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Updates on the *Cronobacter* Genus

Stephen J. Forsythe

foodmicrobe.com, Adams Hill, Keyworth, Nottinghamshire, NG12 5GY, United Kingdom; email: steve.forsythe@foodmicrobe.com

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Abstract

There has been considerable concern related to *Cronobacter* spp. in foods, especially due to their highlighted association with neonatal infections through the ingestion of reconstituted powdered infant formula (PIF). This concern resulted in improved microbiological criteria recommendations by the Codex Alimentarius Commission and revised WHO advice on the preparation of infant feeds. In recent years, the diversity of the genus has been well described, and various detection and typing methods have been developed. This review considers our current knowledge of the genus and how DNA-sequence-based methods have contributed considerably to research into improved detection methods and more reliable identification procedures, genotyping schemes, and genomic analysis. The broader occurrence of *Cronobacter* in food ingredients, finished products, and food manufacturing environments is covered. This review also highlights the significance of clonal lineages in microbial source tracking and the use of CRISPR-*cas* array profiling.

INTRODUCTION TO THE CONTROL OF *CRONOBACTER* SPP. IN THE FOOD INDUSTRY

This review concerns the bacterial genus *Cronobacter*, which, although initially publicized for its connections to neonatal infections, is now recognized as predominantly causing infections in adults. The *Cronobacter* genus came to the attention of regulatory authorities as a result of its association with outbreaks of life-threatening infections in neonates (necrotizing enterocolitis, septicemia, and meningitis) (Block et al. 2002, Himelright et al. 2002, Van Acker et al. 2001). As neonates are frequently fed reconstituted powdered infant formula (PIF), which is not a sterile product, PIF became the focus of attention for reducing infection risk to neonates. This review addresses sources of *Cronobacter*, the need to differentiate *Cronobacter* isolates from closely related organisms, and issues regarding the differentiation of strains for microbial source tracking.

Cronobacter species can be grouped according to their clinical relevance: Group 1 comprises *Cronobacter sakazakii* and *Cronobacter malonaticus*, which form the majority of clinical isolates in all age groups, and Group 2 comprises *Cronobacter turicensis* and *Cronobacter universalis*, which have been rarely reported. The other three species (*Cronobacter dublinensis*, *Cronobacter muytjensii*, and *Cronobacter condimenti*) are primarily environmental commensals and are probably of little or no clinical significance.

Cronobacter infections are not unique to neonates. They occur in all age groups, albeit with a greater incidence in the very young and elderly, who are typically more immunocompromised (FAO-WHO 2008, Holy & Forsythe 2014, Patrick et al. 2014). Neonates, particularly those of low birth weight, are the major identified group at risk of mortality. Symptoms in neonates include necrotizing enterocolitis (NEC), septicemia, and meningitis. The former is noninvasive, whereas in septicemia and meningitis the organism has attached and invaded the host body, presumably through the intestinal epithelial layer. It needs to be noted that NEC is a common multifactorial, gastrointestinal illness in neonates and can be caused by a variety of bacterial pathogens. It is not solely associated with *Cronobacter* spp.

To date, control of *Cronobacter* has focused on the reduction of exposure through specific microbiological criteria for PIF and revised hygienic reconstitution procedures. The need for good hygienic practice before feeding is essential because PIF is not produced as a sterile product, and there are other extrinsic routes of contamination, such as the preparation equipment. A number of neonatal intensive care unit (NICU) outbreaks have been associated with the lack of adequate hygienic preparation and temperature control of the reconstituted formula (Caubilla-Barron et al. 2007, Himelright et al. 2002).

The first two FAO-WHO (2004, 2006) risk assessment meetings on the microbiological safety of PIF categorized bacterial pathogens associated with PIF into three groups:

- Category A. Clear evidence of causality. *Salmonella* serovars and *Cronobacter* spp.
- Category B. Causality plausible but not yet demonstrated. Enterobacteriaceae: *Escherichia coli*, *Escherichia vulneris*, *Citrobacter koseri*, *Enterobacter cloacae*, *Hafnia alvei*, *Pantoea agglomerans*, *Klebsiella pneumoniae*, and *Klebsiella oxytoca*. Non-Enterobacteriaceae: *Acinetobacter* spp.
- Category C. Causality less plausible or not yet demonstrated. *Clostridium botulinum*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Bacillus cereus*.

Therefore, only *Salmonella* serovars and *Cronobacter* spp. were formally recognized as causing neonatal infection through contaminated PIF. However, it is understood that control of these two organisms could also control other potentially infectious non-spore-forming bacteria. To achieve this, the FAO-WHO expert committees proposed that PIF should be reconstituted at temperatures no cooler than 70°C and used immediately (FAO-WHO 2004, 2006). Consequently, the bacterial load of the feed is reduced, and, by using the feed immediately, there is limited growth of any

surviving organisms. A second outcome from the FAO-WHO meetings was the production of an online risk model (**http://tools.fstools.org/esakmodel/ESAKRAModelWizard.aspx**). The model allows the user to compare the level of risk between different levels of contamination and reconstitution practices. The model was based on growth and death kinetic data for a limited number of *Cronobacter* strains. Forsythe et al. (2009) extended the risk model to cover all organisms in Categories A and B. The data were generated using casein- and whey-based formulas, as the type of formula affects bacterial lag times and growth and death rates.

Despite the emphasis on *Cronobacter* infections of infants, it should be noted that such neonatal infections are rare, and not all have been associated with reconstituted formula ingestion. Breast milk has been a suspect source in a number of cases (Barreira et al. 2003, Stoll et al. 2004). In fact, the *C. malonaticus*–type strain LMG23826^T was isolated from a breast abscess. *Cronobacter* species have also been isolated from hospital air, dust, and human intestines and throats. Thus, the improved microbiological safety of PIF does not necessarily totally remove the risk of neonatal exposure to the organism.

Cronobacter infections in the adult population show a wide range of symptoms: conjunctivitis, biliary sepsis, urosepsis, appendicitis, wound infections, and pneumonia. Adult patients at increased risk include those previously treated with antibiotics, immunocompromised and elderly patients, and those patients with medical implants or acute, chronic, or serious illnesses (Lai 2001, Patrick et al. 2014, Pitout et al. 1997). Sources of adult infections are unknown but could be through increased susceptibility to commensal *Cronobacter*. In particular, strains of *C. malonaticus* clonal complex (CC) 7 appear to be more associated with adult than neonatal infections, although the reason for the increased incidence is unknown (Forsythe et al. 2014, Joseph & Forsythe 2011).

