

## Review

*Cronobacter* spp. – opportunistic food-borne pathogens. A review of their virulence and environmental-adaptive traits

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The genus *Cronobacter* consists of a diverse group of Gram-negative bacilli and comprises seven species: *Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter muytjensii*, *Cronobacter turicensis*, *Cronobacter dublinensis*, *Cronobacter universalis* and *Cronobacter condimenti*.

*Cronobacter* are regarded as opportunistic pathogens, and have been implicated in newborn and infant infections, causing meningitis, necrotizing enterocolitis and bacteraemia or sepsis.

*Cronobacter* virulence is believed to be due to multiple factors. Some strains were found to produce diarrhoea or cause significant fluid accumulation in suckling mice. Two iron acquisition systems (*citCBAD* and *iucABCD/iutA*), *Cronobacter* plasminogen activator gene (*cpa*), a 17 kb type VI secretion system (T6SS), and a 27 kb filamentous haemagglutinin gene (*fhaBC*) and associated putative adhesins locus are harboured on a family of RepFIB-related plasmids (pESA3 and pCTU1), suggesting that these are common virulence plasmids; 98 % of 229 tested *Cronobacter* strains possessed these plasmids. Even though pESA3 and pCTU1 share a common backbone composed of the *repA* gene and *citCBAD* and *iucABCD/iutA* gene clusters, the presence of *cpa*, T6SS and FHA loci depended on species, demonstrating a strong correlation with the presence of virulence traits, plasmid type and species. Other factors were observed, in that *Cronobacter* form biofilms, and show unusual resistance to heat, dry and acid stress growth conditions. The outer-membrane protein A is probably one of the best-characterized virulence markers of *Cronobacter*. Furthermore, it was reported that *Cronobacter* employ phosphatidylinositol 3-kinase/Akt signalling, which activates protein kinase C- $\alpha$  and impairs the host cell's mitogen-activated protein kinase pathway, in order to invade cells.

*Cronobacter* can also use immature dendritic cells and macrophages to escape the immune response. This review addresses the various virulence and environmental-adaptive characteristics possessed by members of the genus *Cronobacter*.

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## Introduction

The genus *Cronobacter* consists of a diverse group of Gram-negative bacilli and comprises seven species: *Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter muytjensii*, *Cronobacter turicensis*, *Cronobacter dublinensis*, *Cronobacter universalis*, and *Cronobacter condimenti* (Iversen *et al.*, 2007, 2008a; Joseph *et al.*, 2012b, 2013a; Masood *et al.*, 2013a, b, c). Recently, Brady *et al.* (2013) suggested that three non-pathogenic *Enterobacter* spp. (*Enterobacter pulveris*, *Enterobacter helveticus* and *Enterobacter turicensis*), originally excluded by Iversen *et al.* (2008b), be included as members

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**Abbreviations:**  $a_w$ , water activity; BBB, blood–brain barrier; CNS, central nervous system; CSF, cerebrospinal fluid; FHA, filamentous hemagglutination; GIT, gastrointestinal tract; HBMEC, human brain microvascular endothelial cell; iNOS, inducible nitric oxide; MAPK, mitogen-activated protein kinase; MLST, multilocus sequence typing; NEC, necrotizing enterocolitis; PI3K, phosphatidylinositol 3-kinase; PIF, powdered infant formula; PKC, protein kinase C; ST, sequence type; T6SS, type VI secretion system; TGF, transforming growth factor.

of *Cronobacter*, but the current biological basis for this suggestion does not support further revision of the taxon at this time. As a result of this posited taxonomic uncertainty and the fact that no virulence attributes have been ascribed to these organisms, this review will not treat these *Enterobacter* spp. as members of the genus *Cronobacter*.

All *Cronobacter* spp., except for the single species epithet *C. condimentii*, have been associated with human infections (Cruz-Córdova *et al.*, 2012). Historically, *Cronobacter* have been implicated in newborn and infant infections, causing meningitis, necrotizing enterocolitis (NEC) and bacteraemia or sepsis (Healy *et al.*, 2010). However, not all *Cronobacter* spp. are linked to infantile infections and it is thought that virulence among strains may vary. *C. sakazakii*, *C. malonicus* and *C. turicensis* are the three species most often isolated from infantile cases (Joseph & Forsythe, 2011). For an understanding of recent unresolved issues remaining with respect to taxonomy, sources and clinical relevance, and for suggestions on how to safely feed premature neonates, see Holý & Forsythe (2013).

Infections in elderly and immunocompromised adults have also been reported (Healy *et al.*, 2010), and the epidemiology of these cases suggests that other potential sources of contamination exist, such as the home environment (Kandhai *et al.*, 2004; Kilonzo-Nthenge *et al.*, 2008), retail foods (e.g. dried milk powder, dried meats, legumes, nuts, dried flours and spices) (Friedemann, 2007; Hochel *et al.*, 2012) or drinking water (Lui *et al.*, 2013). Although *Cronobacter* spp. have been detected in this wide assortment of foods, only contaminated powdered infant formula (PIF) has been linked epidemiologically with infant infections and outbreaks caused by *Cronobacter* (Himelright *et al.*, 2002; Hunter & Bean, 2013). The source of this contamination is thought to be PIF manufactured under poor Good Manufacturing Practice; however, extrinsic contamination of opened cans and human carriage may also be possible. Some physiological traits exhibited by *Cronobacter*, such as the expression of an extracellular polysaccharide, the ability to produce a yellow pigment and the ability to resist desiccation during long dry periods, suggest that an environmental niche may be the source of these organisms (Schmid *et al.*, 2009). A further understanding of the molecular mechanisms involved in survival and persistence of *Cronobacter* would contribute significantly towards relating food contamination to its food matrix or environmental food production source, and will mitigate the spread of infectious disease occurring through food vehicles and allow for a reduction of illnesses caused by *Cronobacter*.

*Cronobacter* exhibit unusual resistance to heat, dry and acid stress growth conditions compared with other members of the family *Enterobacteriaceae* (Nazarowec-White & Farber, 1997; Breeuwer *et al.*, 2003; Edelson-Mammel *et al.*, 2005; Dancer *et al.*, 2009). They also form biofilms, which serve as a physical protective barrier from these environmental stresses as well as host immune surveillance mechanisms.

However, little is known about these virulence properties and antigenic determinants. The outer-membrane protein A (OmpA) is probably the best-characterized virulence marker of *Cronobacter* (Nair *et al.*, 2009; Mittal *et al.*, 2009a, b; Kim *et al.*, 2010). Furthermore, it was reported that *Cronobacter* employ phosphatidylinositol 3-kinase (PI3K)/Akt signalling to initiate actin microfilaments' rearrangement and the subsequent invasion of host cells. Protein kinase c (PKC)- $\alpha$  activation and impairment of the mitogen-activated protein kinase (MAPK) pathway are also important steps involved in the invasion process (Singamsetty *et al.*, 2008; Li *et al.*, 2010; Liu *et al.*, 2012b).

The fact that *Cronobacter* can exploit immature dendritic cells and persist within human macrophages indicates that *Cronobacter* possess some immune evasion properties that enable them to avoid the host's immune response, and reach and penetrate the blood-brain barrier (BBB) (Townsend *et al.*, 2007b, 2008; Mittal *et al.*, 2009b; Emami *et al.*, 2011; Joseph & Forsythe, 2011).

