Multilocus sequence typing of *Cronobacter* spp. from powdered infant formula and milk powder production factories

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Abstract

This study applied the *Cronobacter* spp. multilocus sequence typing (MLST) scheme to three strain collections, then known as *Enterobacter sakazakii,* which had been isolated between 1988 and 2009 from 14 countries. The results revealed the predominance (85%) of *C. sakazakii* (72 strains) in all three collections. The remaining strains were *C. turicensis* (10%), *C. malonaticus* (4%), and *C. muytjensii* (1%). No strains of *C. dublinensis*, *C. universalis* or *C. condimenti* were identified. Twenty-one out of seventy two *C. sakazakii* strains were in the clinically significant ST4 clonal complex, and were found in all three strain collections. These results confirm *C. sakazakii* ST4 is one of the predominant clonal complexes over the past 20 years in several parts of the world. Further understanding of the ecosystem and sources of the organism may be used for the development of improved intervention strategies in the diary industry.

Introduction

Cronobacter spp. (formerly known as *Enterobacter sakazakii*) is a diverse genus in the family *Enterobacteriaceae*. It is composed of seven species; *C. sakazakii, C. malonaticus*, *C. muytjensii, C. turicensis, C. dublinensis, C. universalis,* and *C. condimenti* (Iversen et al., 2007; Joseph et al., 2012a). Three of these species have been isolated from neonatal infections: *C. sakazakii, C. malonaticus,* and *C. turicensis* (Forsythe, 2005; Joseph et al., 2012c). Whole genome sequencing of all seven species has revealed that the organism encodes a range of virulence traits comprising of adhesins, phage sequences, type four and six secretion systems, multidrug efflux systems, and a range of iron acquisition genes (Joseph et al., 2012b; Kucerova et al., 2010).

Although the majority of *Cronobacter* spp. infections are in adults (FAO/WHO, 2008), this bacterial genus has come to the attention of regulatory authorities and the public due to its association with severe neonatal infections (Bowen & Braden, 2006; Codex Alimentarius Commission, 2008). Such infections have a high fatality rate, of 40 to 80%, and survivors often suffer from severe neurological disorders (Caubilla-Barron et al., 2007; Lai, 2001; van Acker, de Smet, Muyldermans, Bougatef, & Naessens, 2001). Epidemiological studies of outbreaks in neonatal intensive care units led to the recognition of reconstituted powdered infant formula (PIF) as a route of infection (Himelright, Harris, Lorch, & Anderson, 2002; van Acker et al., 2001).

A multilocus sequence typing (MLST) curated database has been established for the entire *Cronobacter* genus and has open access at http://www.pubMLST.org/cronobacter (Baldwin, et al., 2009; Joseph & Forsythe, 2012; Joseph et al., 2012c). The scheme is based on seven housekeeping genes (*atpD, fusA, glnS, gltB, gyrB, infB, ppsA*) with a concatenated length of 3036 nucleotides that can be used for phylogenetic analysis. The *Cronobacter* MLST scheme has been applied to over 400 isolates. There are currently 136 defined sequence types (ST), with 55 STs in *C.* sakazakii (Joseph & Forsythe, 2012; Joseph et al., 2012c). Recently, Joseph and Forsythe (2011) compared *C. sakazakii* ST profiles with severity of infection by compiling patient details, isolation site and clinical presentation for strains isolated from around the world up to 2008. *C. sakazakii* ST1 strains are primarily isolates from infant formula, whereas *C. sakazakii* ST8 is primarily composed of isolates from clinical sources. Of

special significance is *C. sakazakii* ST4 which has a high propensity for neonatal meningitis (Joseph & Forsythe, 2011; Joseph et al., 2012c). This appears to be a very stable lineage as clinical and non-clinical ST4 strains have been isolated from seven countries for over 50 years. This retrospective association was supported in December 2011 in the US with highly publicised *Cronobacter* neonatal infection cases (Centers for Disease Control and Prevention, 2012), in which *C. sakazakii* ST4 was isolated from the neonatal meningitis cases (Hariri, Joseph, & Forsythe, 2012). In addition, *C. malonaticus* ST7 is associated with adult infections though the source has not been identified (Joseph & Forsythe, 2011; Joseph et al., 2012c).

