Genotyping and Source Tracking of *Cronobacter sakazakii* and *C. malonaticus* Isolates from Powdered Infant Formula and an Infant Formula Production Factory in China

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Cronobacter spp. (formerly defined as *Enterobacter sakazakii*) are opportunistic bacterial pathogens of both infants and adults. In this study, we analyzed 70 *Cronobacter* isolates from powdered infant formula (PIF) and an infant formula production facility in China to determine possible contamination routes. The strains were profiled by multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), PCR-based O-antigen serotyping, and *ompA* and *rpoB* sequence analyses. The isolates were primarily *Cronobacter sakazakii* (66/70) or *Cronobacter malonaticus* (4/70). The strains were divided into 38 pulsotypes (PTs) using PFGE and 19 sequence types (STs) by MLST. In contrast, *rpoB* and *ompA* sequence analyses divided the strains into 10 overlapping clusters each. PCR serotyping of the 66 *C. sakazakii* and 4 *C. malonaticus* strains resulted in the identification of four *C. sakazakii* serotypes (O1, O2, O4, and O7) and a single *C. malonaticus* serotype, O2. The dominant *C. sakazakii* sequence types from PIF and an infant formula production factory in China were *C. sakazakii* clonal complex 4 (CC4) (*n* = 19), ST1 (*n* = 14), and ST64 (*n* = 11). *C. sakazakii* CC4 is a clonal lineage strongly associated with neonatal meningitis. In the process of manufacturing PIF, the spray-drying, fluidized-bed-drying, and packing areas were the main areas with *Cronobacter* contamination. *C. sakazakii* strains with the same pulsotypes (PT3 and PT2) and sequence types (ST1 and ST64) were isolated both from processing equipment and from the PIF finished product.

*C*ronobacter (formerly defined as *Enterobacter sakazakii*) is a diverse genus in the family *Enterobacteriaceae*. These organisms are Gram-negative, motile, facultatively anaerobic, non-spore-forming peritrichous rod-shaped opportunistic bacterial pathogens (1–3). The genus consists of seven species: *Cronobacter sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii*, *C. dublinensis*, *C. universalis*, and *C. condimenti* (4). Of these, *C. sakazakii* has been associated primarily with neonatal infections and *C. malonaticus* with adult infections (5–7). *Cronobacter* spp. are adapted to a wide range of environments, such as water, soil, plant material (wheat, rice, herbs, and spices), food production environments, and various food products (8–10). More importantly for neonatal health, *Cronobacter* spp. have been isolated from powdered infant formula (PIF) and milk powder production factories (11–13). Consequently, they can cause severe neonatal infections through the ingestion of contaminated PIF, especially in low-birth-weight infants (14, 15). Once infected by *Cronobacter* spp., patients may suffer from fatal necrotizing enterocolitis, septicemia, or meningitis, which can cause severe neurological sequelae, developmental disorders, and even death (16, 17).

Understanding the genetic diversity of the genus *Cronobacter* can ensure that accurate detection methods are applied for its detection and control, as well as for reliable microbial source tracking of contaminated foods such as infant formula (16–19). Consequently, the genetic diversity of the *Cronobacter* genus has been a considerable focus of study. An international multilocus sequence typing (MLST) database, containing >350 defined sequence types and metadata for >1,000 strains, has been established for *Cronobacter* (5, 20) (http://pubmlst.org/cronobacter/). The standard method uses the DNA sequences of 7 loci and recently has been expanded to 53-locus ribosomal MLST as well as

1,865-locus core genome MLST (5, 20). Joseph and colleagues applied the 7-locus method to *Cronobacter* sp. isolates obtained between 1950 and 2009 and showed that *C. sakazakii* sequence type 4 (ST4) was the predominant sequence type associated with severe cases of neonatal meningitis but not necrotizing enterocolitis (15, 20). This association between clinical presentation and sequence type was confirmed in 2011, when the isolates from a number of *Cronobacter* meningitis cases in the United States were shown to belong to *C. sakazakii* clonal complex 4 (CC4) (21). Furthermore, *Cronobacter* spp. from PIF and milk powder production factories in several countries had been characterized by MLST. The results showed that the main *C. sakazakii* sequence type in factories was CC4 (25%), followed by ST1, ST40, ST9, and ST3 (22). However, understanding of the genetic diversity of *Cronobacter* isolates recovered from PIF and infant formula production factories in China is still very limited. Lu et al. identified and profiled *Cronobacter* strains isolated from PIF in China by

