis* grouped with *C. muytjensii*.

The differences in gene content that contributed to the separation of *Cronobacter* species into different branches were further analysed. Thirteen gene clusters that were present in all *C. sakazakii* strains but absent in some or all other *Cronobacter* species were identified and denoted SR1–SR13. The presence of these regions in different *Cronobacter* species is summarized in Table 2. SR 1 (ESA_00257–ESA_00258) encodes a putative toxin/antitoxin pair RelE/RelB, which, if encoded on plasmids, may help to maintain a plasmid in a bacterial population. When encoded on a chromosome, however, the toxin/antitoxin system probably represents selfish DNA. SR2 (ESA_01116–ESA_01119) is a cluster of genes encoding a complete ABC-type multidrug efflux system. ESA_01116 encodes a multidrug efflux pump, ESA_01117 encodes an outer membrane efflux protein from a family including TolC, ESA_01118 is the permease component of the ABC-type system and ESA_01119 encodes the ATPase component of the efflux system. SR3 (ESA_01448–ESA_01450) encodes three proteins from the family of fatty acid desaturases. Members of this family are involved in cholesterol biosynthesis and biosynthesis of a plant-like cuticular wax, but may be implicated in other biosynthetic pathways. SR4 (ESA_02125–ESA_02129) encodes a diverse group of proteins where no common assignment to a pathway or mechanism could be found; it includes acetyltransferases, a transcriptional regulator from the lysR family and a putative esterase/lipase. ESA_02129 encodes a serine protease inhibitor ecotin; ecotins from species that come into contact with the mammalian immune system like *E. coli*, *Yersinia pestis* and *Pseudomonas aeruginosa* have been shown to protect bacteria against the effects of neutrophil elastase (Eggers *et al.*, 2004). SR5 (ESA_02538–ESA_02542) is a cluster of fimbrial genes (described in 5.3). The genes in SR6 (ESA_02544–ESA_02547) are involved in the metabolism of β-glucosides. ESA_02544 is a transcriptional antiterminator from the BglG family, which is involved in positive control of the utilization of different sugars by transcription antitermination (Bardowski, Ehrlich & Chopin, 1994). ESA_02545 encodes a kinase than converts β-glucosides to 6-phospho-β-glucosides and ESA_02546 encodes a 6-phospho-β-glucosidase (EC 3.2.1.86) specific to arbutin-6 phosphate and salicilin-6-phosphate. ESA_02547 also encodes 6-phospho-β-glucosidase (EC 3.2.1.86), which may have the same or similar function as ESA_02546. SR7 (ESA_02549–ESA_02553) may encode a complete ABC

Table 3 *C. sakazakii* BAA-894 fimbrial clusters and their presence in other *Cronobacter* strains