Attachment of *Cronobacter* to certain cell lines and the subsequent invasion were the focus of several reports in an attempt to understand the pathogenicity mechanisms of this pathogen (Mange *et al.*, 2006; Kim & Loessner, 2008; Singamsetty *et al.*, 2008; Townsend *et al.*, 2008; Giri *et al.*, 2012). Therefore, the objective of this review is to address the various virulence characteristics of members of the genus *Cronobacter* that contribute to its contamination of foods, and survival in the environment and host.

## Diseases and outbreaks caused by *Cronobacter*

### Infantile or neonatal infections

These organisms are regarded as opportunistic pathogens linked to life-threatening infections predominantly in neonates (infants <4 weeks old) (Bar-Oz *et al.*, 2001; Mullane *et al.*, 2008). Clinical presentation of *Cronobacter* infections in infants include NEC, bacteraemia and meningitis, with case fatality rates ranging between 40 and 80% being reported (Bowen & Braden 2006; Friedemann, 2007). Infections in older infants have also been noted (Bowen & Braden, 2006).

The Food and Agriculture Organization of the United Nations/World Health Organization statistics for 2006 estimated that the annual incidence rate in the USA among low-birth-weight infants who weighed <2500 g and were <1 year old was 8.7 per 100 000 (FAO/WHO, 2006). Globally, there is no active surveillance system for tracking this pathogen; however, in their 2008 report, the WHO Expert Panel tracked cases from 1961 to 2008, and found 120 recorded cases of *Cronobacter* among infants and children <3 years old (FAO/WHO, 2008). Although only ~120 cases have been reported worldwide, the actual number of cases is considered far higher (CDC, 2009; Friedemann, 2009; Teramoto *et al.*, 2010). Stoll *et al.* (2004) reported that only one case of sepsis caused by *Cronobacter* was diagnosed among 10 660 neonates, indicating that outside the epidemic outbreaks, *Cronobacter* are very rare in

low-birth-weight infants. Even though septicaemia was the diagnosis in this single case, the infant was breast fed, further emphasizing that other routes of nosocomial transmission may be present. Nonetheless, it is not the number of infections caused by *Cronobacter* that is the issue; it is rather the 80 % fatality rate associated with illness. In addition, it was reported that all patients recovering from central nervous system (CNS) infections develop chronic neurological and developmental disorders (Lai, 2001).

An early hypothesized source of *Cronobacter* infection was the birth canal. However, this source now seems to be unlikely, as none of the vaginally delivered infants developed signs of infection until several days after birth and it is also unlikely that *Cronobacter* constitute a part of the vaginal microflora (Yan *et al.*, 2012; Hunter & Bean, 2013). In addition, Biering *et al.* (1989) observed that infants who showed *Cronobacter* meningitis had all received reconstituted powdered-milk formula before falling ill. Furthermore, after a review of feeding procedures, a significant association was found between the consumption of certain brands of PIF, the development of NEC in neonates and the isolation of *Cronobacter* (van Acker *et al.*, 2001). Several *Cronobacter* outbreaks have occurred in neonatal intensive care units that were linked epidemiologically to contaminated PIF (Himelright *et al.*, 2002; Teramoto *et al.*, 2010). In a recent survey, the majority of the infant formula packages were found completely devoid of any micro-organisms (Jaradat *et al.*, 2009). However, extrinsic contamination of opened PIF cans or formula preparation utensils has also been reported (Noriega *et al.*, 1990; Friedemann, 2009; Jaradat *et al.*, 2009). Table 1 shows a list of the various foods and environments where *Cronobacter* have been isolated. Accordingly, the risk of infection may rely on different factors, including the number of bacterial cells contaminating the product, the source of *Cronobacter*, handling after preparation and the health status of the infants (e.g. low birth weight, prematurity, or immunosuppression) (Himelright *et al.*, 2002).

## NEC

It is generally thought that *Cronobacter* gain entrance to the human body through the gastrointestinal tract (GIT) where they may cause NEC (Liu *et al.*, 2012b). Grishin *et al.* (2013) point out that the development of NEC requires a susceptible host, typically a premature infant with physiological impairment (i.e. hypoxia, hypothermia, intestinal ischaemia), administration of enteral formula feeds (which lack the beneficial protective components normally found in breast milk) and uncontrolled bacterial colonization. These conditions lead to an increased degree of mucosal inflammation, which subsequently results in the production of high levels of host inflammatory factors, including cytokines, nitric oxide, platelet-activating factor and prostanooids, which further damage the apical GIT epithelium. These authors and others also suggest that the virulence of *Cronobacter* spp. is dose-dependent, but not due to a

property of a particular bacterial species as a whole, rather a characteristic(s) of certain strains, which may be innocuous in a full-term, yet pathogenic in a pre-term infant (Hamby *et al.*, 2011; Cetinkaya *et al.*, 2013). *In vitro* models of infection also suggest that the organism may also gain entrance into the systemic circulation through transcytosis of the GIT epithelium (Giri *et al.*, 2012).

## CNS infections

Once the organism has entered the systemic circulation, it has a tropism toward the CNS, thus increasing the propensity to cause meningitis among low-birth-weight neonates and infants, whilst causing bacteraemia or sepsis among slightly higher-birth-weight infants (Yan *et al.*, 2012) or adults. Once the pathogen crosses the BBB and enters the brain, it might cause ventriculitis and form cysts or brain abscesses, which later may develop into hydrocephalus – a condition where excessive accumulation of cerebrospinal fluid (CSF) in the brain occurs (Bowen & Braden, 2006; Chenu & Cox, 2009). The disproportionate accumulation of CSF results in an abnormal enlargement of the spaces in the brain called ventricles. The enlarged CSF-filled ventricles create a situation where the balance between CSF production and absorption is disturbed, which potentially leads to an increase of cranial pressure on the tissues of the brain.

## Infections associated with adults

Compared with infant cases, *Cronobacter* are more commonly detected in adults, with most case reports describing infections related to elderly patients who were previously ill or immunocompromised. Up to 50 % of adults with *Cronobacter* infection had an underlying malignancy (Lai, 2001; See *et al.*, 2007). Interestingly, *Cronobacter* have been linked to or should be considered as a pathogen that may cause sepsis, conjunctivitis, aspiration pneumonia in stroke patients, osteomyelitis, diarrhoea, acute cholecystitis, wound infections, abscesses associated with indwelling catheters and urinary tract infections (Gosney *et al.*, 2006; Friedemann, 2009; Flores *et al.*, 2011; Yan *et al.*, 2012; Tsai *et al.*, 2013).

Nosocomial infections by *Cronobacter*, such as aspiration pneumonia, urinary tract infections and conjunctivitis, are unlikely to be related to contaminated PIF, so medical equipment surfaces, water outlets and intrapersonal contacts may serve as possible sources of these infections (Friedemann, 2009; Flores *et al.*, 2011). However, other environmental sources of contamination are also possible. Jaradat *et al.* (2009) isolated *Cronobacter* from household vacuum dust in addition to isolating it from spices, indicating the strong association of the pathogen with a variety of dried food products and environmental factors. See *et al.* (2007) described a case of *Cronobacter* bacteraemia with multiple splenic abscesses in a 75-year-old institutionalized woman. It was the first reported case of *Cronobacter* with splenic abscesses and was the first case in a non-immunocompromised adult.