SOURCES OF *CRONOBACTER* SPP.

Iversen & Forsythe (2003) were the first to hypothesize that a common ecosystem for the *Cronobacter* species might be plants. This association with plants was due to the notable production of a polysaccharide capsule and of a yellow carotenoid-based pigment and its desiccation resistance. Together, these traits could enable the organism to attach to plant leaves, protect against oxygen radicals generated from sunlight exposure, and survive dry periods, including autumn. Subsequent microbiological surveys of food and related food ingredients have supported the proposal that plants are a probable major eco-system for *Cronobacter* spp. *Cronobacter* is rarely isolated from foods commonly associated with other foodborne pathogens, e.g., meat, eggs, and milk. Instead, *Cronobacter* is present in a wide variety of processed foods and fresh produce (**Table 1**). These

Table 1 Surveys of food products and food ingredients for *Cronobacter* spp.[a]

Food product or ingredient	Number of positive samples	Total number of samples	%
Follow-up formula	10	74	14
Cereal-based follow-up formula	6	100	6
Dry infant food	5	49	10
Dry infant food	22	179	12
Dry infant cereals	2	6	33
Milk powder	3	72	4
Milk powder	2	20	10
Milk powder	3	50	6
Milk-based products	5	20	25
Milk powder and derived products	1	55	2

(*Continued*)

Table 1 (*Continued*)

Food product or ingredient	Number of positive samples	Total number of samples	%
Starches	40	1,389	3
Corn, soy, wheat, and rice	14	78	18
Rice flour	6	16	38
Dry food ingredients	15	66	23
Herbs and spices	40	122	33
Spices	13	21	62
Spices	14	71	20
Spices and dried herbs	7	26	27
Sprouts and fresh herbs and spices	14	23	61
Dried powdered vegetables	1	50	2
Ready-to-eat salad	19	109	17
Salad	1	15	7
Nuts	2	2	100
Instant soups	2	13	15
Instant soups	6	10	60
Lentils	1	11	9
Vegetables	5	12	42
Vegetables	19	128	15
Semolina[b]	1	3	33
Cereal[b]	8	50	16
Oat flakes[b]	1	10	10
Wheat sprout[b]	1	9	11
Fruit	3	41	7
Tea	3	5	60
Pastries	5	9	56
Confectionary	3	42	7
Chocolate products	11	37	30
Raw meat	1	64	2
Raw meat	3	15	20
Raw meat (spiced or marinated)	17	48	35
Meat by-products (ready to eat)	9	81	11
Seeds	14	34	41
Desiccated coconut	1	10	10
Coconut biscuits	1	1	100
Dried fish	13	50	26
Sunsik	17	36	47
Tofu	4	11	36
Cheese	2	62	3
Eggs	1	20	5
Shellfish	3	8	38
Seaweed	3	24	13

[a]Updated from Forsythe (2015). Data have been collated from a wide range of publications. Please contact author for original references.
[b]Cereal products.

sources include cereals, wheat, corn, soy, pasta, rice, cake mixes, packet soup, flavored teas, herbs and spices, vegetables, and salads as well as PIF and infant-weaning foods (Friedemann 2007, Hochel et al. 2012, Iversen & Forsythe 2004, Vojkovska et al. 2016). Fresh and dried herbs and spices are a particularly productive source of *Cronobacter* strains, with an ~30% incidence (Iversen & Forsythe 2004). Ironically, a food thickener extracted from *Cronobacter* spp. isolated from Chinese tea has been patented (Harris & Oriel 1989, Scheepe-Leberkuhne & Wagner 1986).

Because *Cronobacter* spp. is plant-associated, it is not surprising that it can be isolated from a wide range of environments. It has been isolated from domestic environments (i.e., household vacuum cleaning bags) and also from household utensils (Kandhai et al. 2004, Killer et al. 2015). Rats and flies are also additional sources of *Cronobacter* spp. (Kuzina et al. 2001, Hamilton et al. 2003, Pava-Ripoll et al. 2012). Nevertheless, it is the widespread presence and persistence of the organism at milk powder and infant formula manufacturing sites that need to be appreciated and controlled due to the potential contamination of the finished product. The organism has been isolated from such manufacturing plants worldwide (Australia, China, Germany, Ireland, Switzerland, and the United States) and may therefore be a widespread issue for industry (Craven et al. 2010, Fei et al. 2015, Jacobs et al. 2011, Reich et al. 2010, Sonbol et al. 2013, Yan et al. 2013). Isolation sites include roller dryers, drying towers, air filters, air particles, tanker bays, and factory roofs. The organism can survive spray drying (Arku et al. 2011). Genotyping has shown the long-term persistence of indistinguishable strains in the factories (Müller et al. 2013, Sonbol et al. 2013, Yan et al. 2013). The organism's natural resistance to desiccation may account for its persistence in factories and PIF ingredients (i.e., starches). Despite one reported serious infant infection attributable to the reconstitution of PIF with water containing *Cronobacter*, water as a source of the bacterium has not received much attention (Hariri et al. 2013, Liu et al. 2013).

There is asymptomatic human carriage of *Cronobacter* spp., with the organism having been isolated from teeth, saliva, feces, breast milk, and skin (Baltimore et al. 1989, Gosney et al. 2006, Zogaj et al. 2003). In an age profile of *Cronobacter* isolated using throat swabs from more than 45,000 outpatients during the period 2005–2011, the organism was isolated from every age group, with a higher frequency from children less than 14 years of age (Holy et al. 2013). The bacterium has been isolated from the hospital environment, e.g., hospital air, and clinical samples, e.g., cerebrospinal fluid, blood, bone marrow, sputum, urine, inflamed appendix, and neonatal enteral feeding tubes and conjunctivae (Masaki et al. 2001).

