Desiccation resistance and persistence of *Cronobacter* species in infant formula

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Abstract

Cronobacter is a newly described genus which includes opportunistic pathogens formerly known as '*Enterobacter sakazakii*'. These organisms have been isolated from a wide variety of sources, including powdered infant formula (PIF). This review focuses on the desiccation survival of *Cronobacter*, and its relevance to vehicles of infection. Due to its probable natural habitat of plant material, the organism has an array of survival mechanisms which include resistance to desiccation and osmotic stresses. The organism can survive for long periods of time (>2 years) in the desiccated state, and can be recovered from a large number of powdered foods in addition to powdered infant formula. On reconstitution, the organism may rapidly multiply and present a risk to immunocompromised infants. It is expected that an improved understanding of the nature of *Cronobacter* persistence may aid in further improved control measures and eliminate the bacterium from the critical food production environments.

1. Introduction

Cronobacter is well known to be resistant to osmotic and dry stresses. This high tolerance to desiccation may provide a competitive advantage in dry environments, such as would be found in PIF and other manufacturing plants of powdered food products. This physiological trait has also been used as a selective agent in various enrichment broths such as modified lauryl sulphate broth (0.5M NaCl), and *Enterobacter sakazakii* enrichment broth (10 % sucrose) (Iversen and Forsythe, 2007). This review considers the studies to date on this important survival trait for this emergent pathogen.

1.1 Prevalence of Cronobacter in powdered infant formula and other desiccated foods.

The microbiological safety of infant foods is very important due to infants lacking a developed immune system, or a competing intestinal flora (Townsend and Forsythe, 2008). The WHO (2007) have issued guidance on the preparation of infant formula. Additionally, improved detection methods have been developed to support improved control measures in the production of infant formulas, via the recently revised international microbiological criteria (CAC, 2008b). However it is not the purpose of this review to consider these detection methods, and the reader is directed to the review chapter by Fanning and Forsythe (2008). Instead the focus is on the desiccation survival of the organism, which may enable it to persist in powdered products including PIF.

Unlike commercially available ready to feed liquid formula, dried infant formula milk powders (hitherto known as 'powdered infant formula', PIF) are not sterile and must conform to national and international microbiological criteria (CAC

2008a,b). It is of interest to note that when Farmer et al. (1980) defined the former '*Enterobacter sakazakii*' species they included a national culture collection strain NCTC 8155 isolated from dried milk by Thornley (1960). Therefore, the presence of *Cronobacter* spp. in dried milk products can be traced back for many decades, and overlaps with the first meningitis case attributed to *Cronobacter* in 1958 (Urmenyi and Franklin, 1961). However, at that time, there was no evidence of any link with infant formula, which considerably differs from milk powder.

A number of surveys of PIF have been reported and are summarised in Table 1. However it should be noted that these surveys were undertaken at the time of the previous Codex Alimentarius Commission (CAC, 1979) microbiological criteria, and may not reflect current prevalence of *Cronobacter* spp. under the more stringent guidelines (CAC, 2008a,b). Also a number of these surveys used non-specific methods for *Cronobacter* (i.e. FDA protocol) in which the organism could be outnumbered on the violet red bile glucose agar (VRBGA) plates, and non-pigmented *Cronobacter* isolates on tryptic soy agar (TSA) would be overlooked.

The first reported large survey of PIF samples for *Cronobacter* spp. and other *Enterobacteriaceae* was by Muytjens et al. (1988) who studied 141 samples, from 35 countries. They reported that 52.2% of samples were contaminated with *Enterobacteriaceae*, and 14% (13 countries) contained '*E. sakazakii* '. The level of contamination ranged from 0.36 to 66.0 cfu/100 g. This low level (<1 cfu/g) of contamination has been confirmed in numerous further studies. Simmons et al. (1989) reported 8 *Cronobacter* cfu/100 g for an open can of powdered milk formula used during the time of an outbreak on an neonatal intensive care unit. Nazarowec-White and Farber (1997b) analysed 120 cans of PIF from five different companies in Canada and found that 6.7% contained *Cronobacter* spp. at levels of 0.36 cfu/100 g.