**Table 1.** Foods of animal and plant origin and environments where *Cronobacter* was isolated

| Food/environment                                                                                               | Reference                                                 |
|----------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------|
| <b>Infant food, beverages and processed foods</b>                                                              |                                                           |
| Infant food                                                                                                    | Jaradat <i>et al.</i> (2009); Chap <i>et al.</i> (2009)   |
| Follow-up formula                                                                                              | Chap <i>et al.</i> (2009)                                 |
| Rice flour                                                                                                     | Lampel & Chen (2009)                                      |
| Chocolate and cakes, vanilla cream bars                                                                        | Baumgartner <i>et al.</i> (2009)                          |
| Herbal tea, iced tea                                                                                           | Chap <i>et al.</i> (2009); Osaili & Forsythe (2009)       |
| Weaning foods                                                                                                  | Osaili & Forsythe (2009)                                  |
| Rice, corn, soy, grain, starches, potato flour, pasta, cereals                                                 | Osaili & Forsythe (2009)                                  |
| Tofu, sodium casinate                                                                                          | Osaili & Forsythe (2009)                                  |
| <b>Plants and spices</b>                                                                                       |                                                           |
| Liquorice, thyme, anise, chamomile, fennel, sage                                                               | Jaradat <i>et al.</i> (2009)                              |
| Mixed spices                                                                                                   | Jaradat <i>et al.</i> (2009)                              |
| Attieke, barley, biscuits, cereals, cowpea paste, dry nuts, grains, herbs and spices, red algae, sorghum, peas | Beuchat <i>et al.</i> (2009)                              |
| Dried herbs                                                                                                    | Molley <i>et al.</i> (2009)                               |
| Mixed spices, black pepper and white pepper, curry and mixed herbs                                             | Baumgartner <i>et al.</i> (2009)                          |
| Oats, barley, wheat                                                                                            | Molley <i>et al.</i> (2009)                               |
| Dried vegetables                                                                                               | Osaili & Forsythe (2009)                                  |
| <b>Fresh produce</b>                                                                                           |                                                           |
| Salads, and herbs                                                                                              | Molley <i>et al.</i> (2009)                               |
| Mixed vegetables, salad                                                                                        | Osaili & Forsythe (2009)                                  |
| Sprouts, fresh herbs and salads, parsley, dill, coriander, celery, basil                                       | Baumgartner <i>et al.</i> (2009)                          |
| <b>Animal products</b>                                                                                         |                                                           |
| Camel, eggs, cheese, milk, pork, fish and products, poultry, sausages, shellfish, shrimp                       | Beuchat <i>et al.</i> (2009)                              |
| Beef and products, pork, burgers, minced meat from beef and pork                                               | Molley <i>et al.</i> (2009)                               |
| <b>Environment</b>                                                                                             |                                                           |
| Milk powder and environment                                                                                    | Lampel & Chen (2009)                                      |
| Water, soil and grass                                                                                          | Lampel & Chen (2009); Molley <i>et al.</i> (2009)         |
| Household vacuum dust                                                                                          | Jaradat <i>et al.</i> (2009); Molley <i>et al.</i> (2009) |

## Molecular characterization of strains

### Multilocus sequence typing (MLST) sequence types (STs)

The reclassification scheme described by Iversen *et al.* (2007, 2008a) was supported subsequently by both optical mapping and genome sequencing data, which confirmed the revision of the taxonomic positions of the seven species (Yan *et al.*, 2012; Kotewicz & Tall, 2009; Kucerova *et al.*, 2010; Stephan *et al.*, 2011; Joseph *et al.*, 2012; Grim *et al.*, 2013). Although *C. sakazakii* and *C. malonaticus* were found to be closely related and difficult to distinguish by 16S rDNA gene sequence analysis, a MLST scheme reported by Baldwin *et al.* (2009) was shown to support the separation of these two species (Yan *et al.*, 2012). Other supporting evidence which suggested that *C. malonaticus* and *C. sakazakii* are separate species were the single nucleotide polymorphisms identified for two genes, *rpoB* and *cgcA*, as reported by Stoop *et al.* (2009) and Carter *et al.* (2013). Their work led to the development of two species-specific end-point PCR assay schemes that are now widely used for speciation of isolates. The sequencing of another 15 *Cronobacter* strains by Grim *et al.* (2013) fully corroborates both the taxonomic scheme described by Iversen *et al.* (2008a) and the MLST scheme

described by Baldwin *et al.* (2009). Grim *et al.* (2013) concluded that, in particular, *C. sakazakii* and *C. malonaticus* must have evolved or acquired accessory genes that have enhanced their virulence capacity and host species adaptation, and thus augmented their overall pathogenicity. Furthermore, the MLST scheme has defined over 136 STs and *C. sakazakii* ST4 was the predominant ST found associated with CSF isolates from cases of neonatal meningitis (Joseph *et al.*, 2012a). In fact, the MLST typing method has alone defined 55 STs for *C. sakazakii* (Sonbol *et al.*, 2013). As suggested by Holý & Forsythe (2013), the clonality and prevalence of the ST4 strains are clearly associated with meningitis, but not NEC. Fortunately, this information gives direction for further meningitis research with the bacterium.

Nevertheless, *C. sakazakii* has been implicated in most of the infant and neonatal meningitis cases. Similarly, in another study by Hariri *et al.* (2013), the authors found that most serious meningitis cases were caused by *C. sakazakii* ST4. Furthermore, they also found that all the five CSF isolates were ST4. In another study, Joseph & Forsythe (2011) showed that half of the studied *Cronobacter* strains from all clinical sources over 50 years from seven countries were *C. sakazakii* ST4. These findings indicated the

predominance of this particular clonal or clonal complex in causing neonatal meningitis and provided evidence that ST4 may be a stable virulent clone. Furthermore, it was suggested that *C. sakazakii* ST4 may be composed of multiple STs and comprises a clonal complex, and ST4 was predominantly involved in infant meningitis and was frequently isolated from milk powder processing facilities. *C. malonaticus* contained other STs such as ST8 that were associated with clinical cases, but not necessarily associated with neonates, whilst ST7 was associated with adult infections (Sonbol *et al.*, 2013). Sonbol *et al.* (2013) described the occurrence of different *Cronobacter* MLST STs in global surveys and their findings suggest that 24 % of strains from PIF manufacturing sites are the ST4 clone. In their study, Müller *et al.* (2013) reported that 132 of 141 *Cronobacter* isolates obtained from an infant formula production factory were *C. sakazakii*, whilst only seven were *C. malonaticus*. Furthermore, when the isolates were analysed by MLST, *C. sakazakii* ST4 was the predominant type among them.

### Molecular O-antigen typing

The O-antigen is a component of the LPS structure located on the outer surface of Gram-negative bacteria and is responsible for serological diversity. As a Gram-negative pathogen, the cell surface of *Cronobacter* is covered with a dense area of LPS (endotoxin) with a lipid A core, the toxic moiety forming ~8 % of the total LPS weight (MacLean *et al.*, 2009). It was found that different strains of *Cronobacter* have different LPS structures, and show differences in pathogenicity based on differences in structure (linear versus branched) and composition of LPS (MacLean *et al.*, 2009). It was found that the LPS of *C. sakazakii* and *C. malonaticus* was branched, whilst LPS from other *Cronobacter* strains such as *C. muytjensii* was unbranched (Joseph *et al.*, 2012c). Indeed, *C. muytjensii* LPS failed to provoke the production of mAbs due to its unbranched structure (Jaradat *et al.*, 2011).