using phenotyping (API 20E), ribotyping, and matrix-assisted laser desorption ionization–time of flight mass spectrometry (23). However, phenotyping and physiochemical techniques do not enable determination of the relatedness of isolates, and commonly used phenotyping databases have not been updated with the changes in *Cronobacter* taxonomy (5, 24). In contrast, Cui et al. used the 7-locus MLST and multilocus sequence analysis (MLSA) to identify and genotype *Cronobacter* spp. from clinical, food, and environmental sources in China from 2010 to 2012 (25, 26).

Alternative methods to MLST for *Cronobacter* typing have been proposed. Although they are not as discriminatory as MLST, they can make significant contributions to our understanding of the organism. Seventeen O-antigen serotypes have been described across the genus, seven of which belong to *C. sakazakii*; of particular interest is *C. sakazakii* serotype O2, which often corresponds to *C. sakazakii* ST4 (5, 27). The associated lipopolysaccharide (LPS) structure could be responsible for the proinflammatory host response to infection. The outer membrane protein OmpA of *Cronobacter* spp. has been shown to be a potential virulence factor in the crossing of the blood-brain barrier prior to the onset of meningitis, and the sequencing of this gene has been used for identification purposes (28, 29). Sequencing of the housekeeping gene *rpoB* has also been used for the identification of *Cronobacter* species (30). However, the method is reported to give false-positive results, including misidentifying strains as *Cronobacter* during an outbreak (31, 32). Hence, a comparison of the four genotyping methods MLST, O-antigen serotyping, *ompA* analysis, and *rpoB* analysis is warranted. The online *Cronobacter* MLST databases (http://pubmlst.org/cronobacter/) facilitate such comparison, since all four typing schemes are included, and data from previous publications are available through open access.

In our study, 70 *Cronobacter* strains were isolated from PIF in different parts of China and an infant formula production factory from 2009 to 2012. We determined the genetic diversity of these strains by MLST, pulsed-field gel electrophoresis (PFGE), and O-antigen serotyping, analyzed the phylogenetic relationships of the *ompA* and *rpoB* genes, and revealed the possible routes of *Cronobacter* contamination during PIF manufacture. These results are important for understanding the genetic diversity of *Cronobacter* spp. in China and for enabling microbial source tracking to control the occurrence of *Cronobacter* spp. in the PIF processing chain.

MATERIALS AND METHODS

Bacterial strains. A total of 70 *Cronobacter* strains were isolated from PIF ($n = 43$) and an infant formula production factory ($n = 27$) in China between 2009 and 2012. Details about the isolates are given in Tables 1 and 2. Forty-three *Cronobacter* sp. isolates had been recovered previously from 1,228 PIF samples from different areas of China. The 27 *Cronobacter* strains for which data are shown in Table 2 had been isolated from 375 samples taken over a 4-year period from an infant formula factory environment and finished products from the factory. All the isolates had been provisionally identified as *Cronobacter* spp. using API 20E, and this identification had been confirmed using 16S rRNA gene sequencing (23). All the strains were grown in Luria-Bertani (LB) broth at 37°C for 12 h, streaked onto tryptic soy agar (TSA) plates, and then cultivated at 37°C for 24 h. Single colonies in the TSA plates were inoculated into the LB medium and were cultivated at 37°C for 18 h.

For accurate determination of the interspecific phylogenetic relationships, *C. sakazakii* ATCC 29544[T], ATCC BAA-894, ATCC 29004, and ATCC 12868 were used as the *C. sakazakii* species reference strains. *C.*

malonaticus CDC 105877[T], *C. dublinensis* LMG 23823[T], *C. turicensis* LMG 23827[T], *C. universalis* NCTC 9529[T], *C. condimenti* LMG 26250[T], and *C. muytjensii* ATCC 51329[T] were used as the type strains of the remaining species.