Although its presence in PIF fed to newborn babies has attracted the most attention, *Cronobacter* spp. have been isolated from foods such as cheese and meat and from hospital environments including air, formula-mixing utensils, and enteral feeding tubes (Hurrell et al., 2009; Kucerova, Joseph, & Forsythe, 2011).

Many studies have shown that *Cronobacter* spp. can be isolated from milk powder and PIF manufacturing facilities (Craven, McAuley, Duffy, & Fegan, 2010; Jacobs, Braun, & Hammer, 2011; Mullane, Whyte, Wall, Quinn, & Fanning, 2007). The organism may persist in these environments due to its ability to survive spray drying, desiccation, and osmotic stress (Arku, Mullane, Fox, Fanning, & Jordan, 2008; Breeuwer, Lardeau, Peterz, & Joosten, 2003; Osaili & Forsythe, 2009). *Cronobacter* spp. have been shown to survive more than two years in desiccated PIF (Caubilla-Barron & Forsythe, 2007). Mullane et al. (2007) used pulsed-field gel electrophoresis (PFGE) to profile *Cronobacter* spp. isolates from a production site, and demonstrated the persistence of specific bacterial clones in the industrial facilities, and periodically these could be isolated from air samples. Minimising the presence of *Cronobacter* spp. in milk powder production facilities is achieved by environment control including zoning to physically separate high and low hygiene areas, maintaining a low moisture environment (reducing water ingress), effective cleaning routines and control of dust and waste powder. Together these reduce the survival, growth and colonization opportunities for the organism (Cordier, 2008). Nevertheless, PIF should not be considered a sterile product.

In the 1980's, Muytjens et al. (1983) and Muytjens, van Der Ros-van de Repe, & van Druten (1984) reported several cases of *E. sakazakii* infection in neonates, which could be linked to

100 contaminated milk powders (these days more commonly known as PIF) and preparation
101 equipment. The group undertook an international survey of PIF for the presence of
102 *Enterobacteriaceae,* which were identified phenotypically. They isolated *E. sakazakii* from
103 20 out of 141 (14.2%) PIF samples from 35 countries (Muytjens, Roelofs-Willemse, & Jaspar,
104 1988). This highly cited study was used in the FAO/WHO risk assessments of *E. sakazakii* in
105 PIF (FAO/WHO, 2004, 2006, 2008). However, given this seminal work was before the 2007
106 taxonomic revision, the strains lack *Cronobacter* species attribution, therefore genotyping
107 the strains would considerably increase the value of these older studies. In addition,
108 Townsend, Hurrell, Caubilla-Barron, Loc-Carrillo, and Forsythe (2008) reported that one of
109 the strains of Muytjens et al. (1988) was a mis-identified strain of *E. hormaechei*. Therefore
110 the reinvestigation of the available strains is warranted using MLST to assign the
111 *Cronobacter* species and sequence types (STs).
112 Similarly, this study has determined the *Cronobacter* species and STs of isolates from two
113 studies of six milk powder processing factories (Craven et al., 2010; Jacobs et al., 2011). The
114 isolates had not been identified at the *Cronobacter* species level as, despite the year of
115 publication, they had been isolated and identified before the taxonomic revision. Craven et
116 al. (2010) identified 49 *E. sakazakii* pulsetypes, according to *Xba*I restriction digestion,
117 representing 126 isolates from 100 locations in the non-processing and processing
118 environments of five milk powder factories in Australia. These had been sampled between
119 November 2006 and March 2007. In addition, three strains could not be profiled by PFGE.
120 Jacobs et al. (2011) analysed environmental and final product samples from a milk powder
121 manufacturing plant over a four year period (2005–2009) in Germany. Eighty-one *E.*
122 *sakazakii* strains were isolated from the spray-drying area and the roller-drying area. These
123 were divided into 13 pulsetypes, following PFGE analysis with *Xba*I restriction digestion.
124 This study applied MLST to these previously published sets of strains, then known as *E.*
125 *sakazakii* , from PIF and milk powder processing plants in order to up-date those earlier
126 studies by speciating the strains and determining their sequence types. The profiles of
127 *Cronobacter* spp. isolates before and after the raised concern over the microbiological
128 content of PIF are also compared. This new information has been obtained to increase the
129 understanding of the ecology and distribution of significant strains of the organism which

may be used in the development of improved and targeted intervention strategies for the control of the organism in the dairy industry.