As a useful method of characterizing Gram-negative bacteria, O-antigen typing and studies describing the nature of the O-antigen associated with *Cronobacter* spp. have been reported (Mullane *et al.*, 2008; Jarvis *et al.*, 2011, 2013). Mullane *et al.* (2008) initially developed a molecular serotyping method, based on amplification of the *rfb* encoding locus (in Gram-negative enteric bacteria this is located between *galF* and *gnd*) followed by *Mbo*II digestion. Using this approach, a PCR-RFLP profile was generated that could be compared across several isolates. Based on this approach, the first two O-antigen serotypes were characterized, and denoted as O1 and O2. More recently, Sun *et al.* (2011) added five additional *C. sakazakii* O-antigen serotypes that correlated with the PCR-RFLP profiles. Jarvis *et al.* (2011) extended the original Mullane molecular characterization scheme to include other *Cronobacter* spp. and defined eight new molecular O-serotype gene clusters. Two of these O-serotype gene clusters were shared among

*C. sakazakii*, *C. muytjensii*, *C. malonaticus* and *C. turicensis* strains (Jarvis *et al.*, 2011, 2013). The structural composition of several O-serotypes has now been described (MacLean *et al.*, 2009; Arbatsky *et al.*, 2010, 2011; Shashkov *et al.*, 2011). Presently, there are 17 recognized serotypes among the seven species. Over a 1 year period (2011–2012), Müller *et al.* (2013) found that *C. sakazakii* serotype O2 was the predominant serotype observed among 141 confirmed *Cronobacter* isolates obtained during a surveillance study of a Swiss PIF manufacturing facility. Correlations between ST4 and *C. sakazakii* serotypes O2/O3 and between ST83 and *C. sakazakii* serotype O7 were observed, suggesting that a ST may contain multiple serotypes.

### Pathogenicity and virulence of *Cronobacter*

Despite the exponential increase in research on *Cronobacter* over the last decade, the exact mechanism of *Cronobacter* pathogenesis is still unknown. One of the first attempts to understand the pathogenicity of *Cronobacter* was the work of Pagotto *et al.* (2003), who investigated enterotoxin production among different *Cronobacter* strains using the suckling mice assay. To date, a number reports have been published on the putative virulence factors, such as toxin production, adhesion and invasion, adaptation to stress factors, presence of virulence genes, and antibiotic resistance. These factors are discussed separately throughout this review.

### Toxins and proteolytic enzymes

Historically, enterotoxin production has been a major virulence factor that marks the pathogenicity of microbes. Pagotto *et al.* (2003) were the first to describe putative enterotoxin activity in *Cronobacter*. Using the suckling mice assay, they were able to show an enterotoxin effect by four of the 18 tested *Cronobacter* isolates. When tested on cultured mammalian cells, filtrates from one strain were toxic to Vero and Y-1 cells, causing rounding and cell lysis. Boiling the filtrates for 20 min did not affect the toxin activity in suckling mice, but it reduced the cytopathic effect of the toxin on Vero cells. Yang *et al.* (2009) showed that filtrates from two out of eight strains studied were toxic to Vero cells. Raghav & Aggarwal (2007) were the first to purify and characterize a putative enterotoxin from *Cronobacter* isolates. The molecular mass of the toxin was determined to be 66 kDa, which is similar to the 62 kDa toxin of *Shigella dysenteriae* 1 and to the 65 kDa toxin of *Pseudomonas aeruginosa* with optimum activity observed at pH 6. Additionally, the activity of the toxin was unaffected by holding it at 50 or 70 °C and it was stable at 90 °C for 30 min, implying that it is a moderately heat-stable toxin. This indicates that the enterotoxin could be resistant to commercial milk pasteurization treatments and thus stays active in PIF.

However, it seems that the discovery of these toxins is still in its infancy, as the specific genes need to be identified to

shed light on the importance of these enterotoxins (Chenu & Cox, 2009). Molecular studies using genome sequencing will help with the further characterization of these toxins.

In addition to enterotoxins, *Cronobacter* also secrete proteolytic enzymes that lyse cells and create tissue damage at the site of infection in mice (Pagotto *et al.*, 2003). Kothary *et al.* (2007) reported the presence of a zinc metalloprotease that deforms CHO cells, eventually causing their rounding and leading to cell damage. The genes for a haemolysin (*hly*) have been identified, but no activity has been found (Kucerova *et al.*, 2010; Cruz *et al.*, 2011; Grim *et al.*, 2013).

Townsend *et al.* (2007a) provided evidence that the presence of endotoxin along with *C. sakazakii* in infant formula enhanced the translocation of *C. sakazakii* from rat guts through the BBB. One possible mechanism is thought to be the disruption of the tight junctions by LPS, thus increasing the permeability of host barriers to the pathogen (Kim & Loessner, 2008). In addition, endotoxin was found to impair enterocyte migration and epithelial restitution, and therefore inhibited tissue repair following bacterial infection, which leads to the translocation of the pathogen from the gut into the blood and internal tissue (Cetin *et al.*, 2004). As the endotoxin is heat stable at 100 °C, its presence in formulas might also play an important role in enhancing the pathogenesis of *Cronobacter* in human infants (Townsend *et al.*, 2007a).

## Attachment and invasion

**Attachment.** Many meningitis-causing bacteria, such as *Neisseria meningitidis*, *Haemophilus influenzae* and *P. aeruginosa* utilize type IV pili to colonize intestinal tissues and to later overcome the shear forces of the capillary blood flow before crossing the BBB (Kucerova *et al.*, 2010; Joseph & Forsythe, 2011; Grim *et al.*, 2013). *Cronobacter* have been mostly implicated in meningitis in infants following the consumption of reconstituted and temperature-abused PIF (Hunter & Bean, 2013). Hence, it is thought that the main colonization site of *Cronobacter* is the GIT, where it first colonizes the mucous membranes, gastric and intestinal epithelial or endothelial tissues prior to their internalization within enterocytes, or translocation through the lamina propria into the systemic blood flow and then invasion of the brain.

Several *in vitro* studies using human-derived cell lines investigated the behavioural characteristics of *Cronobacter*. Mange *et al.* (2006) examined non-fimbrial adhesive characteristics of *Cronobacter* with human brain microvascular endothelial cells (HBMECs), which make up the BBB, and with human epithelial cell lines (HEp-2 and Caco-2). They reported that neither mannose-sensitive type 1 fimbriae nor type 3 fimbriae were involved in the adhesion of *Cronobacter* to the cells. Comparatively, one striking difference among *Cronobacter* as reported by Kucerova *et al.* (2010), Joseph & Forsythe (2011) and Stephan *et al.* (2011) is the finding that *C. sakazakii* does not possess the genes encoding

curli, whereas *C. turicensis* does. However, the implications of these findings in virulence have not been fully studied.

Later, Townsend *et al.* (2008) showed that there was no direct correlation between rates of attachment to and invasion of *Cronobacter* by Caco-2 cells. In fact, this was the case with other pathogens, such as *Listeria monocytogenes*, where there was no relation between the attachment and invasion, and the most highly attaching isolates showed low or moderate invasion capabilities (Jaradat & Bhunia, 2003).