		C. sakazakii									
Locus tag	Gene Product	1[1]	2	20	701	767	696	*C. malonaticus*	*C. turicensis*	*C. muytjensii*	*C. dublinensis*
ESA_01976	Pilin FimA	−1	1	−1	−1	−1	−1	−1	−1	−1	−1
ESA_01975	Chaperone FimC	−1	0	−1	−1	−1	−1	−1	−1	−1	−1
ESA_01974	Usher FimD	−1	1	−1	−1	−1	−1	−1	−1	−1	−1
ESA_01973	Pilin FimA	−1	1	−1	−1	−1	−1	−1	−1	−1	−1
ESA_01972	Pilin FimA	−1	1	−1	−1	−1	−1	−1	−1	−1	−1
ESA_01971	Pilin FimA	−1	1	−1	−1	−1	−1	−1	−1	−1	−1
ESA_01970	Pilin FimA	−1	1	−1	−1	−1	−1	−1	−1	−1	−1
ESA_02538	Pilin FimA	1[2]	1	1	1	1	1	−1	−1	−1	−1
ESA_02539	Chaperone FimC	0	1	1	0	0	1	−1	−1	−1	−1
ESA_02540	Usher FimD	1	1	1	1	1	1	−1	−1	−1	−1
ESA_02541	Pilin FimA (FimH)	1	1	1	1	1	1	−1	−1	−1	−1
ESA_02542	Putative minor component FimG	1	1	1	1	1	1	−1	−1	−1	−1
ESA_02799	Putative fimbrial protein	1	1	0	0	v0	0	0	−1	−1	−1
ESA_02798	Chaperone FimC	1	1	1	1	1	1	−1	−1	−1	−1
ESA_02797	Usher FimD	1	1	1	1	1	1	−1	−1	−1	−1
ESA_02796	Pilin FimA	1	1	1	1	1	1	−1	−1	−1	−1
ESA_02795	Fimbrial protein	1	1	1	1	1	1	−1	−1	−1	−1
ESA_04067	Putative fimbrial protein	0	1	1	1	0	1	−1	−1	−1	−1
ESA_04068	Fimbrial protein	−1	1	0	−1	−1	0	−1	−1	−1	−1
ESA_04069	Fimbrial protein	0	1	1	1	1	1	−1	−1	−1	−1
ESA_04070	Fimbrial protein	0	1	1	1	1	1	−1	−1	−1	−1
ESA_04071	Usher FimD	1	1	1	1	1	1	−1	−1	−1	−1
ESA_04072	Chaperone FimC	1	1	1	1	1	1	0	0	−1	−1
ESA_04073	Fimbrial protein	0	1	1	1	1	1	−1	−1	−1	−1

[1]*C. sakazakii* strain number, see Kucerova *et al*. (2010) for details.
[2]According to CGH analysis 1 = present, 0 = intermediate, −1 = absent.

multidrug transport system. SR8 (ESA_02616–ESA_02618) contains genes related to mannose metabolism. ESA_02616 encodes an α-mannosidase, ESA_02617 is a gene taking part in the mannosyl-D-glycerate uptake via the phosphotransferase system and ESA_02618 encodes a mannosyl-D-glycerate transport/metabolism system repressor. SR9 (ESA_02795–ESA_02799) is a fimbrial cluster (described in 5.3). Genes in SR10 (ESA_03301–ESA_03305) encode proteins involved in the metabolism of fructose and mannose via the PTS and a putative porin KdgM. ESA_03301 encodes an isomerizing glucosamine-fructose-6-phosphate aminotransferase. ESA_03302, located on the opposite strand to the rest of the genes in this cluster, encodes an oligogalacturonate-specific porin protein (KdgM). ESA_03303 encodes a fructose-specific II component of the PTS system FruA, which converts fructose to fructose-1-phosphate. ESA_03304 encodes an α-mannosidase involved in mannose degradation. ESA_03305 encodes a phosphomannose isomerase, which converts D-mannose to β-D-glucose-6-phosphate. SR11 (ESA_03609–ESA_03613) is a cluster of genes also putatively involved in metabolism of mannose and other sugars. ESA_03609 encodes a putative β-galactosidase. Genes ESA_03610 and ESA_03612 encode genes involved in the *N*-acetylneuraminate and *N*-acetylmannosamine degradation pathway. ESA_03610 encodes a *N*-acetylmannosamine kinase and ESA_03611 encodes a *N*-acetylneuraminate lyase. Gene ESA_03612 encodes a transcriptional regulator from the GntR family. SR 12 (ESA_04067–ESA_04073) is a cluster of fimbrial genes. SR13 (ESA_04101–ESA_04106) encodes genes that may be involved in the O-PS biogenesis. ESA_04102 encodes a glycosyltransferase involved in cell wall biogenesis, ESA_04103 encodes a putative O-antigen ligase or a related enzyme. Genes ESA_04104 and ESA_04105 encode glycosyltransferases and ESA_04105 encodes a putative lipopolysaccharide heptosyltransferase III. This cluster is a part of a larger cluster of genes involved in LPS biogenesis, however, it is not related to the O-antigen cluster defined by Mullane *et al.* (2008). The genes from this cluster were absent in *C. malonaticus*, but present or intermediate in all other *Cronobacter* species, but the putative O-