Materials and Methods

Bacterial strains

A total of 85 strains were analyzed in this study. This was composed of 20 available strains from Muytjens et al. (1988; Table 1), 52 strains from Craven et al. (2010; Table 2) and 13 strains from Jacobs et al. (2011; Table 3). The latter two strain sets were representatives of the pulsetypes described in the original publications. Further details of the strains are given in Tables 1 to 3.

MLST and sequence analysis

The DNA extraction and MLST protocol was performed as described by Joseph et al. (2012c). All allele profiles and ST assignments were in accordance with the open access, curated database entries at http://www.pubmlst.org/cronobacter. Phylogenetic analysis of the concatenated sequences of the seven loci (3036 nucleotides concatenated length) was performed using the Maximum-Likelihood algorithm in MEGA 5, with 1000 bootstrap replicates (Tamura et al., 2011).

Results

A total of 85 strains were genotyped by MLST, and submitted to the pubMLST.org/cronobacter database. The majority (n=72) of strains were identified as *C. sakazakii*, followed by *C. turicensis* (n=9), *C. malonaticus* (n=3), *C. muytjensii* (n=1). No strains of *C. dublinensis*, *C. universalis* or *C. condimenti* were identified. Details of the *Cronobacter* spp. sequence type profiles are given in Tables 1 to 3, and are summarized in Table 4. In addition, one strain from India was re- identified as *E. hormaechei*. The phylogenetic tree based on the concatenated 7 loci of MLST sequences (Fig 1) shows clear clustering across the *Cronobacter* genus with the 85 strains in four out of the seven species,

and also the predominance of *C. sakazakii* ST4 and ST1 strains. The tree also shows the relatedness between the sequence types.

The older strain collection (Muytjens et al., 1988) was comprised of *C. sakazakii* (17/20), *C. malonaticus* (2/20), and *C. muytjensii* (1/20) (Table 1). These strains had been isolated from PIF produced from Australia, Belgium, Canada, Denmark, France, Germany, New Zealand, Russia, The Netherlands, Uruguay and USA (Table 1). Five out of 17 of the *C. sakazakii* isolates were *C. sakazakii* ST4 strains. These had been isolated from PIF samples purchased in Canada, Russia, West Germany and The Netherlands. Three strains of *C. sakazakii* ST1 were isolated from PIF from The Netherlands and Russia, and two strains of *C. sakazakii* ST3 were from products from Belgium and The Netherlands. One *E. hormaechei* strain, previously identified as *E. sakazakii*, was also identified and had been isolated from PIF purchased in India.

The Australian strains (Craven et al., 2010) were primarily comprised of *C. sakazakii* (42/52), followed by *C. turicensis* (9/52) and *C. malonaticus* (1/52)(Table 2). The *C. sakazakii* strains were different pulsetypes of 116 isolates from 5 milk processing factories. Twelve of these pulsetype representatives were *C. sakazakii* ST4. The *C. sakazakii* ST4 strains had been isolated between 2006-2007, from various locations of all five sampled manufacturing plants; tanker bay, factory roofs, milk powder processing environment and outside grounds. Two isolates of *C. sakazakii* ST97 were from a tanker bay at one factory. This ST is within clonal complex 4, differing by 1 nucleotide (position 321, G:A) in the *gltB* allele from the ST4 profile. The close relatedness between ST4 and ST97 is also shown in the phylogenetic tree; Figure 1. The *C. sakazakii* ST1 strains represented 9 pulsetypes which comprised of 33 isolates. These had been isolated from similar milk powder manufacturing areas in 3/5 factories sampled (Table 2). Two strains which could not be profiled using PFGE were *C. sakazakii* STs 3 and 133. A third strain which also could not be profiled using PFGE was *C. turicensis* ST132.

The original study by Jacobs et al. (2011) isolated 81 *E. sakazakii* isolates from one German manufacturing plant, and these were divided into 13 pulsetypes. In our study, all representative strains of these pulsetypes were identified as *C. sakazakii* (Table 3). The strains were primarily in ST1 (n=4), ST4 (n=3) and ST99 (n=4). The *C. sakazakii* ST1 strains were isolated from a roller dryer which had been sampled in 2009. The *C. sakazakii* ST4

strains were isolated from a roller dryer (sampled in 2009), and from a drying tower in 2006. The *C. sakazakii* ST99 strains had been collected from the filter powder and routine testing from two towers in 2006. Additionally, one strain (1530) was ST101. This sequence type is in clonal complex 10 with ST99; differing in one nucleotide of the *fusA* allele (position 378, G:A). The close relatedness of ST99 and ST101 is shown in Figure 1. Strain 1530 (ST101) had been isolated from filter powder collected from the same drying tower as had some of the closely related ST99 strains.