However, with the advent of whole-genome sequencing, Grim *et al.* (2013) reported that the *Cronobacter* genome contains genes for the type IV pili in addition to a P pilus homologous to that found in uropathogenic *Escherichia coli* that causes meningitis in infants. Interestingly, not all *Cronobacter* spp. contain the genes for these pili. For instance, *C. sakazakii* BAA-894 and *C. malonaticus* LMG 23826 harbour a unique type 1 fimbriae (GR82), which was absent in other genomes. This may explain the variation in the pathogenicity observed among the different *Cronobacter* spp. Furthermore, some *Cronobacter* genomes contain genes for other types of fimbriae that are found in other well-known pathogens. A gene for the curli fimbriae homologous to that of *E. coli* and another gene related to the tafi fimbriae of *Salmonella* were also identified (Grim *et al.*, 2013).

Other adherence mechanisms might be involved in the colonization of GIT cells by *Cronobacter* spp. *Cronobacter* use host surface proteins such as fibronectin – a major glycoprotein component of the extracellular matrix – to adhere to the intestinal epithelial or endothelial cells as an initial step before their internalization (Mange *et al.*, 2006; Nair & Venkitanarayanan, 2007; Mittal *et al.*, 2009a). Blocking fibronectin binding reduced the number of *Cronobacter* cells attaching to INT-407 epithelial cells (Nair & Venkitanarayanan, 2007), whilst it had no significant impact on the adherence of *C. sakazakii* to HBMECs (Nair *et al.*, 2009). These differing results indicate that *Cronobacter* attachment mechanisms mediated by fibronectin are likely host cell-type specific, where some *Cronobacter* spp. use their OmpA, not fimbriae, to adhere to the fibronectin receptors as an initial step in colonization. These results also emphasize the critical role of this outer-membrane protein in bacterial invasion (Mange *et al.*, 2006; Kim *et al.*, 2010; Liu *et al.*, 2012b; Yan *et al.*, 2012).

**Invasion.** After the successful attachment of *Cronobacter* to intestinal cells, the pathogen invades these cells in a process that ends up with the pathogen translocating across the intestinal tissue layers to enter the systemic blood flow, resulting in extra-intestinal infection, such as sepsis and meningitis (Nair & Venkitanarayanan, 2007). The invasive capability of *Cronobacter* was studied by several researchers using mammalian cell lines. Singamsetty *et al.* (2008) suggested that HBMECs are more susceptible to *Cronobacter* invasion, demonstrating a higher invasion frequency in HBMECs than endothelial and epithelial cells from different

tissue origins. Townsend *et al.* (2008) showed a variation in *Cronobacter* invasion capacity with intestinal and BBB cells, indicating that *Cronobacter* invade HBMECs in a strain-dependent fashion, whereas no direct connection between attachment and invasion rates for Caco-2 cells was detected. Kim & Loessner (2008) found that *Cronobacter* entry into and invasion of Caco-2 cells is an active process that needs *de novo* protein synthesis, and depends on exposure time and m.o.i. Recently, Giri *et al.* (2012) demonstrated that *Cronobacter* isolates were able to transcytose across tight monolayers of Caco-2 cells and HBMECs, thus mimicking the *in vivo* ability to cross the intestine and subsequently the CNS BBB to cause meningitis. Not only that, the rate of transcytosis of some strains was equivalent to that of the control meningitis-causing *E. coli* K1 strain. Interestingly, the rate of transcytosis among the isolates varied significantly.

### Role of OmpA in *Cronobacter* invasion of mammalian cells

OmpA appears to play a major role in *Cronobacter* invasion. *In vitro*, it was found that OmpA plays a critical role in the invasion of human intestinal epithelial cells (INT-407), Caco-2 cells and HBMECs (Nair & Venkitanarayanan, 2007; Kim & Loessner, 2008; Singamsetty *et al.*, 2008; Kim *et al.*, 2010). An 87 % reduction of *Cronobacter* invasion of INT-407 cells was observed in the absence of OmpA expression. Similarly, Nair *et al.* (2009) reported that OmpA is a determinant that influences the invasion of *C. sakazakii* into HBMECs, by providing evidence that the absence of OmpA expression resulted in 83 % reduction in *C. sakazakii* invasion. However, adherence of an OmpA mutant did not differ significantly from the WT strain, possibly because there are other surface determinants that contribute to *C. sakazakii* attachment to host cells that may not play a role in the invasion.

Mittal *et al.* (2009a) showed that OmpA expression is important for the onset of meningitis caused by *Cronobacter* in a newborn rat model as OmpA<sup>+</sup> *Cronobacter* were present in high numbers in the brains of infected animals. They also showed that OmpA expression is essential for *Cronobacter* resistance to blood and serum killing (Mittal *et al.*, 2009a). Although both OmpA<sup>+</sup> and OmpA<sup>-</sup> *Cronobacter* were efficiently taken up by dendritic cells, only OmpA<sup>+</sup> *Cronobacter* resisted killing and multiplied in these cells, thus highlighting the critical role of OmpA in protecting the intracellular survival of this pathogen (Mittal *et al.*, 2009b).

Similarly, Kim *et al.* (2010) reported that OmpA and OmpX of *C. sakazakii* are important for both apical and basolateral adhesion to and invasion of host cells, as well as for movement into deeper organs, such as the spleen and liver. Invasion studies proved that OmpA and OmpX are crucial for *C. sakazakii* invasion of INT-407 and Caco-2 cells. However, adherence studies demonstrated that OmpA was essential for binding to Caco-2 cells, but not INT-407 cells. OmpX did not show any significant role in binding to

both types of cells examined. The authors concluded that *C. sakazakii* adherence to Caco-2 cells is mediated by OmpA, but adhesion to INT-407 cells is not, and that the initial interaction of *C. sakazakii* with Caco-2 and INT-407 cells is independent of OmpX. Nonetheless, not all *Cronobacter* spp. were positive for OmpA using the primers described by Nair & Venkitanarayanan (2007), as reported by Jaradat *et al.* (2009) and Giri *et al.* (2012).

### Role of cytoskeleton rearrangement in *Cronobacter* invasion

To enable smooth entrance into the cytoplasm of a host cell, most pathogenic bacteria are able to manipulate the cytoskeleton of the host cell either by the secretion of effector molecules or by interfering with the phosphorylation cascades in the intracellular signal transduction pathways (Singamsetty *et al.*, 2008; Kim & Loessner, 2008; Mittal *et al.*, 2009b).

Results of several studies clearly demonstrated that *Cronobacter* exploit both microfilaments and microtubules to invade host cells. Actin microfilaments are the most well-known structures in this process. This was supported by the fact that treating INT-407 cells, Caco-2 cells and HBMECs with the actin-depolymerizing agent cytochalasin D completely blocked the invasion by *C. sakazakii* of these cell lines (Nair & Venkitanarayanan, 2007; Kim & Loessner, 2008; Li *et al.*, 2010). *C. sakazakii* invasion of HBMECs involves participation of distinct signal transduction processes in which the PI3K/Akt signalling pathway also plays an essential role to initiate the rearrangement of actin filaments and subsequent bacterial internalization (Singamsetty *et al.*, 2008; Li *et al.*, 2010). Liu *et al.* (2012a) found that host cytosolic phospholipase A<sub>2</sub>α is a downstream effector of PI3K/Akt signalling, which is needed for the actin reorganization in HBMECs elicited by *C. sakazakii* infection. In contrast, it was shown that *Cronobacter* severely impair the phosphorylation of p38, extracellular signal-regulated kinase 1/2 and c-Jun N-terminal kinase, which are major components of the MAPK pathway, and prevent the activation and maturation of dendritic cells by disarming the MAPK pathway – a process that may aid the pathogen in evading the immune system (Mittal *et al.*, 2009b).