The prevalence was between 0 and 12% of the samples per manufacturer. Heuvelink et al. (2001), using a present/absence test for 25 g quantities, detected *Cronobacter* spp. in 1 of 40 infant formula powders and 7 out of 170 milk powders. Santos (2006) studied 98 PIF samples and reported levels of *Cronobacter* at 0.22-1.61 cfu/100g product. Hence the organism has never been reported at levels >1 cfu/g.

A detailed analysis of almost 500 food samples including 82 PIF and 49 weaning foods was reported by Iversen and Forsythe (2004). This survey used the chromogenic Druggan-Forsythe-Iversen agar (DFI) to improve the recovery of *Cronobacter* in the presence of other *Enterobacteriaceae*. This medium has better sensitivity (87.2%) and specificity (100%) than the Muytjens et al. (1988) procedure (Iversen et al., 2004a). *Cronobacter* were isolated from 2 of 82 PIF, 5 of 49 weaning foods, 3 of 72 milk powders, 40 of 122 herbs and spices, and 15 of 66 other dry food ingredients. Following the FAO/WHO (2008) call for data on follow up formula, an international consortium of laboratories was formed which analysed a total of 287 samples of follow-up formula and weaning foods which are all desiccated powdered products. A total of 7 countries participated; Brazil, England, Indonesia, Jordan, Korea, Portugal, and Malaysia (Chap et al., 2009). They reported the isolation of *Cronobacter* spp. from 1 of 84 samples of follow up formula and 30 of 203 weaning foods. It was also found that there were differences in national definition of 'follow up formula' which did not necessarily equate with the Codex Alimentarius Commission.

Therefore, it is clear that *Cronobacter* spp. is recoverable from the desiccated state in a number of powdered food products which are given to infants. Linked to this, it is pertinent to remember that the well publicized 2001 Tennessee outbreak of *C. sakazakii* was attributed to the accidental feeding of a non-infant formula to

neonates (Himelright et al., 2002). The intended market for this product was children and adults. Additionally, the prevalence of *Cronobacter* spp. infections in adults is raised in the elderly who are immunocompromised, and may use protein supplements as part of their diet (FAO/WHO, 2008). A common risk factor in reported *Cronobacter* outbreaks in France was the temperature abuse of reconstituted formula (Caubilla-Barron et al., 2007; Coignard et al., 2006). This highlights the need for temperature control to reduce microbial growth in reconstituted formula.

2. Persistence of Desiccated Cultures of *Cronobacter*

The following section reviews the limited number of studies which have been undertaken to investigate the persistence of *Cronobacter* under desiccated conditions. Although the thermotolerance of microorganisms is affected by their physiological states (Lou and Yousef, 1996; Wesche et al., 2005) most studies on thermal inactivation of *Cronobacter* spp. in reconstituted PIF have used non-stressed cultures, grown under optimal laboratory conditions (Breeuwer et al., 2003; Edelson-Mammel and Buchanan, 2004; Iversen et al., 2004b; Nazarowec-White and Farber, 1997a). However in food processing or preparation environments, microorganisms are exposed to a wide range of chemical, physical, and nutritional stresses. Therefore, it is appropriate to study the thermotolerance properties of the pre-stressed i.e. desiccated *Cronobacter* cells, as it could occur prior to the intrinsic contamination of PIF.

It should be noted that many publications prior to 2008 used the name '*E. sakazakii*' and cannot be reinterpreted with respect to specific species, and therefore in this review the general term *Cronobacter* species has been used.

2.1. Persistence of desiccated Cronobacter *cells*

Riedel and Lehner (2007) screened 56 *Cronobacter* spp. for desiccation tolerance by determining the viable count of stationary phase cultures which had been spread on nylon membrane discs, dried and stored at room temperature for 72 h. One strain *C. sakazakii* z236 (species determined by 16S rDNA sequence analysis, Genbank accession number AY752943) was chosen for further detailed proteomic studies. For desiccation stress studies, cells were harvested from 1.5 L of LB broth, washed and dried in a Petri dish for 5 h at room temperature, followed by storage at room temperature for 7 days. Osmotically-stressed cells were grown in LB broth supplemented with 1 M NaCl.