Across the three collections, the majority (28/39) of STs were identified in *C. sakazakii* compared to only 11 in *C. malonaticus*, *C. turicensis* and *C. muytjensii*. The main *C. sakazakii* STs were ST4 (24%), ST1 (19%), ST40 (5%), ST99 (5%) and ST3 (5%); Table 4. *C. sakazakii* ST1 and ST4 were the only STs isolated from all three collections.

Discussion

A total of 85 strains of *Cronobacter* spp., which had only been identified as *E. sakazakii* in previous publications, were genotyped by MLST. The majority (85%) of *Cronobacter* spp. isolates in the three strain collections were *C. sakazakii*, and included strains which could not be profiled using PFGE. The remaining strains were *C. turicensis* (10%), *C. malonaticus* (4%), and *C. muytjensii* (1%). This corresponds with the predominance of *C. sakazakii* in neonatal infections and the few cases associated with *C. turicensis* and *C. malonaticus* (Hariri et al., 2012; Kucerova et al., 2011). To date, no neonatal infections have been attributed to *C. muytjensii, C. dublinensis*, *C. universalis* or *C. condimenti*. The latter three species were not identified from any of the three strain collections.

In the study of Muytjens et al. (1988), 50 strains had been isolated from PIF sourced from 35 different countries. They represent strains isolated before the international concern of neonatal infections through reconstituted infant formula which led to changes in the Codex Alimentarius Commission (2008) microbiological guidelines for PIF manufacturers. However, not all the strains in the Muytjens et al. (1988) study were *Cronobacter* species. A previous publication had shown that one strain identified as *E. sakazakii* from PIF from The Netherlands was *E. hormaechei* (Townsend et al., 2008). In this study, one strain isolated from PIF in India was also re-identified as *E. hormaechei*. The remaining *Cronobacter* spp.

222 strains were identified as *C. sakazakii* (17/20), *C. malonaticus* (2/20) and *C. muytjensii* (1/20)
223 (Table 1). Despite the presence of *C. sakazakii* ST4 in PIF samples, it should be noted that
224 Muytjens et al. (1988) reported that no sample contained the organism at levels >1 cell g^{-1}.
225 Therefore good hygienic practices in the preparation of formula feeds should be used to
226 reduce bacterial multiplication and risk of infection (FAO/WHO, 2006, 2008).
227 It can be seen from table 4 that the main sequence types were *C. sakazakii* ST4 (20/85
228 isolates) and ST1 (16/85 strains). The former value slightly increases when including the
229 single locus variant (ST97) in clonal complex 4 (21/85)(Table 4). This predominance of *C.*
230 *sakazakii* in dairy factory environments matches investigations of previous *Cronobacter* spp.
231 infections and outbreaks. *C. sakazakii* clonal complex 4, including ST4, is the predominant
232 lineage of *Cronobacter* spp. associated with cases of neonatal meningitis (Hariri et al. 2012;
233 Joseph et al., 2011). Furthermore, in the *Cronobacter* MLST database
234 (http://www.pubMLST.org/cronobacter), more than one third of all the *C. sakazakii* isolates,
235 isolated over a 50 year period, belong to these sequence types (Joseph et al., 2012c). The
236 results of this study demonstrate that *C. sakazakii* ST4 can be present in the environment of
237 milk powder factories such as tanker bay, shoes, roof, roller-dryer, spray-drying area and
238 milk powder.
239 MLST and PFGE are genotyping techniques which can be applied to *Cronobacter* spp.
240 isolates, although not all strains give PFGE profiles (Craven et al., 2010). No restriction site
241 for *Xba*I lies within the 7 alleles sequenced and therefore the allele sequences are
242 independent of the PFGE methods used by Craven et al. (2010) and Jacobs et al. (2011).
243 MLST discriminates at the level of one nucleotide in 3036 total sequenced bases, and can be
244 used for phylogenetic construction (Joseph & Forsythe, 2012; Joseph et al., 2012c). Figure 1
245 shows the overall diversity of the *Cronobacter* genus and that the majority of isolates were
246 in a few STs of *C. sakazakii*. The figure also shows the close similarity between certain STs.
247 For example, clonal complex 6 (ST40, ST45 and ST105) comprises of STs that differ by one
248 locus. It is of note that the dominant *C. sakazakii* STs, ST1 and ST4, are not closely 'related'
249 according to this figure, and this has been confirmed by whole genome sequencing (Joseph
250 et al., 2012b). The reason for their predominance in strains collected over a 20 year period
251 from around the world is unknown. The two sequence types also differ in that *C. sakazakii*
252 ST4 is more associated with neonatal meningitis, whereas *C. sakazakii* ST1 is less commonly