### Tight junction disruption

The tight junction constitutes the principal barrier in intestinal epithelial cells. Kim & Loessner (2008) showed that when the tight junctions of Caco-2 cells were disrupted, the efficiency of *C. sakazakii* adhesion and invasion increased substantially. Apparently, after binding of *Cronobacter* to the intestinal enterocyte cell surfaces, *Cronobacter* caused the generation of increased amounts of nitric oxide, which is thought to have led to the disruption of the tight junctions between individual enterocytes. Disruption of the tight junctions subsequently led to apoptosis (Liu *et al.*, 2012b). This, in turn, led to the leakage of the pathogen

through these localized disruptions, eventually reaching the systemic circulation (Liu *et al.*, 2012b).

Interestingly, the inhibition of the PKC- $\alpha$  intracellular signalling pathway abolished the disruption of the tight junctions. These results indicate that PKC- $\alpha$  influences the permeability of the intestinal epithelium to *C. sakazakii* (Liu *et al.*, 2012b). Singamsetty *et al.* (2008) showed that PKC- $\alpha$  activation is also critical for *Cronobacter* invasion of HBMECs. The disruption of tight junctions might be a result of *C. sakazakii* interaction with dendritic cells, which could modulate the expression of the 'tightness' of the tight junctions (Emami *et al.*, 2011).

### Evading the immune response

It is expected that *Cronobacter* possess virulence factors that help in the colonization and invasion of the various human mucosal cells as a first step in the systemic spread of the organism. Furthermore, such pathogens have evolved mechanisms to avoid the immune system lines of defence, such as surviving within macrophages, and possessing resistance to the bactericidal action of complement and serum. *Cronobacter* appear to evade the host's immune system by adapting to a lifestyle of replication in the intracellular environment confined within macrophages. Townsend *et al.* (2007b) showed that *Cronobacter* can persist within human U937 macrophages and survival varied between strains, *Cronobacter* persisting intracellularly for up to 96 h. In another study, most *Cronobacter* strains persisted in macrophages for 48 h (Townsend *et al.*, 2008). These results suggest that *Cronobacter* possess virulence properties that enable them to tolerate the intracellular environment of macrophages.

Studies using both scanning and transmission electron microscopy revealed that *Cronobacter* were taken up by dendritic cells by a conventional phagocytic mechanism and internalized cells were enclosed inside membrane-bound compartments of dendritic cells. However, it was shown that *Cronobacter* could interfere with the maturation of dendritic cells and exploit them as a replication permissive niche. *Cronobacter* targeted DC-SIGN to survive in myeloid dendritic cells (Mittal *et al.*, 2009b). Emami *et al.* (2011) showed that dendritic cell recruitment to the intestine upon infection with *C. sakazakii* is responsible for the intestinal barrier dysfunction. Dendritic cells infected with OmpA<sup>+</sup> *Cronobacter* displayed higher production of IL-10 and transforming growth factor (TGF)- $\beta$ , and very low levels of proinflammatory cytokines – a phenotype associated with tolerogenic dendritic cells. Thus, *Cronobacter* could exploit IL-10- and TGF- $\beta$ -producing tolerogenic dendritic cells to escape potent host immune defence mechanisms temporarily (Mittal *et al.*, 2009b; Emami *et al.*, 2011).

Hunter *et al.* (2009) demonstrated that enterocyte injury caused by *Cronobacter* appears to be dependent on the production of high levels of nitric oxide, as inhibition of inducible nitric oxide synthase (iNOS) by small interfering RNA prevented apoptosis of intestinal epithelial cells.

Blocking TGF- $\beta$  by a specific antibody also reduced iNOS production, and prevented monolayer leakage and apoptosis. The presence of TGF- $\beta$  seems to be required for upregulation of iNOS and subsequent epithelial cell injury (Emami *et al.*, 2011). The increased mucosal cytokine response and nitric oxide production in the absence of polymorphonuclear leukocytes and macrophages may also be responsible for an increase in mucosal injury (Emami *et al.*, 2012).

Once inside phagocytic cells, *Cronobacter* showed an increased level of superoxide dismutase activity. Superoxide dismutases are metalloenzymes that help bacteria resist intracellular oxidative stressed environments within the polymorphonuclear leukocytes (Townsend *et al.*, 2007b). Additionally, overexpression of outer-membrane protease Cpa (plasminogen activator) was reported following the entry of *Cronobacter* systemically. Cpa provides resistance against the bactericidal activity of serum by cleaving complement components C3 and C4b, and also activates plasminogen and inactivates  $\alpha$ 2-AP (plasmin inhibitor) (Franco *et al.*, 2011a; Schwizer *et al.*, 2013). Interestingly, it was reported that, of the different *Cronobacter* spp., *C. sakazakii* was the most tolerable to the killing effects of serum – a fact that might explain its strong pathogenic potential and the possession of pESA3-borne *cpa* by *C. sakazakii* strains. The role of other surface structures, such as LPS, OmpA and exopolysaccharide (capsule), which enable the pathogen to resist the bactericidal activity of serum and avoid the immune system is unknown currently (Schwizer *et al.*, 2013).

### Virulence genes and plasmids

Several virulence genes and plasmids were identified, and found to be specific to *Cronobacter* spp. Kothary *et al.* (2007) identified the gene locus of zinc-containing metalloprotease (*zpx*) – a protein that caused rounding of CHO cells in tissue culture. In addition, the presence of putative *sodA* genes might provide resistance for *Cronobacter* against intracellular macrophage oxidase and acidic conditions, and may contribute to its intracellular persistence (Townsend *et al.*, 2007b). However, these genes and OmpA and OmpX were found in all *Cronobacter* spp., and thus cannot be responsible for the variation in pathogenicity among the different *Cronobacter* strains (Joseph *et al.*, 2012c).

The complete genome sequence of *C. sakazakii* ATCC BAA-894 enabled Cruz *et al.* (2011) to compare other *Cronobacter* genome sequences with the sequence of *C. sakazakii* in their attempt to identify virulence genes. Three putative virulence genes – type III haemolysin (*hly*), siderophore-interacting protein (*sip*) and plasminogen activator (*cpa*) – were identified.

Among the annotated genes inspected, Kucerova *et al.* (2010) also showed that the complete cation efflux system (*cusA*, *cusB*, *cusC* and *cusF*) and its regulatory gene *cusR* were present in strains associated with neonatal infections, but missing in the other strains, which may explain the fact that not all *Cronobacter* spp. are equally pathogenic and cause meningitis.