VIRULENCE MECHANISMS

Cronobacter spp. can invade human intestinal cells, replicate in macrophages, and invade the blood-brain barrier (Almajed & Forsythe 2016; Townsend et al. 2007, 2008). In vitro studies have shown that *Cronobacter* attachment to and invasion of mammalian intestinal cells, macrophage survival, and serum resistance are comparable with those of *E. cloacae* and *Citrobacter freundii* but are less than those of *Salmonella* Typhimurium. *C. sakazakii* produces outer membrane vesicles that cause cytopathogenic effects on host cells (Alzahrani et al. 2015). *C. sakazakii*, and some *C. turicensis* strains, can utilize exogenous sialic acid as a carbon source for growth, and this may have clinical significance. This could be a major evolutionary host adaptation because sialic acid is found in breast milk, mucin, and gangliosides. Sialic acid is also an ingredient in PIF because of its association with brain development. *C. sakazakii* is also able to grow on the ganglioside GM1 as a sole carbon source (Joseph et al. 2013b). The majority of virulence studies using animals have used *C. muytjensii* ATCC 51329^{T}, as this was the Preceptrol^{TM} strain for the former *E. sakazakii* species (Mittal et al. 2009). However, no clinical cases have been reported for this species, and thus the relevance of the studies is uncertain.

The sequenced genomes of *Cronobacter* species have revealed an array of adhesins, outer-membrane proteins, efflux systems, iron-uptake mechanisms, hemolysins, and type VI secretion systems that could contribute to the organism's virulence (Grim et al. 2012; Joseph et al. 2012a; Kucerova et al. 2010, 2011). Other candidate virulence determinants include superoxide dismutase (*sodA*) for macrophage survival (Townsend et al. 2007, 2008), flagella (Cruz et al. 2011), a metalloprotease (Kothary et al. 2007), an enterotoxin (Pagotto et al. 2003), and plasmid-borne virulence factors such as *Cronobacter* plasminogen activator (*cpa*; only encoded in *C. sakazakii* and *C. universalis*) and type VI secretion systems (Franco et al. 2011). OmpA and OmpX possibly have a role in *Cronobacter* penetrating the blood-brain barrier, although the mechanism leading to the destruction of the brain cells is unknown and could, in part, be a host response (Kim & Loessner 2008, Kim et al. 2010). Following a multiple-strain *C. sakazakii* outbreak at a French NICU, it was proposed that not all *C. sakazakii* strains were equally virulent (Caubilla-Barron et al. 2007). This observation contributed to the recognition of *C. sakazakii* CC4 as the major clonal lineage associated with neonatal meningitis cases (Forsythe et al. 2014; Joseph & Forsythe 2011, 2012).

ISOLATION OF *CRONOBACTER* SPP. FROM POWDERED INFANT FORMULA, FOLLOW-UP FORMULA, AND WEANING FOODS

Cronobacter was first associated with contaminated PIF by Muytjens et al. (1988). In one of the first published microbiological surveys of PIF, they reported that 52.2% ($n = 141$) of samples from 35 countries contained Enterobacteriaceae, with 14% containing *Cronobacter* spp. (then known as *Enterobacter sakazakii*). These *Cronobacter* strains have been reidentified using DNA-sequence analysis and comprise *C. sakazakii* (17/20), *C. malonaticus* (2/20), and *C. muytjensii* (1/20) (Sonbol et al. 2013). In addition, one strain identified by Muytjens et al. (1988) as *E. sakazakii* was reidentified as *Enterobacter hormaechei*. It should be noted that the highly publicized *Cronobacter* NICU outbreak at the University of Tennessee was attributed to the use of powdered formula, not PIF (Himelright et al. 2002). The intended target age for the product was non-infants. However, it was used to feed neonates following the instruction of neonatologists. The presence of *Cronobacter* in these products is generally overlooked, as they are not subject to the same microbiological criteria as PIF for intended age <6 months.

The plant association of *Cronobacter* may account for the organism's ability to survive spray drying and persist for long periods of time in dry materials (i.e., starches) as well as its presence in ingredients that are added to PIF without additional heat treatment (FAO-WHO 2004, 2006). Chap et al. (2009) reported an international survey for *Cronobacter* and related organisms in PIF, follow-up formula, and infant foods. The study was conducted by eight laboratories in seven countries in response to the call for data in preparation for the FAO 2008 risk assessment (FAO-WHO 2008). The analysis of 290 products isolated *Cronobacter* spp. from 3% ($n = 91$) of follow-up formulas and 12% ($n = 199$) of infant foods and drinks (Chap et al. 2009).

Although some early surveillance studies used methods that may not have accurately identified *Cronobacter* spp., sufficient studies on the prevalence of the organism in PIF from many countries show the incidence of isolation varies between 2% and 14%. It is notable that there are no published reports of *Cronobacter* spp. in PIF exceeding 1 cell/g (Santos et al. 2013), and a value of <1 cell in 100 g is probably a realistic level.

PHYSIOLOGICAL TRAITS AND PERSISTENCE

One feature often cited regarding *Cronobacter* is that the organism characteristically produces yellow colonies. Although this trait was used as a confirmatory test in the first ISO detection

method, it is no longer accepted as reliable and unfortunately this trait has become a commonly misunderstood myth. Studies of large strain collections show that ~20% of *Cronobacter* strains do not produce the nondiffusible, yellow pigment on tryptone soya agar at 25°C. In addition, pigment expression is dependent on the incubation temperature, with even fewer strains producing it at 37°C (Iversen & Forsythe 2007). Subsequently, ~1 in 5 isolates could be regarded as false negative and the PIF would then be accepted for distribution. Yellow pigmentation is not in the revised ISO detection method (ISO 2017).

Cronobacter spp. produces capsular material that may facilitate its attachment to plant surfaces, biofilm formation, and persistence under desiccated conditions. The capsule can be composed of a number of different compounds, e.g., colanic acid, K-antigen, Enterobacteriaceae common antigen, and cellulose (Ogrodzki & Forsythe 2015, 2017). The variation in capsule composition between strains has not been thoroughly investigated but has been proposed as the molecular basis for a typing scheme, as described below.