MLST analysis. Genomic DNA was extracted by the TIANamp Bacteria DNA kit (Tiangen Biotech [Beijing] Co., Ltd., Beijing, China) and was amplified using the 7 primer pairs described previously (24). The PCR products were sequenced by Life Technologies Limited (Shanghai, China). All allele profiles and ST assignments can be obtained from the *Cronobacter* MLST open-access database (http://pubmlst.org/cronobacter/). The phylogenetic relationship of the concatenated sequences (3,036 bp) of the seven housekeeping genes (*atpD*, *fusA*, *glnS*, *gltB*, *gyrB*, *infB*, and *ppsA*) was analyzed using the maximum-likelihood algorithm in MEGA, version 6, with 1,000 bootstrap replicates. The nucleotide diversity indices were determined using DnaSP software, version 5.0.

PFGE analysis. The pulsed-field gel electrophoresis (PFGE) scheme for *Cronobacter* spp. was that described by previous studies (11, 16). Electrophoresis was performed with the auto algorithm model on a CHEF DR-II electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA) with an initial switch time of 2.16 s, a final switch time of 63.80 s, a condensation temperature of 14°C, and the electric field alternates of 120°. A lambda ladder PFGE marker (New England BioLabs) was used as the DNA size marker for standard analysis. After electrophoresis for 18 h, the gels were stained for 30 min with 10 mg ml^{-1} ethidium bromide and were then destained for 30 min with distilled water. The PFGE gels were visualized and photographed under a UV transilluminator. The gel images were analyzed using the Dice coefficient and the unweighted pair group method with arithmetic means (UPGMA) in GelCompar II software, version 5.1 (Applied Maths, Sint-Martens-Latem, Belgium), with a 1.5% band position tolerance. The intraspecific diversity was reflected with the Shannon-Weiner index.

O-antigen serotype analysis. Using multiplex serotyping PCR, the serotypes of all 70 *Cronobacter* isolates were determined with the primers and reaction conditions given in previous publications (33, 34). The mixed primers for *C. sakazakii* are composed of seven pairs of primers encoding the gene sequences of *C. sakazakii* serotypes O1 to O7, and the mixed primers for *C. malonaticus* consist of two pairs of primers encoding the sequences of *C. malonaticus* serotypes O1 and O2. The resulting PCR products were sequenced (Life Technologies Limited, China) and were analyzed using the neighbor-joining algorithm in MEGA, version 6.

***ompA* and *rpoB* sequence analyses.** The specific PCR amplifications of *ompA* and *rpoB* were performed as described by Mohan Nair and Venkitanarayanan (28) and Li et al. (30), respectively. The PCR products were sequenced (Life Technologies Ltd., China). The *ompA* sequences were submitted to the MLST *Cronobacter* databases for allele designation, and the *rpoB* sequences were submitted to the NCBI. The phylogenetic trees of *ompA* and *rpoB* were constructed using the maximum-likelihood algorithm in MEGA, version 6, with 1,000 bootstrap replicates.

Nucleotide sequence accession numbers. The *rpoB* sequences determined in this study have been submitted to the NCBI under GenBank accession numbers KP192773 to KP192846.

RESULTS

MLST analysis of *Cronobacter* spp. isolated from powdered infant formula and the production environment. Initial experiments using 16S rRNA gene sequencing had identified the *Cronobacter* isolates as either *C. sakazakii* or *C. malonaticus*. By use of 7-locus MLST, the 70 *Cronobacter* strains clustered into 19 sequence types, shown in Tables 1 and 2. Seventeen sequence types were in the species *C. sakazakii*, and the remaining two were in *C. malonaticus*. Overall, *C. sakazakii* was the dominant (66/70 [94.29%]) species isolated from both PIF and its production environment. The main C. *sakazakii* sequence types were *C. sakazakii* ST4 (18/66 [27.27%]), ST1 (14/66 [21.21%]), and ST64

TABLE 1 MLST, PFGE, O-antigen serotyping, and *ompA* and *rpoB* analyses of *Cronobacter* strains isolated from PIF in different parts of China from 2009 to 2012[a]