In addition, Franco *et al.* (2011a) showed that all *Cronobacter* RepFIB plasmids encode two iron acquisition systems – a siderophore-mediated iron acquisition system (*iucABCD/iutA* operon) and ATP-binding cassette transport-mediated iron uptake and siderophore system (*eitCBAD* operon) – suggesting that these plasmids are common important virulence plasmids and may also contribute to systematic survival of *Cronobacter*. The ability to acquire iron is crucial for most pathogens in establishing infection after entering a host cell. Franco *et al.* (2011b) showed that the *iucABCD/iutA* siderophore (cronobactin) is the only functional siderophore possessed by *Cronobacter*. However, Grim *et al.* (2013) demonstrated that cronobactin – a hydroxamate-type, aerobactin-like siderophore – was not the only iron acquisition system possessed among *Cronobacter*. The cronobactin locus consists of five genes homologous to biosynthetic genes *iucABCD* and the receptor gene *iutA*. Indeed, iron acquisition is considered as growth promotion as it is related to utilization of iron in milk, but as iron utilization genes were found in all *Cronobacter* spp. its role is still not understood (Joseph *et al.*, 2012c). Grim *et al.* (2013) reported targeted *in silico* sequence analysis of nine *Cronobacter* genomes, and showed that *Cronobacter* shared iron acquisition systems among the seven species and also possessed iron acquisition genes encoding ferric and ferrous transporters and haem-iron extractors, as well as putative TonB-dependent iron receptors and ferric reductases. For acquisition of ferrous iron, all *Cronobacter* have both the Feo and Efe systems, and for transport of ferric iron, all plasmid-harboring strains (97%) have the aerobactin-like siderophore cronobactin. All *Cronobacter* have the genes encoding the enterobactin-like siderophore, but this siderophore is thought not to be functional. Grim *et al.* (2013) also reported that in addition to receptors for cronobactin and enterobactin, all *Cronobacter* have five common receptors (FhuA, YncD, FoxA, FhuE and PfeA) for siderophores that are also produced by other organisms. The ferric dicitrate transport system was found specifically in a small subset of *C. sakazakii* and *C. malonaticus* strains, most of which were isolated from clinical samples, suggesting that this iron acquisition system plays a role in the virulence of *Cronobacter*. These studies also provided evidence that *C. dublinensis* and *C. muytjensii* have two other receptors, Fct and FcuA, for heterologous siderophores produced by plant pathogens, indicating that these receptors may give an advantage to these *Cronobacter* spp. to compete more successfully for iron in a plant niche. This also supports the hypothesis that *Cronobacter* emerged from a common ancestor that possessed a plant-associated lifestyle prior to its species-level bi-directional divergence.

Secretion systems, referred to as type IV secretion systems, that can transfer proteins and nucleoprotein complexes are considered to be important virulence factors as they were identified in *C. sakazakii* and *C. turicensis* as a plasmid-borne (pESA2/pCTU2) gene cluster (Franco *et al.*, 2011b). In contrast, several newly identified type VI secretion systems (T6SSs) were found in the chromosomes of most

*Cronobacter* spp. as well as on pESA3 carried by *C. sakazakii*, including pathogenic *C. sakazakii* ST4 strains (Joseph *et al.*, 2012c). Interestingly, the T6SS was also found to be important for *E. coli* K1 invasion of the BBB (Joseph *et al.*, 2012c).

### Other virulence characters

Whole-genome studies of *Cronobacter* revealed that *C. sakazakii* is the only *Cronobacter* species that has the *nanAKT* gene cluster encoding utilization of exogenous sialic acid as a carbon source (Joseph *et al.*, 2013b). Other genome-sequencing studies revealed the presence of two genomic regions (GR127 and GR129) that are involved in the utilization of sialic acid in the genome of *C. sakazakii* BAA-894 (Grim *et al.*, 2013). Furthermore, Grim *et al.* (2013) found that 55 out of 57 strains of *C. sakazakii* were able to utilize sialic acid or its derivative *N*-acetylneuraminic acid, whilst no other *Cronobacter* strains were able to do this. Sialic acid is found in breast milk, infant formula, the mucin lining of the intestinal tract and is a component of the brain ganglioside complex (Siqueira Santos *et al.*, 2013). Hence, it is possible that the ability of *C. sakazakii* to utilize sialic acid will enhance its pathogenicity for neonates and young infants. *Cronobacter* might use sialic acid to produce its exopolysaccharide (capsule) which helps it to avoid the immune system, and this behaviour is probably exacerbated in the presence of different milk sources and their products, which also contain sialic acid or its precursors. In addition, *C. sakazakii* sialic acid may also help in colonizing the intestinal tract via interactions with host mucins (Joseph *et al.*, 2012c).

Inositol fermentation has been proposed recently as a marker of pathogenicity for *Cronobacter* based on the presence of the inositol monophosphatase gene (*suhB*) in some pathogenic strains. However, the inositol utilization operon GR29 was found in *Cronobacter* strains isolated from the environment, whilst also being absent in genomes of pathogenic strains (Grim *et al.*, 2013). Therefore, its role in virulence is unclear at present.

### Biofilm formation

*Cronobacter* have been reported to attach to and form biofilms on stainless steel, glass, latex, silicon, polyvinyl chloride and polycarbonate (Iversen *et al.*, 2004; Lehner *et al.*, 2005). Capsular polysaccharides on the bacterial cell surface play a pivotal role in biofilm formation. Colanic acid production is encoded by ESA\_0115-01175, the *wzABCKM* gene in all *Cronobacter* spp. (Joseph *et al.*, 2012c). Bacterial cells appear to attach more rapidly to hydrophobic non-polar materials such as Teflon and plastics than to hydrophilic surfaces such as glass or metals (Lehner *et al.*, 2005). Furthermore, Cruz-Córdova *et al.* (2012) reported that flagella from *C. sakazakii* are involved in biofilm formation. In addition to flagella, two hypothetical proteins (ESA\_00281 and ESA\_00282) were identified as possible adhesin proteins that may contribute in biofilm formation (Cruz-Córdova *et al.*, 2012). Flagella seem to aid *Cronobacter*

cells to adhere to mammalian cells such as Caco-2 and other epithelial cells.

The ability of *Cronobacter* spp. to form biofilms provides protection from environmental stresses that impart resistance to cleaning and sanitizing agents (Ravishankar & Juneja, 2003; FAO/WHO, 2006). Kim *et al.* (2007) showed that resistance of *Cronobacter* spp. to disinfectants was increased when they formed biofilms compared with planktonic cells that were inoculated and dried onto the surface of stainless steel or even planktonic cells in culture. Additionally, a higher UV dose was required to kill *Cronobacter* growing in a biofilm than in the planktonic state (Jo *et al.*, 2010).

Biofilm formation is of special importance in the food industry, because biofilms can act as a source of microbial contamination that might lead to spoilage of foods or contamination of food products undergoing processing (Lehner *et al.*, 2005; Hartmann *et al.*, 2010). Biofilms of *Cronobacter* formed on feeding areas and on equipment surfaces used in formula preparation are thought to have contributed to several neonatal outbreaks (Kim *et al.*, 2006). Indeed, these observations support the increased risk of *Cronobacter* for formula-fed infants.

#### Antibiotic susceptibility and resistance

Prior to 1985, patients with *Cronobacter* infections were frequently treated with ampicillin, gentamicin and/or chloramphenicol. Willis & Robinson (1988) recommended the combination of gentamicin and ampicillin for the treatment of meningitis caused by *Cronobacter*. In contrast, Lai (2001) found that all *Cronobacter* isolates were uniformly resistant to ampicillin, cefazolin and extended-spectrum penicillins, whereas they were uniformly susceptible to trimethoprim/sulfamethoxazole and aminoglycosides. Recently, Al-Nabulsi *et al.* (2011) showed that streptomycin, gentamicin, kanamycin and ciprofloxacin are effective against both stressed and unstressed *C. sakazakii* cells, and thus these antibiotics might be appropriate choices for patient treatment regimes. Nevertheless, Nazarowec-White & Farber (1999) found that two *Cronobacter* strains out of eight were resistant to tetracycline and chloramphenicol. Kim *et al.* (2008) described *Cronobacter* strains recovered from foods that were resistant to cephalothin or ampicillin and susceptible to tetracycline. Hochel *et al.* (2012) found that all *Cronobacter* isolates varied in their susceptibility to tetracycline.