Strains of *Cronobacter* may produce so much capsular material that on milk agar plates the colonies drip onto the lid of inverted Petri dishes (Caubilla-Barron et al. 2007). In fact, the capsular material can be so profuse that it has been patented for use as a thickening agent in foods (Harris & Oriel 1989, Scheepe-Leberkuhne & Wagner 1986). Combined with a tolerance to desiccation, the capsule gives the organism the environmental fitness to colonize and persist on plant material in vivo. These traits may also contribute to the organism's presence in starches used in the manufacture of PIF and other foods, and persist during the manufacturing process. The organism can survive spray drying, albeit with a considerable reduction in viability, and the surviving cells may be severely damaged (Arku et al. 2011, Forsythe et al. 2009). Caubilla-Barron & Forsythe (2007) reported that *Cronobacter* can persist in PIF for two years.

The capsule may also enable *Cronobacter* to adhere to a range of innate materials: silicon, latex, polycarbonate, stainless steel, glass, and polyvinyl chloride (Iversen et al. 2004b, Lehner et al. 2005). These materials are commonly used for infant-feeding and food-preparation equipment and hence their contamination may increase the risk of infection due to increased exposure. Compounding the issue is that such biofilms are typically resistant to cleaning agents and disinfectants (Kim et al. 2007). In addition to PIF preparation surfaces and materials, the organism is part of the mixed flora biofilm in neonatal enteral feeding tubes, even those of neonates not fed reconstituted formula (Hurrell et al. 2009b). In laboratory studies, it has been shown that one contaminated feed passing through the tube could cause subsequent feeds to be contaminated (Hurrell et al. 2009a). Therefore, the ability of *Cronobacter* to attach to surfaces, form biofilms, and resist dry stress conditions contributes to the increased risk of *Cronobacter* exposure and ingestion.

TEMPERATURE RESPONSE AND THERMAL STRESS

Growth and death rates of *Cronobacter* have been determined in reconstituted PIF and breast milk (Lenati et al. 2008). The organism can grow over a wide temperature range. The lowest permissible growth temperature is near refrigeration (~5°C) and therefore the organism may grow during prolonged cold storage or following temperature abuse (Xu et al. 2015). At room temperature (21°C), *Cronobacter* has a doubling time of 40–94 minutes. The optimal temperature for growth is ~37–39°C, with a maximal temperature for growth at 44–47°C. It should be noted that although the *C. sakazakii* type strain ATCC 29544^{T} does not grow above 42°C (Nazarowec-White & Farber 1997), this temperature was required in the 2006 ISO/TS 22964 isolation method. Like the yellow pigmentation trait, the current ISO (2017) method does not use this raised temperature, as it can lead to missing some strains of *Cronobacter*.

Decimal reduction times and z-values vary considerably between strains, i.e., D_{55} ranges from 2 to 49 minutes and z-values range from 2°C to 14°C (Forsythe et al. 2009). Although initial studies referred to the organism as being thermotolerant, subsequent work clarified that the organism was less thermotolerant than *L. monocytogenes* and therefore susceptible to normal temperature control (Nazarowec-White & Farber 1999). For more detailed proteomic analysis of *Cronobacter* temperature and stress responses, the reader should consult Riedel & Lehner (2007) and Osaili & Forsythe (2009).

ADDITIONAL ASPECTS OF GROWTH AND SURVIVAL OF *CRONOBACTER* SPP. IN RECONSTITUTED POWDERED INFANT FORMULA

WHO (2007) guidelines for hygienic preparation of PIF are aimed at reducing the number of bacteria in the reconstituted product by using hot water to kill vegetative bacteria and limiting the time available for any survivors to multiply. However, a wider perspective is that neonates are frequently fed via nasogastric (NG) feeding tubes. Electron micrographs of used NG tubes from NICUs show that there is a considerable biofilm present that is composed of bacteria and fungi (Hurrell et al. 2009a,b). *Cronobacter* and other Enterobacteriaceae have been isolated from NG tubes of neonates at levels of up to 10^7 cfu per tube (Hurrell et al. 2009a,b). This scenario is applicable to all neonates with NG tubes and not only those on reconstituted PIF. Therefore, hygienic practices and avoidance of temperature abuse are vitally important regardless of the type of feed.

CRONOBACTER TAXONOMY AND IDENTIFICATION

Taxonomy has been a major topic with regard to *Cronobacter* because accurate bacterial taxonomy is essential for reliable regulatory control. Detection methods must be based on a thorough understanding of the diversity of the target organism and be able to distinguish it from closely related organisms that may also occur in the test material. It is necessary to be aware of the various taxonomic revisions, as some detection methods, especially those based on polymerase chain reaction (PCR) probes, were validated according to the taxonomy of *Cronobacter* at that particular time and may no longer be fit for purpose.

Because members of the *Cronobacter* genus were formerly known as the single species *Enterobacter sakazakii*, this name was used in publications before mid-2007, and such strains were typed using phenotypic methods. This led to the initial description of 15 biogroups (Farmer et al. 1980), with a 16th biogroup added later (Iversen et al. 2006).

The *Cronobacter* genus was defined with four recognized species in 2007, with a fifth species added in 2008 (Iversen et al. 2007, 2008). These five species were *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii*, and *C. dublinensis*. Two of the species, *C. sakazakii* and *C. malonaticus*, are so closely related that they could not be reliably distinguished at the time using 16S rDNA sequence analysis. Differentiation between the species was based on a combination of genotypic analysis and biotyping: *C. sakazakii* (biotypes 1–4, 7, 8, 11, and 13), *C. malonaticus* (biotypes 5, 9, and 14), *C. turicensis* (biotypes 16, 16a, and 16b), *C. muytjensii* (biotype 15), and *C. dublinensis* (biotypes 6, 10, and 12).