Breeuwer et al. (2003) and Shaker et al. (2008) used similar techniques to prepare desiccated cultures of *C. sakazakii* and *C. muytjensii*. Overnight cultures of the *Cronobacter* strains were divided into 50 μL portions in a sterile Petri dish. The plate was placed, without a lid, in a 40°C incubator for drying along with dehydrated silica gel. After 2 h, the plate was covered and kept at 21°C for 4 d. Initial studies showed that the drying procedure decreased the *Cronobacter* viability by 1 $\log_{10}$ and the 4 d-storage period decreased the level of the cells by ≤ 1 $\log_{10}$/mL.

Caubilla-Barron and Forsythe (2007) prepared desiccated cultures of 10 *Cronobacter* strains and 17 strains of other *Enterobacteriaceae* for a long-term

persistence study of 2.5 years. The *Enterobacteriaceae* included *E. cloacae, Salmonella* Enteritidis, *Citrobacter koseri, Cit. freundii, E. coli, E. vulneris, Pantoea* spp., *K. oxytoca,* and *K. pneumoniae.* Such a large study required a less labour intensive method per strain than that of Breeuwer et al. (2003) and Shaker et al. (2008) described above. A miniaturised method of desiccation was designed which was based on the 'most probable number' approach to estimate microbial viability. Nearly all *Enterobacteriaceae* were grown on milk agar plates at 37°C for 48 h, except *Salmonella* Enteritidis (non–lactose fermentor) which was grown on (TSA). Cells were harvested in sterile liquid infant formula to a cell density of approximately 10^{11} cfu/ml, and then aliquots were transferred into six-well ELISA tray plates and air dried overnight in a class II cabinet at room temperature. After desiccation, bacterial cell suspensions were prepared in sterile liquid infant formula in serial tenfold dilutions. Ten-microliter aliquots of each diluted cell suspension were dispensed into 96-well microtiter plates. Two microtiter trays were prepared per strain for each time point, giving a total of 16 replicates per dilution. To determine the culture viability at 20 time points over the study period, forty 96-well microtiter plates per strain were prepared. This resulted in the preparation of 1,080 microtitre plates for all 27 strains. Uninoculated infant formula was used as the negative control. The plates were dried in a class II cabinet at room temperature for 4 h before being sealed with microtiter lids and stored at room temperature. At known time intervals, each microtiter tray well was rehydrated with 200 µl of sterile liquid infant formula and incubated for 48 h at 37°C. Growth in each well was detected by the addition of bromocresol purple to detect changes in the infant formula pH. The viability of each strain was determined by the most-probable-number (MPN) estimation based on 16 replicates per strain per time point using the BAM-MPN Excel software (FDA, 2006).

Caubilla-Barron and Forsythe (2007) reported that the *Enterobacteriaceae* could be divided into four groups with respect to their long-term survival in the desiccated state. Group 1 was composed of *Cit. freundii, Cit. koseri,* and *E. cloacae.* These organisms were no longer recoverable after 6 months. Group 2 organisms were *S.* Enteritidis, *K. pneumoniae,* and *E. coli* and could not be recovered after 15 months. The third group consisting of *Pantoea* spp., *K. oxytoca,* and *E. vulneris* persisted over 2 years, and some capsulated strains of *C. sakazakii* which were still recoverable after 2.5 years. The recovery of *Cronobacter* spp., under desiccated conditions, decreased an average of 0.58 $\log_{10}$ cycles (range, 0.26 to 1.15 $\log_{10}$ CFU/ml) during the first month. This result was similar to previous published values of 0.5- and 0.6-$\log_{10}$ reductions per month (Edelson-Mammel et al., 2005; Gurtler and Beuchat, 2005). A larger decrease was observed during the first 6 months, when the recovery declined by 3.34 log cycles. During the next 24 months, the average recovery decreased a further 1.88 $\log_{10}$ cycles, resulting in a total decline in viable counts of 4.52 $\log_{10}$ cycles in the desiccated state. *C. sakazakii* type strain (NCTC 11467^T) differed from the other *Cronobacter* strains in that it was no longer recoverable after 1 year. As previously reported this type strain has atypical growth characteristics (Iversen et al., 2004b), and it is advisable not to use it as representative of the species for growth and survival studies. Five of the 10 *Cronobacter* strains were still recoverable after 2 years. The rate of loss of viability decreased after 6 months for all strains except strain (NCTC 11467^T). It is plausible that the cultures were composed of two distinct subpopulations. The minority subpopulation being more resistant to prolonged desiccated storage. After 2 years of storage, four of the five *Cronobacter* strains recovered were capsulated, and the only strains recoverable after 2.5 years were two capsulated strains. Therefore,