Cronobacter species	Strain no.	ID	Region[b]	ST	CC	PT	OT	*ompA* allele designation	*rpoB* cluster
C. sakazakii	CE9	874	NE China	4	4	1	O2	6	1
C. sakazakii	CE10	875	NE China	4	4	1	O2	6	1
C. sakazakii	CE11	876	E China	4	4	1	O2	6	1
C. sakazakii	CE12	877	E China	4	4	1	O2	6	1
C. sakazakii	CE17	878	N China	4	4	1	O2	6	1
C. sakazakii	CE19	880	N China	4	4	1	O2	6	1
C. sakazakii	CE20	881	N China	4	4	1	O2	6	1
C. sakazakii	CE22	882	N China	4	4	1	O2	6	1
C. sakazakii	CE23	883	N China	4	4	1	O2	6	1
C. sakazakii	CE18	879	N China	4	4	1	O2	21	1
C. sakazakii	CE1	872	NE China	4	4	15	O2	6	1
C. sakazakii	CE7	873	NE China	4	4	16	O2	6	1
C. sakazakii	CE27	884	NE China	4	4	17	O2	6	1
C. sakazakii	CE48	885	NE China	4	4	19	O2	6	1
C. sakazakii	CE49	886	NE China	4	4	20	O2	6	1
C. sakazakii	CE69	865	NE China	1	1	3	O1	3	2
C. sakazakii	CE21	858	NE China	1	1	8	O1	3	2
C. sakazakii	CE24	859	NE China	1	1	6	O1	3	2
C. sakazakii	CE43	860	NE China	1	1	10	O1	3	2
C. sakazakii	CE47	861	NE China	1	1	11	O1	54	2
C. sakazakii	CE25	905	NE China	64	64	33	O2	6	3
C. sakazakii	CE34	910	NE China	64	64	35	O2	6	3
C. sakazakii	CE54	912	NE China	64	64	37	O2	6	3
C. sakazakii	CE51	897	NW China	21	21	5	O1	6	6
C. sakazakii	CE53	899	NW China	21	21	5	O1	6	6
C. sakazakii	CE52	898	NW China	21	21	28	O1	6	6
C. sakazakii	CE41	892	NE China	12		4	O2	5	4
C. sakazakii	CE44	893	NE China	12		4	O4	5	4
C. sakazakii	CE38	894	NE China	12		25	O4	5	4
C. sakazakii	CE16	707	N China	259		24	ND	6	1
C. sakazakii	CE28	895	NE China	17	17	26	O2	6	6
C. sakazakii	CE15	900	N China	22		29	O2	6	1
C. sakazakii	CE50	901	NE China	22		30	O2	6	1
C. sakazakii	CE56	902	NE China	31		31	O2	23	5
C. sakazakii	CE29	903	NE China	40	45	32	O4	6	7
C. sakazakii	CE8	917	N China	83	83	38	O7	6	5
C. sakazakii	CE26	708	NE China	268	4	36	O2	6	1
C. sakazakii	CE55	709	NE China	260		ND	O1	6	2
C. sakazakii	CE13	890	N China	8	8	ND	O1	5	5
C. sakazakii	CE14	891	N China	8	8	ND	O1	5	5
C. malonaticus	CMa2	706	S China	258		7	MaO2	24	8
C. malonaticus	CMa35	918	NE China	201	7	9	MaO2	8	9
C. malonaticus	CMa3	919	NE China	258		18	MaO2	24	9

[a] ID, strain identification code in the *Cronobacter* MLST databases; ST, sequence type; CC, clonal complex (defined as clusters of sequence types with single-locus variants); PT, pulsotype; OT, O-antigen serotype; ND, not detected.
[b] E, east; N, north; S, south; NE, northeast; NW, northwest.

(11/66 [16.67%]). Only four strains of *C. malonaticus* were isolated: three strains from PIF (*C. malonaticus* ST258) and one from the manufacturing plant (*C. malonaticus* ST258). Of the 19 sequence types isolated, 6 had not been reported before and therefore were assigned new sequence types in the *Cronobacter* MLST databases: ST258, ST259, ST268, ST260, ST269, and ST261. The new allele numbers assigned were *atpD89*, *glnS107*, *glnS108*, *gltB127*, *gltB128*, *gyrB125*, *ppsA160*, and *ppsA161*.