16S rDNA sequencing can be of limited use for very closely related organisms because of the minor differences in the rDNA sequence (Iversen et al. 2004c). For example, there are seven copies of the rDNA gene in *Cronobacter* and intrageneric differences can lead to uncertain and inconsistent base calls. In contrast, Joseph et al. (2012b, 2013a) used *Cronobacter* strains selected

by multilocus sequence analysis (MLSA) (Baldwin et al. 2009) of seven housekeeping genes as representatives across the genus and therefore overcame the preconceived grouping of strains based on phenotyping. The *Cronobacter* 7-loci multilocus sequence typing (MLST) scheme requires the partial sequence analysis of seven housekeeping genes: *atpD*, *fusA*, *glnS*, *gltB*, *gyrB*, *infB*, and *ppsA* (Baldwin et al. 2009). Comparing the loci DNA sequences with the *Cronobacter* PubMLST reference database (**http://pubmlst.org/cronobacter**) generates a seven-digit allele code, and the strain's sequence type (ST). Hence, just a single base pair difference results in a different ST allocation. STs that have five or more loci in common are grouped together in a CC. When concatenated together, the seven allele sequences form a 3,036-nucleotide length for phylogenetic analysis. These studies led to the naming of two further *Cronobacter* species, *C. universalis* and *C. condimenti* (Joseph et al. 2011), as well as the recognition of specific pathovars associated with particular neonatal and adult infections: *C. sakazakii* CC4 with neonatal meningitis, *C. sakazakii* ST12 with NEC, and *C. malonaticus* CC7 with adult infections (Forsythe et al. 2014, Hariri et al. 2013, Joseph et al. 2011 & 2012).

Brady et al. (2013) caused confusion when they used only four loci (*atpD*, *gyrB*, *infB*, and *rpoB*) to support their proposed reclassification of *Enterobacter helveticus*, *Enterobacter pulveris*, and *Enterobacter turicensis* as three new *Cronobacter* species (*Cronobacter helveticus*, *Cronobacter pulveris*, and *Cronobacter zurichensis*). This was below the normally accepted level of sequence analysis for naming new species and was quickly corrected by more detailed accurate analysis by Stephan et al. (2014). The latter authors proposed that the three *Enterobacter* species (*E. helveticus*, *E. pulveris*, and *E. turicensis*) be reclassified into two new genera: *Franconibacter helveticus* and *Franconibacter pulveris* and *Siccibacter turicensis*. Their relatedness to *Cronobacter* is shown in **Figure 1**. This differentiation is important, as these genera can be coisolated from the same samples as *Cronobacter*.

It is not surprising that the confusion generated from relying on limited phenotypic and PCR-probe-based methods have resulted in several isolate misidentifications. This includes some outbreak cases that were due to *Enterobacter hormaechei* but were misidentified at the time as being caused by *C. sakazakii* (Caubilla-Barron et al. 2007, Jackson et al. 2015, Townsend et al. 2008).

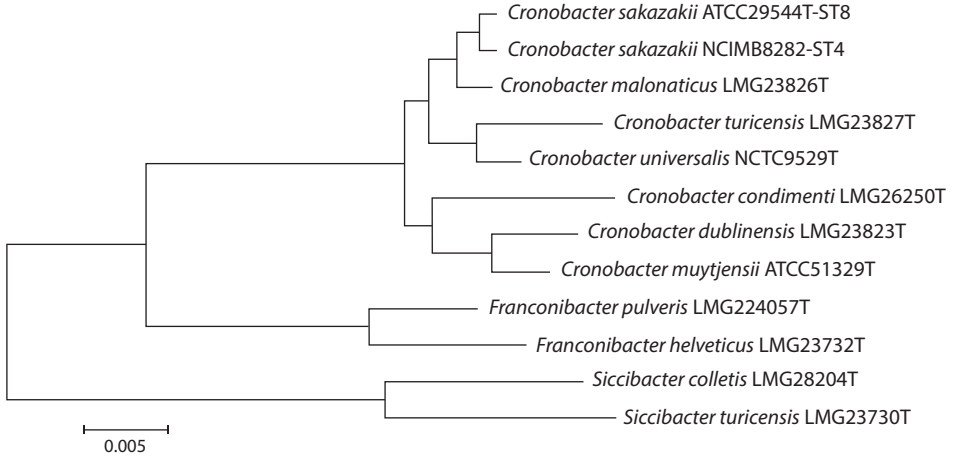

Figure 1

Phylogenetic analysis of the *Cronobacter* genus and closely related organisms using ribosomal–multilocus sequence typing (53-loci; 21,195-nt concatenated sequence). The tree was drawn using MEGA 6.05 (**http://www.megasoftware.net/**) with 1,000 bootstrap replicates.

CRONOBACTER DETECTION SCHEMES

Prior to the concerns of neonatal infections through the ingestion of *Cronobacter*-contaminated reconstituted PIF, the Codex Alimentarius Commission (CAC) microbiological criteria for PIF at point of manufacture had not been revised since 1979. Although the 1979 CAC criteria did not include *Cronobacter* spp. as a named pathogen, they did specify the direct enumeration for Enterobacteriaceae or coliforms, which would theoretically include *Cronobacter* spp. However, such tests may not always detect *Cronobacter* in PIF samples (Iversen & Forsythe 2004) because of the low cell number and injury to the organism during processing.

In 2008, the CAC revised their microbiological criteria for PIF for intended use by infants aged <6 months to include specific testing for *Cronobacter* spp. (CAC 2008). As the organism has only been reported at low numbers (<1 cfu/g), the CAC requirement is to test thirty 10-g quantities. Therefore, the presence/absence testing approach, rather than direct enumeration, is used for *Cronobacter* spp. Along with testing the finished product, ingredients and environmental samples are commonly taken in the production area and are usually tested using the same protocols as those for PIF. Although the organism has been recovered from follow-up formulas (infant formulas with an intended target age of >6 months) and weaning foods, there is currently insufficient epidemiological evidence to support the implementation of criteria for these products (FAO-WHO 2008).

Owing to the stressed state of the cells, the generalized isolation procedures involve resuscitation, enrichment, and plating on a chromogenic agar. *Cronobacter* enrichment broths, e.g., modified lauryl sulfate broth containing 0.5-M NaCl and *Cronobacter* screening broth with 10% sucrose (resulting in low water activity), are often based on the organism's resistance to osmotic stresses. The isolation on chromogenic agar is primarily based on the α-glucosidase reaction, which differentiates *Cronobacter* (positive reaction) from other Enterobacteriaceae (Muytjens et al. 1984). Commonly used *Cronobacter* chromogenic agars are based on the DFI (Druggan, Forsythe, Iversen) formulation, which has been improved since its initial design (Iversen et al. 2004a). The DFI formulation is named after Patrick Druggan (who designed the agar), Stephen Forsythe (myself, principle investigator), and Carol Iversen (my PhD student at the time). The DFI formulation has a dual purpose, as it also detects H_2S-producing *Salmonella* as black colonies (*Cronobacter* colonies are blue-green); hence, it is applicable for both organisms of concern in PIF.