Table 4 Iron uptake systems in *C. sakazakii* BAA-894 and other *Cronobacter* strains

			C. sakazakii									
Gene	Esak homologue (BLAST)	Locus Tag	1[1]	2	20	701	767	696	*C. malonaticus*	*C. turicensis*	*C. muytjensii*	*C. dublinensis*
Enterobactin synthesis – non-ribosomal peptide synthesis pathway												
entA	2,3-dihydroxybenzoate-2,3-dehydrogenase	ESA_00799	1[2]	1	1	1	1	1	1	1	0	1
entB	Hypothetical protein ESA_00798	ESA_00798	1	1	1	1	1	1	1	1	1	1
entC	Hypothetical protein ESA_00796	ESA_00797	0	1	0	0	0	0	1	1	1	1
entD	Hypothetical protein ESA_02731	ESA_02731	1	1	1	1	1	0	0	1	0	0
entE	Enterobactin synthase subunit E	ESA_02729	0	1	0	0	1	1	0	1	0	1
entF	Enterobactin synthase subunit F	ESA_02727	1	1	0	0	1	0	1	1	0	1
entS	Enterobactin exporter EntS	ESA_00794	1	1	1	1	1	1	1	1	1	1
Enterobactin receptor and transporters												
fepA	Outer membrane receptor FepA	ESA_02730	1	1	1	1	1	1	1	1	1	1
fepB	Iron-enterobactin transporter	ESA_00796	1	1	1	1	1	0	1	1	1	1
fepC	Hypothetical protein ESA_00791	ESA_00791	1	1	1	1	1	0	1	1	1	1
fepD	Iron-enterobactin transporter	ESA_00793	1	1	0	0	1	0	1	1	1	1
fepE	Ferric enterobactin transport protein FepE	ESA_00459	1	1	1	1	1	1	0	0	0	−1
fepG	Iron-enterobactin transporter permease	ESA_00792	1	1	0	1	1	0	1	1	1	1
Salmochelin synthesis												
iroB	Salmochelin siderophore system	No match	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
iroC	Salmochelin siderophore system	No match	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
iroD	Enterobactin/ferric enterobactin esterase	No match	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
iroE	IroE protein	No match	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
iroN	Outer membrane receptor FepA	ESA_01552	1	1	1	1	1	1	0	0	1	1
Aerobactin synthesis and receptor												
iucA	Hypothetical protein ESA_pESA3p05547	ESA_pESA3p05547	1	1	0	0	1	1	1	1	−1	1
iucB	Hypothetical protein ESA_pESA3p05548	ESA_pESA3p05548	1	1	0	0	0	0	1	1	−1	1
iucC	Hypothetical protein ESA_pESA3p05549	ESA_pESA3p05549	0	0	0	0	0	0	1	1	−1	0
iucD	Hypothetical protein ESA_pESA3p05550	ESA_pESA3p05550	1	1	0	0	1	1	1	1	−1	1
iutA	Hypothetical protein ESA_pESA3p05551	ESA_pESA3p05551	1	1	1	1	1	1	1	0	−1	0
Other iron uptake genes												
feS	Enterobactin/ferric enterobactin esterase	No match	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
fhuA	Ferrichrome outer membrane transporter	ESA_03190	1	1	1	1	1	−1	1	0	−1	1
fhuB	Iron-hydroxamate transporter permease	ESA_03187	1	1	1	1	1	0	1	1	1	1
fhuD	Iron-hydroxamate transporter	ESA_03188	1	1	1	1	1	1	1	1	1	1
fpvA	Ferrichrome outer membrane transporter	No match	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
fur	Ferric uptake regulator	No match	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
ibpA	Heat shock protein IbpA	ESA_03960	1	1	1	1	0	1	1	1	1	1
ibpB	Heat shock chaperone IbpB	ESA_03959	1	1	1	1	1	1	1	1	1	1

[1] *C. sakazakii* strain number, see Kucerova et al. (2010) for details.
[2] According to CGH analysis 1 = present, 0 = intermediate, − 1 = absent.

antigen ligase (ESA_04103) was absent from all *Cronobacter* species except *C. sakazakii*.