Multilocus sequence analysis (MLSA). The nucleotide diversity (π) of the seven housekeeping genes was analyzed using DnaSP software, version 5.0 (Table 3). The GC contents of all alleles ranged from 53.61% (*fusA*) to 62.84% (*ppsA*), averaging

58.84%, a level similar to the whole-genome GC content of *C. sakazakii* BAA-894 (57%) (2). The number of alleles ranged from 10 (*atpD*) to 16 (*gyrB* and *ppsA*). The proportion of fragments found as polymorphic sites (expressed as a percentage) ranged from 2.74% (*fusA*) to 9.71% (*gyrB*), averaging 6.13% (186 polymorphic sites) of the concatenated 7 alleles (total length, 3,036 nucleotides). The nucleotide diversity of the 3,036 nucleotides was 0.0176, ranging from 0.0084 (*atpD*) to 0.0300 (*gyrB* and *ppsA*) per individual gene, suggesting that the nucleotide diversity of *atpD* was the lowest and the nucleotide diversity values of *gyrB* and *ppsA* were higher than those for other housekeeping genes. The ratio of nonsynonymous to synonymous mutations (K_a/K_s) of the concat-

TABLE 2 MLST, PFGE, O-antigen serotyping, and *ompA* and *rpoB* analyses of *Cronobacter* strains isolated from an infant formula production factory in China from 2009 to 2012[a]

Cronobacter species	Strain no.	ID	Source	ST	CC	PT	OT	*ompA* allele designation	*rpoB* cluster
C. sakazakii	CE61	887	Final product	4	4	21	O2	55	1
C. sakazakii	CE64	888	Final product	4	4	22	O2	6	1
C. sakazakii	CE67	889	Final product	4	4	23	O2	6	1
C. sakazakii	CE60	863	U valve tube	1	1	3	O1	3	2
C. sakazakii	CE63	864	Powder lumps after spray drying	1	1	3	O1	3	2
C. sakazakii	CE70	866	Final product	1	1	3	O1	3	2
C. sakazakii	CE71	867	Final product	1	1	3	O1	3	2
C. sakazakii	CE72	868	Final product	1	1	3	O1	3	2
C. sakazakii	CE73	869	Final product	1	1	3	O1	3	2
C. sakazakii	CE74	870	Powder lumps on fluidized bed	1	1	3	O1	3	2
C. sakazakii	CE59	862	Raw material	1	1	12	O1	3	2
C. sakazakii	CE79	871	Powder lumps on fluidized bed	1	1	14	O1	3	2
C. sakazakii	CE30	906	Final product	64	64	2	O2	6	3
C. sakazakii	CE31	908	Final product	64	64	2	O2	6	3
C. sakazakii	CE36	911	Powder lumps on fluidized bed	64	64	2	O2	6	3
C. sakazakii	CE62	913	Powder lumps on fluidized bed	64	64	2	O2	6	3
C. sakazakii	CE68	914	Powder lumps on fluidized bed	64	64	2	O2	6	3
C. sakazakii	CE77	915	Final product	64	64	2	O2	6	3
C. sakazakii	CE78	916	Final product	64	64	2	O2	6	3
C. sakazakii	CE75	711	Final product	261	64	2	O2	6	3
C. sakazakii	CE76	922	Final product	261	64	2	O2	6	3
C. sakazakii	CE33	909	Raw material	64	64	34	O2	6	3
C. sakazakii	CE58	896	Raw material	17	17	27	O2	22	6
C. sakazakii	CE32	904	Final product	50	2	2	O2	21	3
C. sakazakii	CE65	710	Fixed bed	269		13	O7	6	10
C. sakazakii	CE66	921	Fixed bed	269		6	O7	6	10
C. malonaticus	CMa5	920	Raw material	258		7	MaO2	24	9

[a] ID, strain identification code in the *Cronobacter* MLST databases; ST, sequence type; CC, clonal complex (defined as clusters of sequence types with single-locus variants); PT, pulsotype; OT, O-antigen serotype.

enated sequences was 0.0014 and ranged from 0 (*atpD*, *gltB*, *gyrB*, and *infB*) to 0.0471 (*fusA*). The K_a/K_s ratios of all seven housekeeping genes were less than 1.