capsulation may play an important role in recovery after extended periods. The importance of the capsule in desiccation survival is supported by the persistence of capsulated strains of *K. oxytoca, E. vulneris,* and *Pantoea* spp. over the 2-year period.

2.2. Recovery of desiccated Cronobacter *spp.*

Although the current method for *Cronobacter* spp. detection involves an initial pre-enrichment step to resuscitate desiccated stressed cells, a number of researchers have studied direct plating methods onto selective agars. Gurtler and Beuchat (2005) compared the recovery of *Cronobacter* spp. on non-selective, differential and selective media. They used a cocktail culture of four strains which had been desiccation stressed. They found that the recovery of *Cronobacter* cells which had survived the desicaction process on TSA supplemented with 0.1% sodium pyruvate (TSAP), Leuschner, Baird, Donald, and Cox (LBDC) agar, fecal coliform agar (FCA), and OK agar (Oh and Kang) was significantly higher than on Druggan-Forsythe-Iversen (DFI) medium, VRBGA, or *Enterobacteriaceae* enrichment (EE) agar. Similarly *Cronobacter* spp. exposed to heat, freeze, acid, and alkaline stressed cells were recovered better on TSAP and LBDC than differential, selective media. The authors stated that LBDC can be used as a direct-plating medium for detecting injured *Cronobacter* spp. in dry infant formula containing a low number of background microflora.

Al-Holy et al. (2008) compared an overlay method and selective-differential media (OK, violet red bile agar, DFI, EE and FCA) for the recovery of desiccation stressed *Cronobacter* spp. from dry infant milk formula. The overlay method involved

plating 0.1 ml samples of reconstituted infant milk formula onto TSAP and incubating for 2 h at 37 °C to allow injured *Cronobacter* cells to resuscitate. Afterwards, a thin layer (8 ml) of each of the selective-differential media was overlaid onto TSAP and the plates were incubated for additional 22 h at 37°C. Their results showed that the use of the overlay method was efficient for detecting low levels of desiccation stressed *Cronobacter* spp. in dry infant milk formula without compromising the selectivity of the medium. The highest recovery of desiccated stressed cells was on TSAP, TSAP+VRBA, TSAP+DFI, and TSAP+FCA and the lowest recovery was on EE medium.

Osaili et al. (unpublished work) further evaluated the value of the thin agar layer (TAL) method to recover stressed *Cronobacter* spp. The TAL method involves pouring a molten TSA (40 to 45°C) on selective differential media (VRBGA or DFI) prior to inoculation. There were no significant differences among the recovery of desiccation stressed of *Cronobacter* spp. on TSA, VRBGA+TSA and DFI+TSA. The recovery of desiccated stressed *Cronobacter* spp. on VRBGA was significantly lower than on DFI.