associated with clinical isolates (Joseph & Forsythe, 2011). Nevertheless, severe clinical infections of neonates by *C. sakazakii* ST1 do occur. The most well-known was an outbreak in a neonatal intensive care unit in Tennessee (USA) which was reported by Himelright et al. (2002). The isolate (strain ATCC BAA-894, ST1) from the associated formula has been genome sequenced (Kucerova et al., 2010, 2011).

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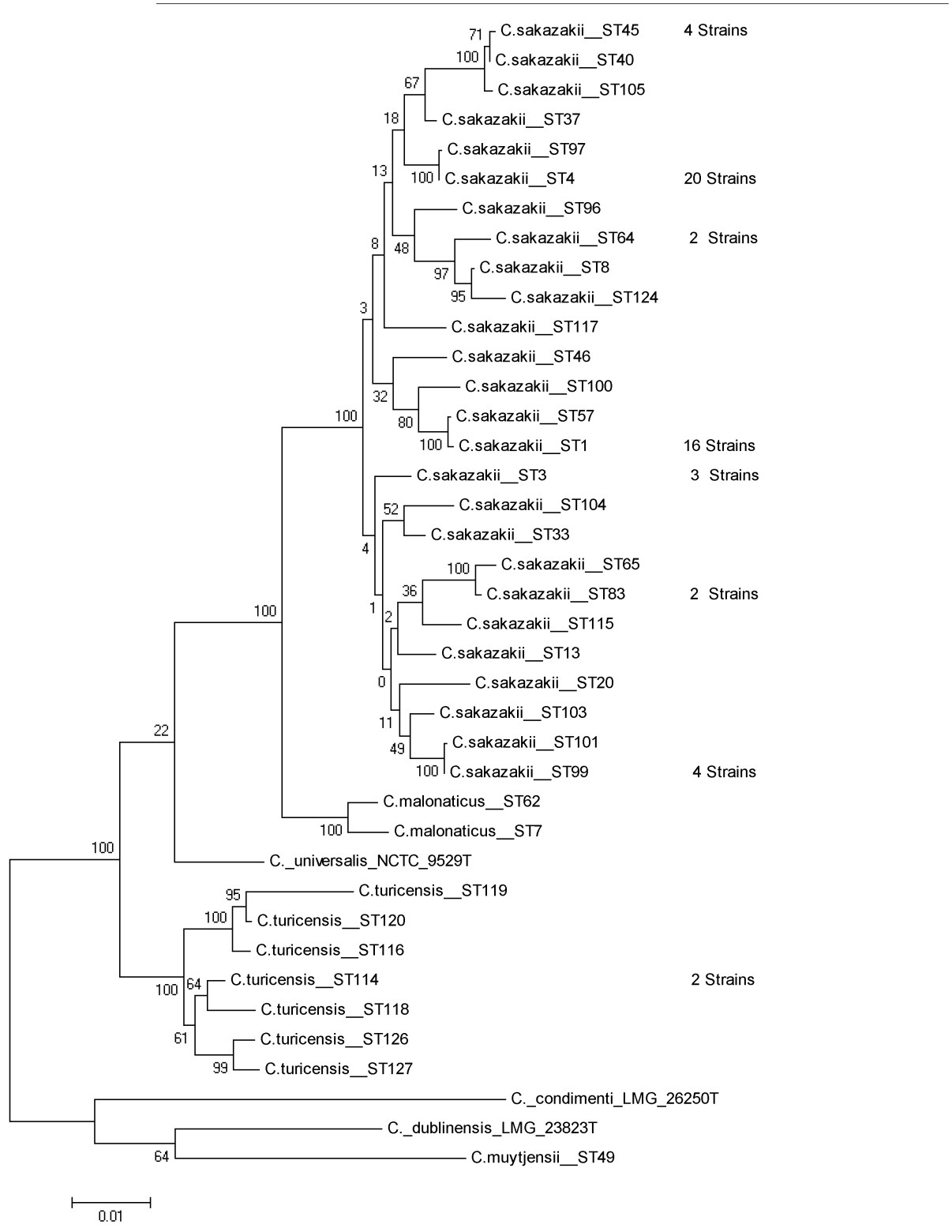
382