The combined use of chromogenic agars, along with DNA-sequence-based identification techniques have added to the better detection and control of *Cronobacter* spp. (Chen et al. 2012, ISO 2017).

CRONOBACTER TYPING METHODS

Because *Cronobacter* is ubiquitous, typing schemes are required for both epidemiological and environmental investigations of strain source. As summarized in **Table 2**, the most useful techniques are based on DNA-sequence-typing methods ranging from 7-loci MLST and ribosomal MLST (53 loci) to core-genome MLST (1,836 loci) and CRISPR (clustered regularly interspersed short palindromic repeat)-*cas* (CRISPR-associated genes) array profiling. These are supported by an open access database (**http://pubmlst.org/cronobacter**), enabling international collaboration and surveillance.

Biotyping

Initially, 15 biogroups of *Cronobacter* were defined based on 10 biochemical tests and used for speciation and differentiation of strains (Farmer et al. 1980). However, improved speciation of strains using DNA-sequence-based methods has revealed that this biotyping approach is severely flawed, with no more than 50% of strains being correctly assigned to a *Cronobacter* species (Cetinkaya et al.

Table 2 Comparison of typing methods for *Cronobacter* spp.

Method	Number of categories	Central database	Comments	References
Phenotyping				
Biotyping	16, plus 14 subdivisions	No	Biotypes do not accurately correlate with separate *Cronobacter* species	Farmer et al. 1980, Iversen et al. 2006
Commercial kits	NA	Yes	Not accurate for speciation	Jackson & Forsythe 2016
Genotyping				
PCR primers: serotyping	24	No	Speciation required before determination	Blažková et al. 2015, Mullane et al. 2008
Pulsed-field gel electrophoresis	NA	Yes	May not differentiate unrelated strains within clonal complex	Caubilla-Barron et al. 2007, Van Acker et al. 2001
DNA-sequence based				
Serotyping: *galF* and *gnd* alleles	34	Yes	Expansive method	Ogrodzki & Forsythe 2015
fusA allele	167	Yes	Expansive method	Baldwin et al. 2009, Forsythe et al. 2014
Multilocus sequence typing 7-loci (also rMLST & cgMLST)	602	Yes	Recognizes major pathovars and clonal groups	Baldwin et al. 2009, Forsythe et al. 2014
Capsule profiling: K-antigen and colanic acid biosynthesis genes	4	Yes	K2:CA2 profile may relate to severe neonatal infections	Ogrodzki & Forsythe 2015
Cas3 allele	52	Yes	Follows *fusA* profiling	Ogrodzki & Forsythe 2016
CRISPR-cas array profiling	20	Yes	To date, only applied to major *Cronobacter sakazakii* pathovars	Ogrodzki & Forsythe 2016

Abbreviations: MLST, multilocus sequence typing; NA, not applicable; PCR, polymerase chain reaction.

2013, Jackson & Forsythe 2016, Joseph et al. 2013a). This is in part due to the initial use of biotype index strains that were attributed to the wrong *Cronobacter* species (Baldwin et al. 2009). Subsequently, the biotyping approach is not regarded as reliable for speciation or accurate definition of isolates (**Table 2**).

Initial Genotyping Methods for *Cronobacter* spp.

A number of DNA-based methods for identification, speciation, and profiling have been proposed for *Cronobacter* spp. (Joseph & Forsythe 2014). Initial procedures used plasmid profiling, chromosomal restriction endonuclease analysis, and multilocus enzyme electrophoresis (Clark et al. 1990, Nazarowec-White & Farber 1999). This was followed by the application of random amplified polymorphic DNA (Drudy et al. 2006), ribotyping, and multiple-locus variable-number tandem-repeat analysis (Mullane et al. 2008), amplified fragment length polymorphism (RFLP) (Turcovsky et al. 2011), and PCR-restriction fragment length polymorphism (Vlach et al. 2017). However, there does not appear to have been a re-evaluation of these methods after 2007 following the taxonomic revisions, and there is no evidence that they can be acceptably used for end-product testing. Hence, these methods are not considered in any further detail here.

Pulsed-Field Gel Electrophoresis

For epidemiological analysis of *Cronobacter* infections (i.e., tracing source and dissemination during an outbreak), pulsed-field gel electrophoresis (PFGE) with two restriction enzymes (*Xba*1

and *Spe*1) has been a commonly used method (Brengi et al. 2012, Caubilla-Barron et al. 2007, Himelright et al. 2002, Van Acker et al. 2001). The method has also been used for microbial source tracing in milk-powder and PIF manufacturing plants (Craven et al. 2010, Jacobs et al. 2011, Mullane et al. 2008). However, the method neither speciates isolates nor determines the relatedness of strains (a common misunderstanding). In addition, due to intrinsic DNase activity, some strains do not give profiles and are therefore nontypable (Alsonosi et al. 2015, Craven et al. 2010). Also, PFGE may not differentiate between unrelated strains within the same CC, a phenomenon common with the *Cronobacter* pathovars (Alsonosi et al. 2015).

Owing to the limitations of PFGE, the CDC is transitioning to using whole-genome sequencing as the basis for PulseNet surveillance, as PFGE is being phased out in favor of the analysis of whole-genome sequences (Carleton & Gerner-Smidt 2016, Nadon et al. 2017).

Genotyping Using Polymerase Chain Reaction Probes

PCR probe methods are dependent on the accuracy of their initial primer design to minimize false-negative and false-positive results. PCR probes have been designed for the *ompA*, *dnaG*, *rpsU*, *cgcA*, *fliC*, and *rpoB* genes in *Cronobacter* (Carter et al. 2013, Nair & Venkitanarayanan 2006, Proudy et al. 2008). However, although PCR probes are useful for small-scale studies, their application is limited, as they often have not been validated against a robust *Cronobacter* strain collection of the seven species and cross-react with closely related organisms (Jackson et al. 2014). Some are very laborious in that they require different PCR primer pairs for each species and have not been designed using sufficiently diverse strain sequences (Lehner et al. 2012, Stoop et al. 2009). PCR-based probes for *ompA* and *rpoB* are independent of the corresponding allele sequencing analyses, which have been included in the *Cronobacter* PubMLST database (**Table 2**).