Phylogenetic relationship of *C. sakazakii* and *C. malonaticus* isolates. A phylogenetic tree based on the concatenated sequences of the seven housekeeping genes (total length, 3,036 bp) for the *C. sakazakii* and *C. malonaticus* isolates and 10 reference strains was constructed (Fig. 1). The 66 *C. sakazakii* strains clustered in the same clade with >95% similarity. *C. malonaticus* was closer to *C. sakazakii* than the other five *Cronobacter* species.

PFGE analysis of *C. sakazakii* and *C. malonaticus* isolates. A total of 74 *Cronobacter* sp. strains, comprising the 70 *Cronobacter* isolates and 4 *C. sakazakii* reference strains, were analyzed using PFGE. Three *C. sakazakii* strains (CE13, CE14, and CE55) could not be digested with XbaI; therefore, only 67 isolates were analyzed using UPGMA in GelCompar II software, version 5.1 (Fig. 2). By using 95% similarity as the critical threshold, the 67 *Cronobacter* strains formed 38 pulsotypes. The major pulsotypes were PT1 (10/71 [14%]), PT2 (10/71 [14%]), and PT3 (8/71 [11%]). Strains belonging to the same sequence type corresponded to a number of pulsotypes. For example, *C. sakazakii* ST4 was further

TABLE 3 Polymorphism of the 7 MLST housekeeping genes for 66 *C. sakazakii* and 4 *C. malonaticus* isolates

Locus	Size (bp)	No. of alleles	GC content (%)	No. (%) of polymorphic sites	K_s[a]	K_a[b]	K_a/K_s	π[c]
atpD	390	10	59.51	11 (2.82)	0.0305	0	0	0.0084
fusA	438	12	53.61	12 (2.74)	0.0705	0.0033	0.0471	0.0183
glnS	363	14	57.56	21 (5.79)	0.0727	0.0005	0.0071	0.0163
gltB	507	15	61.72	42 (8.28)	0.0918	0	0	0.0219
gyrB	402	16	56.95	39 (9.71)	0.1309	0	0	0.0300
infB	441	13	58.52	14 (3.17)	0.0542	0	0	0.0129
ppsA	495	16	62.84	47 (9.49)	0.1391	0.0003	0.0022	0.0300
Concatenated sequence	3,036	19	58.84	186 (6.13)	0.0739	0.0001	0.0014	0.0176