383

384 Figure 1.

385

386



387

388 Table 1 Multilocus sequence typing profiles of *Cronobacter* strains isolated from powdered infant formula, and reported by Muytjens et al. (1988)

Bacterial species	ST[a]	Clonal complex[b]	ID[c]	Country
C. sakazakii	1	1	541	The Netherlands
	1	1	543	The Netherlands
	1	1	537	Russia
	3		528	Belgium
	3		545	The Netherlands
	4	4	529	Canada
	4	4	538	Russia
	4	4	540	The Netherlands
	4	4	544	The Netherlands
	4	4	548	West Germany
	8	15	HPB-3284[d]	Uruguay
	124	15	539	The Netherlands
	13	8	532	East Germany
	45	6	536	Russia
	65	9	547	USA
	57		531	Denmark
	64		533	France
C. malonaticus	7	2	535	New Zealand
	62		527	Australia
C. muytjensii	49		530	Denmark
Total	20			

389

390 a ST=Sequence type

391 b Clonal complex defined as clusters of sequence types with single locus variants, as given by Joseph et al. (2012b).

392 c Strain identification code

393 d Multilocus sequence type profile deposited in http://www.pubMLST.org.cronobacter database by other collaborators.

394

395 Table 2 Multilocus sequence typing profiles of *Cronobacter* strains isolated from five milk powder manufacturing plants in Australia between 2006-2007,
396 and reported by Craven et al. (2010).

Cronobacter species	ST[a]	Clonal complex[b]	ID[c]	Isolation environment	Pulsetype	Number of isolates	Factory
C. sakazakii	1	1	1466	Milk powder	1	5	B
	1	1	1479	Milk powder	14	1	C
	1	1	1492	Roof, milk powder	27	12	B
	1	1	1493	Other processing (butter)	28	1	B
	1	1	1494	Tanker bay, milk powder	29	3	E
	1	1	1495	Milk powder	30	7	E
	1	1	1496	Milk powder	31	1	E
	1	1	1499	Milk powder	34	1	B
	1	1	1502	Roof	37	2	C
	117	1	1497	Milk powder	32	8	A
	3		1503	Tanker bay	38	1	C
	3		1899	Tanker bay	NP[d]	1	C
	4	4	1476	Milk powder, other processing (evaporator), other processing	11	6	B, E
	4	4	1477	Roof, milk powder	12	7	B
	4	4	1480	Milk powder	15	4	A, D
	4	4	1481	Other processing (evaporator)	16	1	A
	4	4	1482	Tanker bay	17	1	A
	4	4	1483	Milk powder	18	1	D
	4	4	1484	Roof, milk powder	19	4	C, E
	4	4	1485	Other external (outside grounds)	20	1	C
	4	4	1486	Other external (outside grounds)	21	1	C
	4	4	1487	Tanker bay	22	1	A
	4	4	1488	Milk powder	23	1	D
	4	4	1489	Milk powder	24	2	A
	97	4	1490	Tanker bay	25	2	B