Polymerase Chain Reaction–Probe O-Antigen Serogrouping

An early method of typing bacterial strains was serotyping using antibodies raised in animals. A common antigenic site was the somatic or O-antigen, leading to schemes for *Salmonella* serovars and *E. coli* (i.e., *E. coli* O157). The use of animals is no longer required for serotyping, as PCR probes can be designed for long-range PCR amplification of the O-antigen region (*rfb* locus). The PCR amplification product is then subject to restriction enzyme digestion to generate a recognizable banding pattern. This approach has been used by a number of researchers for *Cronobacter* spp. (Blažková et al. 2015; Jarvis et al. 2011, 2013; Mullane et al. 2008; Sun et al. 2011, 2012). Some strains of *C. malonaticus* were misidentified as *C. sakazakii* by Sun et al. (2011) and were incorrectly assigned *C. sakazakii* serotypes O:5 and O:6. Blažková et al. (2015) expanded the scheme with additional recognition of seven new and two reassigned serotypes. The total number of designated O serogroups across the *Cronobacter* genus is 24, with 7 in *C. sakazakii* and 3 in *C. malonaticus*. However, the PCR serotyping technique suffers from a number of limitations. First, only seven serogroups have been recognized as *C. sakazakii*, which is not useful for microbial source tracking and therefore of limited use given this species predominates in neonatal outbreaks. Second, PCR amplification products of the O-antigen region are not generated for all *Cronobacter* strains (Jarvis et al. 2011). This indicates that further unrecognized serogroups exist. Third, three structures for O-PS have been determined for *C. sakazakii* strains with the same O:2 profile (Arbatsky et al. 2010, Czerwicka et al. 2010, Maclean et al. 2010). This is probably due to variants in the O-antigen region, which occur outside the target region of the PCR primers. Finally, some *Cronobacter* serogroups occur across more than one species and even more than one genus: *C. sakazakii* O:4 = *E. coli* O103, *C. sakazakii* O:3 = *C. muytjensii* O:1; and *C. malonaticus* O:1 = *C. turicensis* O:1,

S. dysenteriae D11, and *E. coli* O29. Therefore, *Cronobacter* isolates need to be speciated prior to serotyping, which takes extra time and labor.

The PCR-primer pair approach for O-serotyping has consequently been superseded by allele profiling of *gnd* and *galF*, which flank the *rfb* region (Ogrodzki & Forsythe 2015). This DNA-sequence-based method is a more reliable and expansive method for O-antigen determination within *Cronobacter*. It has increased the definable number of serotypes in the *Cronobacter* genus from 24 to 34 (Ogrodzki & Forsythe 2015).

DNA-Sequence-Based Genotyping: Multilocus Sequence Typing

The application of next-generation sequencing (NGS) to *Cronobacter* has led to the establishment of the *Cronobacter* PubMLST genome and sequence definition database (**http://pubmlst.org/cronobacter/**) containing the MLST profiles for more than 2,000 isolates and more than 350 whole genomes. The PubMLST database also contains the metadata for strains that have been compiled from various researchers, sources, and countries. The scheme has revealed stable clones and pathovars within the genus, some of which can be traced back over a 50-year period and from a wide range of countries and sources.

The *Cronobacter* 7-loci MLST analysis is based on seven housekeeping genes: ATP synthase beta chain (*atp*D), elongation factor G (*fus*A), glutaminyl-tRNA synthetase (*gln*S), glutamate synthase large subunit (*glt*B), DNA gyrase subunit B (*gyr*B), translation initiation factor IF-2 (*inf*B), and phosphoenolpyruvate synthase A (*pps*A). The 7 sequenced alleles can be concatenated together to provide more than 3,000 nucleotides for phylogenetic analysis. This is six times the length of the commonly used partial 16S rDNA sequences and has the additional advantage of considerably greater number of variable loci.

Investigating 1,654 isolate entries in the *Cronobacter* PubMLST database at the time of writing (May 2017) reveals the temporal, geographic, and source diversity of the organism (**Table 3**). The earliest isolate was from dried milk powder in 1950 and is one of more than 350 genomes that are accessible for analysis via the PubMLST database (Masood et al. 2013). *Cronobacter* strains have been isolated from 36 countries, and are from clinical (24%), infant formula (15%), food (19%), and environmental (15%) sources as well as other sources such as water (4%) (**Table 3**).

C. sakazakii CC1 is a dominant CC consisting of strains isolated from around the world over a period of more than 25 years. These isolates have been primarily from PIF and clinical cases as well as more recently from milk-powder-processing factories in Australia and Germany (Craven et al. 2010, Jacobs et al. 2011). *C. sakazakii* CC4 is a key CC with respect to *Cronobacter* spp. serious neonatal infection epidemiology (Baldwin et al. 2009, Forsythe et al. 2014). The earliest isolate (*C. sakazakii* NCIMB 8282) from milk powder in 1950 has been genome sequenced, and the first *Cronobacter* genomic-level NICU outbreak of *C. sakazakii* CC4 and ST12 was investigated by Masood et al. (2013, 2015). Why *C. sakazakii* CC4 predominates in neonatal meningitis cases is unclear but could be due to environmental fitness factors as well as virulence traits. It is plausible that adult cases do not occur due to the maturity of the blood-brain barrier. *C. sakazakii* CC4 strains have been isolated from infant formula (Muytjens et al. 1988) and milk-powder manufacturing plants worldwide and therefore may represent a particularly persistent clonal variant resulting in increased neonatal exposure (Craven et al. 2010, Jacobs et al. 2011, Müller et al. 2013, Power et al. 2013, Sonbol et al. 2013). *C. sakazakii* ST12 has been associated with cases of necrotizing enterocolitis (13% of strains) and not neonatal meningitis or septicemia (Masood et al. 2015, Van Acker et al. 2001).