2.3. Effect of desiccation on Cronobacter *thermal tolerance*

Reconstitution with hot water (>70°C) has been recommended by the FAO/WHO (2004, 2006) and WHO (2007) to reduce the risk of *Cronobacter* infections by reducing the bacterial load in PIF. Therefore it is pertinent to study the affect of desiccation on thermal tolerance. Osaili et al. (2008a,c) studied the effect of environmental stresses on the thermal inactivation of *C. sakazakii* and *C. muytjensii* in infant milk formula and found that these stresses decreased the thermal resistance of the microbe. They found that extended dry storage of *Cronobacter* in

infant milk formula increased the susceptibility of the microbe toward heat during rehydration with hot water. Further studies by Shaker et al. (2008) determined the effect of desiccation, as well as other stresses (starvation, heat and cold) on the thermal inactivation of *C. sakazakii* and *C. muytjensii* in reconstituted PIF. Stressed cells in reconstituted PIF were exposed to 52 - 58°C for various time periods, and the subsequent D- and z-values were determined following plating on non-selective agar. D-values for unstressed *Cronobacter* at 52, 54, 56, and 58°C were 15.33, 4.53, 2, and 0.53 min, respectively. Desiccation and heat stresses, but not starvation or cold stress, caused significant ($P < 0.05$) reduction in D-values. The z-values of desiccated, starved, heat stressed, and cold stressed *Cronobacter* were not significantly different from the z-value of unstressed cells (4.22°C). Thermal resistance of *Cronobacter* in reconstituted PIF was affected by desiccation and heat. As given above, these are environmental stresses to which the organism may be exposed to prior to the contamination of infant formula or other foods.

Shaker et al. (2008) calculated the process lethality (*F*), during heating and cooling of reconstituted PIF for desiccated, starved, heat and cold stressed *Cronobacter* strains. Taking the following as an example, when the maximum temperature on reconstitution was 63°C after 4 min of heating, followed by cooling to 40°C within 2 min, this equated to an average process lethality at the reference temperature 58°C of 18 min. This process lethality will result in approximately 60, 27, 67, and 38 $\log_{10}$ reduction (F/D_{58}) desiccated, starved, heat stressed, and cold stressed *Cronobacter* cells and a 34 $\log_{10}$ reduction unstressed *Cronobacter* cells in reconstituted PIF. The authors proposed that due to such a high kill, the presence of *Cronobacter* in powdered infant milk formula was probably due to contamination

after pasteurization during the manufacturing process, and confirmed the high kill when using hot water to reconstituted PIF.

2.4. Effect of desiccation on Cronobacter *ionizing radiation tolerance*

Doses of up to 10 kGy can greatly reduce the number of spoilage organisms and eliminate pathogens without causing toxicological hazards or compromising nutritional and sensory quality (WHO, 1999). The efficacy of reducing the viability of *Cronobacter* spp. in dry infant milk formula by ionizing radiation has been investigated by Hong et al. (2008), Osaili et al. (2007), Lee et al. (2006). Non-desiccated cells were used in these studies.

More recently, Osaili et al. (2008a, b) studied the resistance of environmental stressed *Cronobacter* spp. in PIF to gamma radiation. Four food *C. sakazakii* isolates and *C. muytjensii* type strain were desiccation stressed in PIF for up to 1 year (Osaili et al. 2008a). It was found that extended dry storage in PIF increased the resistance of *Cronobacter* spp. to ionizing radiation. The D_{10}-values of 8 month desiccation stressed cells were higher than those of the controls by 7 to 31%. The D_{10} values (1.08-1.28 KGy) of 8 month desiccated *C. sakazakii* were significantly higher than those of 1 month desiccated cells (0.95-1.0 KGy). Although a 2-$\log_{10}$ cfu/g reduction of the *C. sakazakii* strains in control samples could be achieved by 2 kGy. The latter dose was insufficient to consistently eliminate 1.2 to 1.5 $\log_{10}$ of the same isolates that were desiccation stressed in dry infant milk formula for 12 months. While desiccation enhanced resistance to irradiation treatment, strains varied in terms of the extent of change in resistance development during extended dry storage. For instance, *C. muytjensii* was the most resistant strain after 1 month of

storage. However, this strain was more sensitive than the others after 12 months of storage. In contrast, *C. sakazakii* (PIF isolate) was recovered from dry samples irradiated with 4 kGy after 12 months of dry storage. Osaili et al. (2008b) found that other environmental stresses (starvation, heat, cold, acid, alkaline, chlorine or ethanol) did not significantly change the sensitivity of most *Cronobacter* spp. in dry infant milk formula to ionizing radiation. The D_{10} values of stressed *C. muytjensii* ranged from 1.35 to 1.95, while those for stressed *C. sakazakii* ranged from 0.82 to 1.24 kGy.