Variations in virulence traits as revealed by CGH are covered in more detail in the following section.

The chemical structure of oligo-polysaccharide (O-antigen) in three *Cronobacter* species has been determined and shown to be compositionally and structurally different. It is therefore predictable that the biosynthetic pathways will vary across the genus and this has been confirmed by CGH. The gene cluster corresponding to the O-antigen cluster described by Mullane *et al.* (2008) (ESA_01177–ESA_01189) was examined. The genetic architecture of the O-antigen cluster in the sequenced *C. sakazakii* BAA-894 corresponds to the serotype O:1 as defined by Mullane and colleagues. According to our CGH data, two of the genes in this region, *gal*F (ESA_01177) and *rfb*B (ESA_01178) are conserved among all *Cronobacter* strains tested except *C. sakazakii* 696, whereas the rest of the genes from the O-antigen locus are highly divergent; its genes were not sufficiently similar to be detected by microarray hybridization in any other *Cronobacter* strains. This correlates with the findings of Mullane and colleagues, who showed that both serotypes O:1 and O:2 had the two genes *gal*F and *rfb*B in common, whereas the rest of the gene cluster content differed between the two serotypes.

Virulence traits and survival mechanisms

Because *Cronobacter* is associated with neonates and infants, the availability of iron in milk or formula could be an important virulence trait. A list of known iron assimilation mechanisms was compiled and their presence in different *Cronobacter* species was evaluated based on the available CGH data (Kucerova *et al.*, 2010). All *Cronobacter* strains

examined by CGH possess complete operons for enterobactin synthesis (*entABCDEFS*) and enterobactin receptor and transport (*fepABCDEG*), except *C. dublinensis*, in which *fepE* is absent (Table 4). All *Cronobacter* species except *C. muytjensii* also possess a complete operon for aerobactin synthesis *iucABCD* and its receptor *iutA*. The operon for salmochelin synthesis is missing in all *Cronobacter* species (Table 4). The strong genetic similarity between *C. sakazakii* and *C. koseri*, as well as urinary pathogenic *E. coli* is evident from the presence of all genes for enterobactin and aerobactin synthesis in these organisms. *C. sakazakii* can cause urinary tract infections, though to date this aspect has not been studied in any detail.

The route of infection is probably through attachment and invasion of the intestinal cells, and therefore genes encoding surface appendages such as pili (fimbriae) have been studied. Four putative fimbriae clusters were identified in the genome of *C. sakazakii* BAA-894, some of which were mentioned previously in Healy *et al.* (2009). These are cluster 1 (ESA_01976–ESA_01970), cluster 2 (ESA_02538–ESA_02542), cluster 3 (ESA_02795–ESA_02799) and cluster 4 (ESA_04067–ESA_04073) (Table 3). Further analysis of the comparative hybridization data showed that three of the four putative fimbrial clusters (clusters 2, 3 and 4) were *C. sakazakii* specific, i.e. were classified as present or intermediate in all five strains of *C. sakazakii*, but were absent in *C. muytjensii*, *C. dublinensis*, *C. turicensis* and *C. malonaticus*. Cluster 1 was present only in the reference strain and *C. sakazakii* strain 2, which suggests that it is strain specific. The genetic content of all fimbriae clusters was most similar to the type I chaperone/usher-assembled pilus system as defined in Kline *et al.* (2010). Genes for pilin FimA, chaperone FimC and usher FimD have been found in all four putative fimbriae clusters. These clusters may encode complete and functional pili, as some degree of homology was found between the other genes in the *C. sakazakii* fimbriae clusters and the remaining components necessary for type-I pilus assembly (the minor tip fibrillum FimG and fimbrial adhesin FimH). The presence of the putative fimbriae clusters in *Cronobacter* species according to the CGH data is summarized in Table 3.