Muytjens & van der Ros-van de Repe (1986) examined *Cronobacter* susceptibility to 29 antimicrobial agents and found that *Cronobacter* were the most susceptible among other *Enterobacteriaceae* studied. This was supported by the finding of Stock & Wiedemann (2002) who showed that *Cronobacter* were repeatedly susceptible to  $\beta$ -lactams with no evidence for the expression of  $\beta$ -lactamase. In contrast, Pitout *et al.* (1997) demonstrated that some strains of *Cronobacter* produce  $\beta$ -lactamase at low levels. Caubilla-Barron *et al.* (2007) identified two *Cronobacter* isolates with

extended-spectrum  $\beta$ -lactamase activity. Furthermore, Zhou *et al.* (2011) showed that one *Cronobacter* isolate from PIF produced extended-spectrum  $\beta$ -lactamase. According to the same study, susceptibility results showed that each isolate had different levels of resistance to  $\beta$ -lactam antibiotics (Zhou *et al.*, 2011).

Although multiple antibiotic resistance (*mar*) operons were found in *Cronobacter* (Burgos & Varela, 2002), the overall level of antibiotic resistance was low compared with other food-borne pathogens and the increase of antibiotic-resistant *Cronobacter* could be a result of the overuse of antibiotics (Lee *et al.*, 2012).

#### Environmental adaptation

*Cronobacter* spp. might be exposed to different environmental stresses during processing and preparation of PIF or post-infection after entering the host. The ability of *Cronobacter* spp. to withstand the harmful effects of these stresses is crucial for their survival, persistence and infectivity.

#### Heat adaptation

Some reports indicated that *Cronobacter* are more thermotolerant than most other bacteria belonging to the *Enterobacteriaceae*, whilst other studies reported that some *Cronobacter* isolates have no such tolerance (Nazarowec-White & Farber, 1997; Breeuwer *et al.*, 2003; Osaili *et al.*, 2009). The contradiction between these studies might be attributed to the growth conditions, isolate source (i.e. food or clinical) and growth phase or due to variation among the different *Cronobacter* strains (Arroyo *et al.*, 2009; Walsh *et al.*, 2011). In addition, different properties of food or formula, including low water activity and high fat content, affect thermotolerance of *Cronobacter* (Osaili *et al.*, 2009). Nonetheless, adequate pasteurization conditions are sufficient to inactivate *Cronobacter* spp. during processing and reconstitution of PIF (Al-Nabulsi *et al.*, 2011). Recently, an 18 kbp region containing 22 ORFs that are upregulated under heat adaptation conditions has been reported (Gajdosova *et al.*, 2011). The major feature of the region is a cluster of conserved genes – most of them having significant homologies with known bacterial proteins involved in some type of stress response, including heat, oxidation and acid stress.

#### Desiccation adaptation

*Cronobacter* are considered more resistant to dry and osmotic stresses than other members of the *Enterobacteriaceae* (Feeney & Sleator, 2011). It is thought that this is probably due to the accumulation of trehalose inside the cells, which works as a protectant (Breeuwer *et al.*, 2003). Furthermore, Álvarez-Ordóñez *et al.* (2014) concluded that *de novo* protein synthesis, DNA repair proteins and the maintenance of the structural integrity of cells are vital for the survival of *Cronobacter* in hyperosmotic media. Another

explanation was offered by Barron & Forsythe (2007), who showed that *Cronobacter* were recoverable from PIF even after 2.5 years, assuming that their extracellular polysaccharides could protect the cells against the desiccation effects. Furthermore, Feeney & Sleator (2011) showed bioinformatically that *C. sakazakii* contained multiple copies of the osmotolerance homologues ProP and OpuC, and they concluded that the osmotic stress response of *C. sakazakii* appears to be regulated at the transcriptional, translational and post-translational levels, with RpoS most likely functioning as the global transcriptional regulator of the osmotolerance response.

Gurtler & Beuchat (2007) reported that the reduction in the viability of *Cronobacter* in PIF was less evident in formulas with water activity ( $a_w$ ) values 0.25–0.30 compared with formulas with  $a_w$ =0.43–0.50. Additionally, *Cronobacter* spp. can survive in infant cereals at low populations ( $2 \text{ c.f.u. g}^{-1}$ ) over a wide range of  $a_w$  (0.30–0.83) for up to 12 months with enhanced viability at lower  $a_w$  values (Lin & Beuchat, 2007). Furthermore, Dancer *et al.* (2009) showed that *Cronobacter* grown and dried in PIF survived better throughout drying than those cells grown and dried in tryptic soy broth, highlighting the role of some PIF components in the process of desiccation adaptation. Genes involved in desiccation resistance and osmotic stresses have been identified. For instance, genes encoding osmoprotectant glycine betaine and trehalose were found in all *Cronobacter* (Joseph *et al.*, 2012c). Furthermore, all *Cronobacter* contain genes for  $\beta$ -carotene production, which is believed to protect bacteria against harmful oxygen radicals (Joseph *et al.*, 2012c). Products of these genes are believed to help the bacteria to survive in dry food ingredients utilized for making infant formula preparations. Furthermore, several other genes were identified whose products could help *Cronobacter* to tolerate low water activity environments. The identified genes include cellulose biosynthesis operons, colonic acid exopolysaccharide, the capsular biosynthetic operon, the environmental persistence capsule and the curli GR55. The synergetic expression of these genes might provide resistance to desiccation stress (Grim *et al.*, 2013). Using transposon mutagenesis, Álvarez-Ordóñez *et al.* (2014) identified genes responsible for the desiccation tolerance of *Cronobacter*. They found that the Cpx system, known as an envelope stress response regulator, and the sigma factors RpoN and RpoS seem to be the main signals regulating the bacterial response to hyperosmotic conditions. Furthermore, among the other identified genes, only *dnaK* and *dnaJ*, encoding two molecular chaperones, were important for *C. sakazakii* survival under desiccation.

### Acid adaptation

Dancer *et al.* (2009) reported that 72 *Cronobacter* strains were able to grow at pH 4.5, whilst 98.6, 95.8 and 79.2 % of these tested strains were able to grow at pH 4.3, 4.1 and 3.9, respectively, indicating that *Cronobacter* cells are more acid tolerant than most related enteric pathogens. In

addition, Edelson-Mammel *et al.* (2006) reported that 10 out of 12 strains showed <1 log decline at pH 3.5 over a 5 h incubation at 37 °C, whilst the most acid-sensitive strain showed ~3.5 log decline. Johler *et al.* (2010) showed that *Cronobacter* strains not only survived low pH, but were also able to grow to  $\sim 10^9 \text{ c.f.u. ml}^{-1}$  at pH 4.5 within 24 h. Overall, *Cronobacter* have a good potency to survive in acidic environments that may enable them to cope with stomach acidity. This in turn may explain the increased susceptibility of infants to infection with *Cronobacter* spp., where stomach acidity is not fully developed.

### Conclusions

Although a great deal of research has been conducted over the past decade to understand the nature of *Cronobacter* spp., and to elucidate their mechanisms of pathogenicity, survival and genetics, there is still much to be revealed about their hidden traits. Future work both *in vitro* and *in vivo* will continue to uncover these properties, and will eventually lead to better control of this pathogen and minimize its infections in infants as well as the elderly and immunocompromised.

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