3. Desiccation Stress Survival Mechanisms in *Cronobacter* Species

Milk-based infant formula contains components such as lactose, proteins, and milk fat that may have protective effects on bacteria during drying and reconstitution affecting their ability to survive desiccation. However this does not explain the specific desiccation resistance of *Cronobacter* compared with other *Enterobacteriaceae*. A clue with regard to the noted desiccation resistance may reside in the normal habitat of the organism. As revealed in the various surveys (Table 2), a probable natural habitat of *Cronobacter* is plant material; cereals, wheat, corn, soy, rice, herbs and spices, vegetables, salads. In fact early sources of isolates included sour tea, and Chinese herbs (Scheepe-Leberkühne Wagner 1986; Tamura et al. 1995). Consequently the organism can be present in a number of plant-derived ingredients including starches, and carob powder used in PIF production, pasta, and flour. Hence *Cronobacter* spp. has a number of environmental and plant-related survival mechanisms. These include the production of a yellow pigment in most (but not all) strains to protect against oxygen-radicals,

capsule synthesis to aid attachment, and an array of other survival mechanisms which confer protection against cellular damage due to desiccation and other environmental stresses. This topic has been investigated by Breeuwer et al. (2003), and Riedel and Lehner (2007). It is covered in more detail below.

Prior to the above long-term comparative study of Caubilla-Barron et al. (2007), Breeuwer et al. (2003) aimed to demonstrate that *Cronobacter* spp. were not particularly thermotolerant, but that they adapted following exposure to desiccation and osmotic stresses. D-value estimates showed that the thermotolerance of 22 strains of *Cronobacter* spp. in the exponential phase were comparable with that of other *Enterobacteriaceae*, and lower than the previous reported values of Nazarowec-White and Farber (1997a). However, stationary phase cells were relatively more resistant to dry and osmotic stress than *E. coli*, *Salmonella* and other strains of *Enterobacteriaceae* tested. Given the diverse nature of the *Cronobacter* genus it is plausible that the differences with Nazarowec-White and Faber were due to differences in experimental protocol. For example, Breeuwer et al. (2003) placed the heat-treated cell on ice prior to enumeration, and therefore could have given the cells a cold-shock resulting in lower recoveries. In addition, the two groups could have been analysing different *Cronobacter* species, and it is known that the thermotolerance between *Cronobacter* species differs considerably (Caubilla-Barron et al., 2009). A significant observation by Breeuwer et al. (2003) was that *Cronobacter* cells in the stationary phase accumulated trehalose, and this may be linked with desiccation survival. Under such conditions, the level of trehalose in *Cronobacter* spp. increased >5 fold. This was not observed in exponential phase cells, nor in *E. coli*. The latter being more thermal sensitive than *Cronobacter*. Trehalose is one of a number of compatible solutes; others being glycine, betaine,

proline, ectoine, carnitine, and choline. These are polar, highly soluble compounds which can counteract osmotic pressure and drying stabilizing proteins and membranes. To the authors' knowledge, this observation has unfortunately not been further investigated. The closest is the work by Riedel and Lehner (2007) using a proteomics approach to study stress response in *C. sakazkaii* strain z235 isolated from fruit powder.

Riedel and Lehner (2007) recorded a number of changes in protein synthesis following desiccation and osmotic stress as an adaptive protection mechanism (Table 3). There were similarities, and differences in response to the two stress conditions. Changes in protein profiles in osmotically stressed cells are primarily adaptation to the environment, whereas the response is more protective in desiccated cells. A heat shock protein was detected in desiccated cells, but not osmotically-stressed cells and may have been part of a general stress response. Other proteins which were up-regulated were cold-shock protein CspC, DNA protection and repair proteins Dps and histone-like DNA binding protein, as well as the protective proteins against oxygen radicals; superoxidase dismutase and alkyl hydroperoxide reductase. A number of enzymes involved in glycolysis and fermentation were also up-regulated which might relate to the trehalose accoumulation. Additionally, the induction of OmpC, OmpA and glutamine-binding protein may be linked to the transport into the cell of compatible solutes, similar to trehalose accumulation reported by Breeuwer et al. (2003). The protein Mfla-1165 was also up-regulated in desiccated cells, but not osmotically-stressed cells. This protein was reported to be a biomarker for thermotolerance (Williams et al. 2005), but has not been confirmed by other research groups (Caubilla-Barron et al., 2009).