Type VI secretion system (T6SS) is a newly described system that may be involved in adherence, cytotoxicity, host-cell invasion, growth inside macrophages and survival within the host. Five putative T6SS clusters were identified in the genome of *C. sakazakii* BAA-894, some of which were mentioned in Kucerova *et al.* (2010). Cluster 1 (ESA_00140–ESA_00145) encodes most of the proteins that are conserved across different T6SS clusters (a DotU homolog ESA_00141, Vgr homolog ESA_00141 and a putative lipoprotein from the VC_A0113 family ESA_00145). However, most T6SS clusters typically encode from 12 to 25 proteins (Filloux, Hachani & Bleves, 2008) and also encode a ClpV ATPase, which was not found in this cluster. In some instances, the genes encoding Vgr and DotU proteins are located outside the main T6SS locus, and their products might cooperate with proteins encoded in other loci. Cluster 2 (ESA_02035–ESA_02040) includes genes encoding a Vgr-type protein (ESA_02035), lipoprotein from VC_A0113 family ESA_02038 and other genes homologous to proteins encoded in T6SS clusters. Cluster 3 (ESA_02735–ESA_0240) contains genes encoding SciE-type protein (ESA_02736), Vgr-type protein (ESA_02739) and a protein homologous to phage gp7 protein, all of which are frequently found in T6SS clusters. However, this cluster is adjacent to a prophage fragment described in Kucerova *et al.* (2010) and due to the sequence similarities between T6SS and prophages it is difficult to conclude whether this cluster is a part of a T6SS. Cluster 4 (ESA_03899–ESA_03946) is the longest and the most complete cluster of T6SS genes. Its 48 genes include all the components of T6SS typically conserved among different T6SS systems, such as genes encoding Vgr-type proteins (ESA_03905 and ESA_03917), IcmF-type protein (ESA_03945), DotU-type protein (ESA_03946), ClpV ATPase (ESA_03921), SciE-type protein (ESA_03925), Ser/Thr protein phosphatase (ESA_03927) and Ser/Thr protein kinase (ESA_03920). This cluster may encode a complete and functional T6SS. Cluster 5 (ESA_pESA3p05491–ESA_pESA3p05506) encodes another putative T6SS cluster encoding some of the conserved T6SS proteins. ESA_pESA3p05494 encodes DotU-like protein, ESA_pESA3p05495 encoded a protein with a C-terminal extension with similarity to OmpA, a protein strongly associated with virulence properties of *Cronobacter*. ESA_pESA3p05497 encodes a ClpV ATPase and ESA_pESA3p05500 encodes a Vgr-like protein. The clusters 1–5 described here are putative T6SS clusters. It remains to be determined whether they encode functional type VI secretion systems or functional components of these.

Summary

The FAO/WHO (2004) expert committee recommended that research should be promoted to gain a better understanding of the ecology, taxonomy, virulence and other characteristics of *Cronobacter*. This has largely been undertaken by groups around the world. By understanding the organism better, improved detection systems have been

designed and commercialized. Currently microbiological criteria for *Cronobacter* spp. are required for infant formulas with an intended target age < 6 months. A presence/absence test is applied to large volumes due to the low ($< 1\,\mathrm{CFU\,g^{-1}}$) incidence of the organism in the product. Although the organism has been recovered from follow up formulas (infant formulas with intended target age > 6 months) and weaning foods, there is currently insufficient epidemiological evidence to support the implementation of criteria for these products. Readers should consult the relevant Codex (CAC, 2008) documents for details. With respect to clinical sources, MLST has revealed the organism is highly clonal and sources other than infant formula need to be considered; especially as a number of neonatal cases not attributable to PIF have been reported. *Cronobacter* does cause infections in all age groups. It is found in a wide range of foods, especially those of plant origin. While fortunately *Cronobacter* rarely causes severe meningitic and NEC infections, the heightened interest in the organism has resulted in improved regulatory control of products for the neonates and infants, as well as improved hygienic practices. Together these will reduce the risk of *Cronobacter* infection.

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