The *Cronobacter* PubMLST scheme is expandable beyond the initially described 7-loci approach (Jolley et al. 2012, Maiden et al. 2013). The inclusion of whole genomes has enabled the use of more

Table 3 Summary of *Cronobacter* isolates in the *Cronobacter* PubMLST (multilocus sequence typing) database (Ogrodzki & Forsythe 2017)

Species	Number of strains (%)	Number of Sequence types	Number of genomes	Earliest isolate (year)	Countries	Source				
						Clinical	Infant formula	Food and ingredients	Environmental	Other
Cronobacter sakazakii	1126 (68.1)	236	155	1950	32	14.5[a]	21.5	43.4	17.6	3.0
Cronobacter malonaticus	222 (13.4)	94	55	1973	17	26.4	13.7	44.8	8.5	6.6
Cronobacter dublinensis	155 (9.4)	107	31	1956	12	3.0	5.3	63.9	25.6	2.3
Cronobacter turicensis	76 (4.6)	46	14	1970	13	8.0	4.0	52.0	31.4	6.7
Cronobacter muytjensii	57 (3.4)	28	10	1988	12	1.8	10.7	53.6	1.8	32.1
Cronobacter universalis	16 (1.0)	9	8	1956	6	5.6	0.0	55.6	22.2	16.7
Cronobacter condimenti	2 (0.1)	1	2	2010	1	0.0	0.0	100	0	0
Total	1654	521	275	NA	36	14.2[b]	17.5	46.4	17.2	4.7

[a]Percentage of species total.
[b]Percentage of genus total.

loci; ribosomal-MLST (rMLST) (**Figure 1**) uses 53 loci and core genome–MLST (cgMLST) covers ~1/3 of the genome (1,865 loci) (Forsythe et al. 2014). In addition, the user can define their own scheme. The *C. sakazakii* CC4 clonal lineage is very robust and has been confirmed using the more discriminatory approaches of rMLST and cgMLST (Forsythe et al. 2014). Thus, *C. sakazakii* CC4 represents a major evolutionary lineage associated with neonatal meningitis (Hariri et al. 2013; Joseph & Forsythe 2011, 2012; Joseph et al. 2012b; Masood et al. 2015). Detailed reviews of MLST results for more than 1,600 strains are given by Forsythe et al. (2014) and Ogrodzki & Forsythe (2017).

Capsule Profiling

A capsular profiling scheme for *Cronobacter* based on the K-antigen, colanic acid (CA) biosynthesis encoding genes has been proposed (Ogrodzki & Forsythe 2015, 2017). They reported that all strains of *C. sakazakii* CC4 and ST12 strains associated with severe neonatal infections of meningitis and NEC had the capsular profile K2:CA2. Of particular interest was that this particular capsule profile was also found for the rare non–*C. sakazakii* CC4 cerebral spinal fluid isolates from severe cases of bacterial meningitis, including *C. malonaticus* meningitis isolates (Ogrodzki & Forsythe 2015, 2017).

CRISPR-*cas* Array Profiling

Although the MLST clonal recognition is useful for the identification of *Cronobacter* pathovars, it is counter-productive for microbial source tracking, as unrelated strains occur with the same ST. Additionally, this may explain the observation that the same PFGE pulsotype can be obtained for unrelated clinical *C. sakazakii* strains (Alsonosi et al. 2015, Forsythe et al. 2014). To address this issue, *cas* protein-coding genes (CRISPR-*cas*) array profiling has been applied to *Cronobacter* genomes of strains from the same ST. The CRISPR-*cas* array reflects the exposure of strains to phages and plasmids (Ogrodzki & Forsythe 2016, Zeng et al. 2017). In general, CRISPR-*cas* systems may have up to three sections: (a) *cas* genes, (b) an AT-rich leader sequence upstream of the array, and (c) a CRISPR array composed of short (~24–48 nucleotide) direct repeat sequences separated by similarly sized, unique spacers, which are usually derived from mobile genetic elements such as bacteriophages and plasmids (Grissa et al. 2007, Makarova et al. 2015). Spacers are added sequentially at the leader end, and a given spacer is rarely acquired twice or duplicated. CRISPR arrays may differ between closely related strains because of their different exposures to phages and plasmids, leading to variations in the spacer sequences. These loci can be used as alternative targets for molecular subtyping and may offer higher strain resolution than MLST and PFGE, and therefore their profiles can be useful typing tools for highly clonal organisms such as *Cronobacter*.

Ogrodzki & Forsythe (2016) applied CRISPR-*cas* array profiling to four *C. sakazakii* pathovars: CC1, CC4, ST8, and ST12. They demonstrated that strains in the same ST, and therefore indistinguishable by 7-loci MLST, were distinguishable according to their spacer arrays. All strains encoded for the type I-E subtype CRISPR-*cas* system. Each pathovar had between 2 and 4 CRISPR loci. A total of 32 different direct repeat sequences and 154 different spacer sequences (primarily 29-bp and 32-bp long, respectively) were found. Unrelated strains within the same ST were indistinguishable by differences in their CRISPR-*cas* arrays. For example, all 25 ST4 strains contained the same CRISPR-1 profile, with 8 spacers and direct repeats, and varied in their CRISPR-2 loci. The *C. sakazakii* ST4 strains from the much-studied 1994 NICU outbreak in France (Caubilla-Barron et al. 2007, Masood et al. 2015) contained 23 spacers and direct repeats, which differed

from the remaining ST4 strains. The CRISPR-*cas* arrays were highly varied within other clonal groups, especially ST8. Phylogenetic analysis of *cas3* (signature gene for I-E CRISPR-*cas* loci) across the whole genus showed its diversity (52 variants) and reflected the phylogeny based on *fusA* and whole-genome sequencing and hence the wider use of CRISPR-*cas* array profiling.

These results are highly significant because they demonstrate the usefulness of CRISPR-*cas* profiling for epidemiological purposes, given the highly clonal nature of *Cronobacter* precludes the use of PFGE and MLST alone as genotyping methods (Ogrodzki & Forsythe 2017). CRISPR-*cas* array profiling is readily achievable given the affordability and increasing access to whole-genome sequencing facilities, as already recognized by PulseNet, and reflects the fact that foodborne organisms can be studied in high detail these days (Nadon et al. 2017).

CONCLUSIONS

In 2004, the FAO-WHO requested the establishment of a molecular typing scheme to enable the international control of the organism. Because the *Cronobacter* PubMLST database (**http://pubmlst.org/cronobacter**) contains more than 2,000 isolates, an informed understanding of the diversity and sources of the organism can now be obtained.

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