A number of genes encoding the proteins up-regulated due to desiccation stress in *C. sakazaki* z235 have been sequenced in the *C. sakazakii* strain ATCC BAA-894 (Table 3). There are three putative ABC-type proline/glycine betaine transport systems (ESA_00586 to 00589, ESA_01108 to 0111 and ESA_01738 to 01741), and a number of genes for cold-shock proteins (ESA_02704, ESA_04323, and ESA_02195). In addition, genes related to capsule production have been located ESA_03349 to 03353, but not the gene encoding the protein Mfla-1165. It should be noted that *C. sakazakii* ATCC BAA-894 was not isolated from an infected infant, but from a can of formula associated with the outbreak. It is known that *C. sakazakii* strains may acquire additional traits during infection including antibiotic resistance factors (Caubilla-Barron et al., 2007).

4. Conclusions

Cronobacter spp. have been isolated from a wide variety of sources, including powdered infant formula, powdered ingredients and foods. The organism is more resistant to desiccation than most other *Enterobacteriaceae*, and can persist in the desiccated state for at least 2 years. On reconstitution, the organism can rapidly multiply and hence the reconstituted product can present a risk to the immunocompromised. Therefore temperature abuse should be avoided. Currently neonates are recognised as a vulnerable group to *Cronobacter* infections, however the elderly may also be susceptible. A greater understanding of stress response adaptations, such as to desiccation in the production facility, may contribute to further improvements in the control of this bacterium.

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Table 1. Isolation of *Cronobacter* spp. from powdered infant formula. Adapted from FAO/WHO (2006)

Method	Volume tested (g)	Number of samples	*Cronobacter* spp. positive (%)	Enumeration (cfu/100g)	Reference
BPW, EE, VRBGA	333-555	141	20 (14.2)	0.36-66	Muytjens et al., 1988; Townsend et al. 2007
DW, EE, VRBGA,	333	120	8 (6.7)	0.36	Nazarowec-White and Farber, 1997b
FDA	333	22	5 (22.7)	0.36	FDA, 2003
BPW, EE, VRBGA	25	101	2 (2)	ND	Heuvelink et al., 2003
BPW, EE, ESIA or DFI	300	40	5 (12.5)	ND	Estuningsih et al., 2006
BPW, EE, DFI	25	102	3 (2.9)	ND	Iversen and Forsythe, 2004
FDA and BAX	5 x 100	98	12 (12.2)	0.22-1.61	Santos, 2006

ND not determined

Table 2. Isolation of *Cronobacter* species from non-infant formula powdered foods, plant material and other dry sources. Updated and adapted from Fanning and Forsythe (2008).

Source	References
Follow-on formula (3/89)	Chap et al. 2009
Weaning foods (5/49[a] and 30/203[b])	Iversen and Forsythe, 2004[a]; Shaker et al. 2007; Chap et al. 2009[b];
Preparation equipment (blender, spoons)	Block et al. 2002; Clark et al. 1990; Smeets et al. 1998; Bar-Oz et al. 2001
Milk powder (3/72)[a]	Postupa and Aldová 1984; Muytjens et al. 1988; Heuvelink et al. 2001; Iversen and Forsythe 2004[a]
Rice seed	Cottyn et al. 2001
Dried foods (15/66), herbs and spices (40/122)	Iversen and Forsythe 2004
Dried flour or meal (corn, soy, wheat and rice) (14/78)	Restaino et al. 2006
Dried infant cereals (2/6), adult cereals (2/8)	Restaino et al. 2006
Dried vegetables and spices (1/5)	Restaino et al. 2006
Grain	Jung & Park 2006
Tofu	Fouad & Hegeman 1993; No et al. 2002
Iced tea	Zhao et al. 1997
Mixed salad vegetables	Gaolli et al. 1990; Lack et al. 1999; Weiss et al. 2005
Dried sodium caesinate (4/24)	Restaino et al. 2006