	20	7	1474	Milk powder	9	3	D
	37		1467	Tanker bay	2	1	E
	40	6	1471	Milk powder	6	1	A
	40	6	1505	Milk powder	40	3	D
	40	6	1506	Milk powder, other processing (evaporator)	41	10	D
	40	6	1507	Tanker bay	42	1	C
	105	6	1501	Roof	36	1	C
	46		1504	Milk powder	39	1	C
	64		1473	Milk powder	8	2	E
	83	9	1498	Milk powder, other processing	33	6	E
	83	9	1500	Milk powder	35	1	A
	96		1491	Tanker bay	26	1	A
	100	14	1475	Milk powder	10	2	B
	103		1508	Milk powder	43	5	A
	104		1470	Other external (shoes)	5	1	C
	115		1472	Roof	7	1	B
	133		1900	Floor	NP	1	B
C. malonaticus	102		1514	Milk powder	49	1	E
C. turicensis	114		1468	Other processing (cheese), tanker bay	3	2	B, E
	114		1469	Roof	4	1	A
	116		1478	Tanker bay, other external (shoes), milk powder	13	3	C
	118		1511	Roof	46	1	D
	119	12	1512	Other processing	47	1	E
	120	12	1513	Milk powder	48	1	C
	126		1509	Roof	44	1	D
	127		1510	Milk powder	45	1	A
	132		1898	Floor	NP	1	B
Total	52					129	

397

398 a ST=Sequence type

399 b Clonal complex defined as clusters of sequence types with single locus variants, as given by Joseph et al. (2012b).

400 c ID = Strain identification code

401 d NP = No profile obtained from PFGE

402

403

404 Table 3 Multilocus sequence typing profiles of *Cronobacter* strains isolated from a milk powder manufacturing plant in Germany in 2006 and 2009, and
405 reported by Jacobs et al. (2011).

406

Cronobacter species	ST[a]	Clonal complex[b]	ID[c]	Source	Year	Pulsetype[d]	Number of isolates
C. sakazakii	1	1	1536	Roller Dryer (conc.)	2009	2009-2	2
	1	1	1538	Roller Dryer (conc.)	2009	2009-1	2
	1	1	1540	Roller Dryer (powder)	2009	2009-3	1
	1	1	1541	Roller Dryer (powder)	2009	2009-4	2
	4	4	1537	Roller Dryer (powder)	2009	2009-5	10
	4	4	1542	Roller Dryer (conc.)	2009	2009-6	7
	4	4	1533	Drying tower 1 (environment)	2006	2006-6	2
	33		1534	Drying tower 1 (environment)	2006	2006-7	1
	99	10	1529	Drying tower 1 (MTA)[e]	2006	2006-3	1
	99	10	1531	Drying tower 1 (filter powder)	2006	2006-1	14
	99	10	1532	Drying tower 2 (filter powder)	2006	2006-5	2
	99	10	1535	Drying tower 1 (MTA)	2006	2006-2	29
	101	10	1530	Drying tower 2 (filter powder)	2006	2006-4	8
Total	13						81

407

408 a ST= Sequence type

409 b Clonal complex defined as clusters of sequence types with single locus variants, as given by Joseph et al. (2012b).

410 c ID= Strain identification code

411 d Note, same pulsetype numbers for strains isolated in 2006 and 2009 do not reflect any similarity.

412 e MTA = microbiological trend analysis (from final products).

413

414

415

416 Table 4 Multilocus sequence typing profiles of 85 *Cronobacter* strains collected between 1988 and 2009.

417

Bacterial species	Sequence type (clonal complex)[a]	Number of strains			Total of strains	Percentage (%)
		Muytjens et al. (1988)	Craven et al. (2010)	Jacobs et al. (2011)		
C. sakazakii		17	42	13	72	85
	4 (4)	5	12	3	20	24
	97 (4)	0	1	0	1	1
	1 (1)	3	9	4	16	19
	117	0	1	0	1	1
	40 (6)	0	4	0	4	5
	105 (6)	0	1	0	1	1
	99 (10)	0	0	4	4	5
	101 (10)	0	0	1	1	1
	3	2	2	0	4	5
	8 (15)	1	0	0	1	1
	124 (15)	1	0	0	1	1
	Others	5	12	1	18	21
C. turicensis	114, 116, 118, 119, 120, 126, 127	0	9	0	9	10
C. malonaticus	7, 62, 102	2	1	0	3	4
C. muytjensii	49	1	0	0	1	1
Total		20	52	13	85	100

418

419 a Clonal complex defined as clusters of sequence types with single locus variants, as given by Joseph et al. (2012b).

420

421

Figure 1. Maximum likelihood tree of the seven multilocus sequence typing loci (3036 base pair concatenated length) for the *Cronobacter* genus, showing the sequence type for isolated strains and type strains only for *Cronobacter* species not identified from the strain collections. The tree was drawn using MEGA5 (http://www.megasoftware.net/) with 1000 bootstrap replicates.