Starches (40/1389)	FAO/WHO 2004
Milk powder, chocolate, cereal, potato flour, pasta and spices factories, and household dust	Kandhai et al. 2004
Hospital air	Masaki et al. 2001

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Table 3. Proteins associated with desiccation resistance and osmotic stress adaptation, and location of capsule production genes; based on Riedel and Lehner (2007).

COG functional annotation	Protein	Putative gene in *C. sakazakii* BAA-894[a]	Response to desiccation stress conditions
DNA replication, recombination and repair	DNA protecting protein under starved conditions Dps	ESA_02528	Present
Transcription	Cold shock-like protein CspC	ESA_02195, ESA_02704, ESA_04323	Up-regulated; conversely regulated in desiccated and osmotically stressed cells
Translation, ribosomal structure and biogenesis	50S ribosomal protein	ESA_00203	Up-regulated
	Elongation factor EF-Tu	ESA_03699	Up-regulated
	Elongation factor EF-G	ESA_04401	Up-regulated
Cell division and chromosome partitioning	Cell division and chromosome partitioning MinD	ESA_01458	Down-regulated
Cell envelope biogenesis, outer membrane	Outer membrane protein OmpC	ESA_00974, ESA_013112, ESA_01235, ESA_02413	Up-regulated
	Outer membrane protein OmpA	ESA_02391	Up-regulated
	Capsule production	ESA_03349-03353	Previously reported to be protective[b]
Cell motility and secretion	Flagellin FliC	NF	Down-regulated
	Thermoregulated motility protein	ESA_02188	Up-regulated
Inorganic ion transport and metabolism	Superoxide dismutase	ESA_03843	Up-regulated
Post-translational modification, protein turnover and chaperones	AAA ATPase, central region: Clp, N-terminal	ESA_00662	Up-regulated
	Trigger factor	ESA_02862	Up-regulated
	Chaperonin GroES	ESA_00153	Up-regulated
	HSP	ESA_03959	Up-regulated
	HSP ClpB	ESA_00662	Present
	Alkyl hydroperoxide reductase	ESA_02721	Up-regulated
Amino acid transport and metabolism	Arg 3rd transport system periplasmic binding protein	ESA_02473, ESA_02477	Present

	Gln-binding periplasmic protein	ESA_02529	Conversely regulated
	Glu/Asp-binding periplasmic protein	ESA_02680	Present
	Metalloprotease	ESA_00752	Up-regulated
Carbohydrate transport and metabolism	Enolase	ESA_00523	Up-regulated
	PTS system, glucose-specific IIA component	ESA_00828	Up-regulated
	Phosphoglycerate kinase	ESA_00409	Up-regulated
	a-Glucosidase	ESA_02513, ESA_04054, ESA_04154	Up-regulated
	Maltose-binding periplasmic protein	ESA_00081	Present
	ABC-type proline/glycine betaine transport systems	ESA_00586-00589, ESA_01108-0111, ESA_01738-01741	Compatible solute transpo
Energy production and conversion	Inorganic pyrophosphatase	ESA_00231	Up-regulated
General function prediction only	DNA-binding protein Hns	ESA_01537	Up-regulated
Function unknown	Hypothetical protein Mfla_1164	NF	Present
	Hypothetical protein Mfla_1165	NF	Up-regulated
	Hypothetical protein Psyc_0523	ESA_01369	Present in osmotically stressed cells only

NF not found.

a Protein accession number (GI) from Riedel and Lehner (2007) was used to BLAST the *C. sakazakii* BAA-894 genome (www.ncbi.nlm.nih.gov).

b Caubilla-Barron & Forsythe (2007).