[a] K_s, number of synonymous substitutions.
[b] K_a, number of nonsynonymous substitutions.
[c] π, nucleotide diversity.
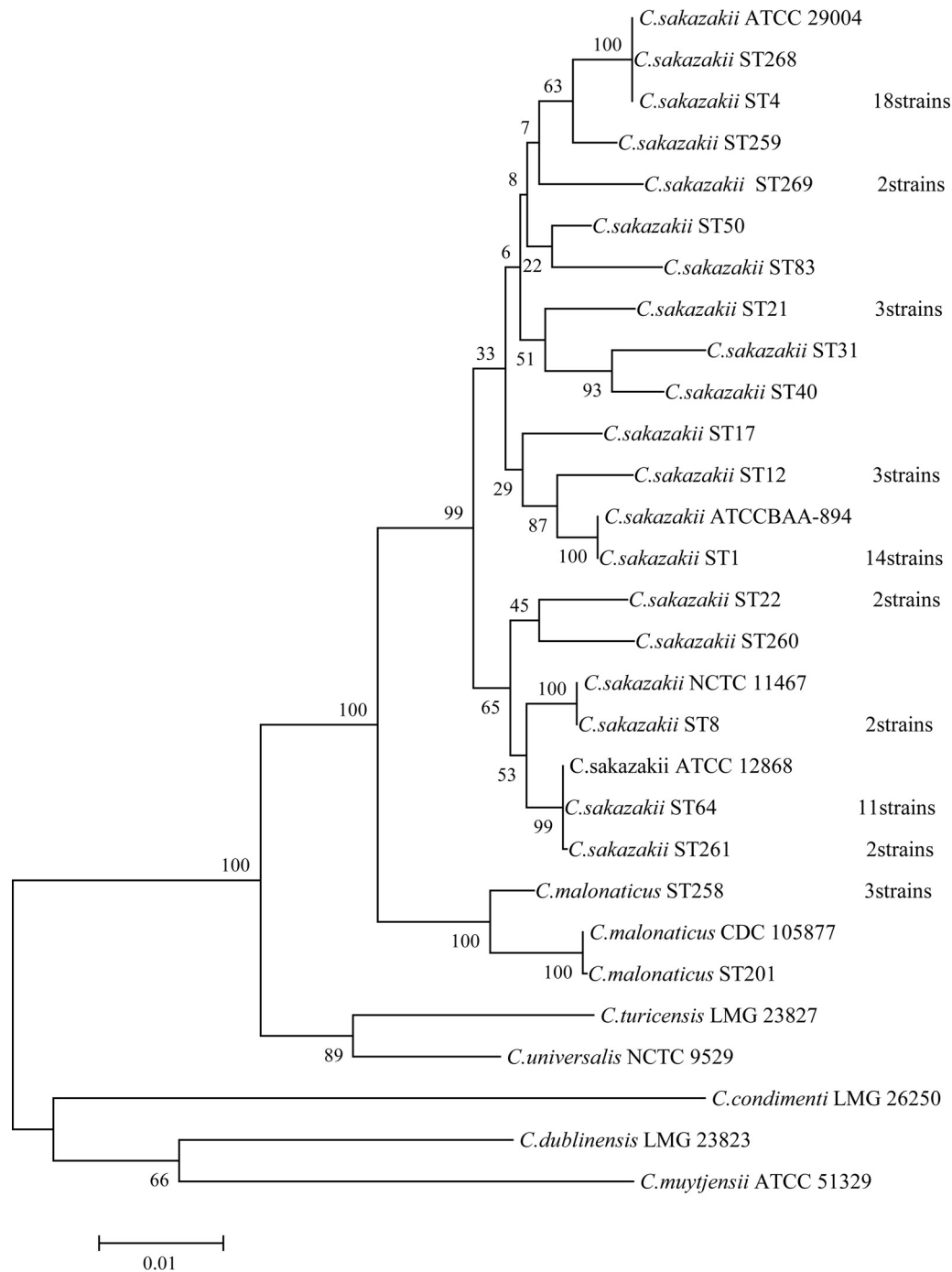
FIG 1 Maximum-likelihood tree of the spliced sequences of the 7 loci (3,036 bp) for the 80 strains. Seventy *Cronobacter* strains isolated from PIF or an infant formula production factory, 4 reference strains (*C. sakazakii* ATCC BAA-894, *C. sakazakii* ATCC 29004, *C. sakazakii* ATCC 29544, and *C. sakazakii* ATCC 12868), and 6 type strains (*C. malonaticus* CDC 105877[T], *C. dublinensis* LMG 23823[T], *C. turicensis* LMG 23827[T], *C. universalis* NCTC 9529[T], *C. condimenti* LMG 26250[T], and *C. muytjensi*i ATCC 51329[T]) were included. The tree was obtained using MEGA, version 6.0, with 1,000 bootstrap replicates.

divided into 10 pulsotypes (Shannon-Weiner index, 2.50); *C. sakazakii* ST1 contained 8 pulsotypes (Shannon-Weiner index, 2.31); and *C. sakazakii* ST64 consisted of 5 pulsotypes (Shannon-Weiner index, 1.58). The *C. sakazakii* PT1 strains were mainly from northern China.

ompA and rpoB analysis of *C. sakazakii* and *C. malonaticus*. *C. sakazakii* and *C. malonaticus* strains were distinguishable by both *ompA* and *rpoB* sequences, each of which formed 10 clusters that overlapped (Tables 1 and 2). The majority of strains had *ompA6* (42/70 [60%]) and belonged to nine sequence types: ST4, ST64, ST17, ST21, ST40, ST259, ST260, ST261, and ST269. There were eight different *rpoB* gene clusters across 67 *C. sakazakii* isolates, and two further clusters for the 3 *C. malonaticus* strains. The *C. sakazakii rpoB* cluster 1 strains (24/70 [34.28%]), which be-

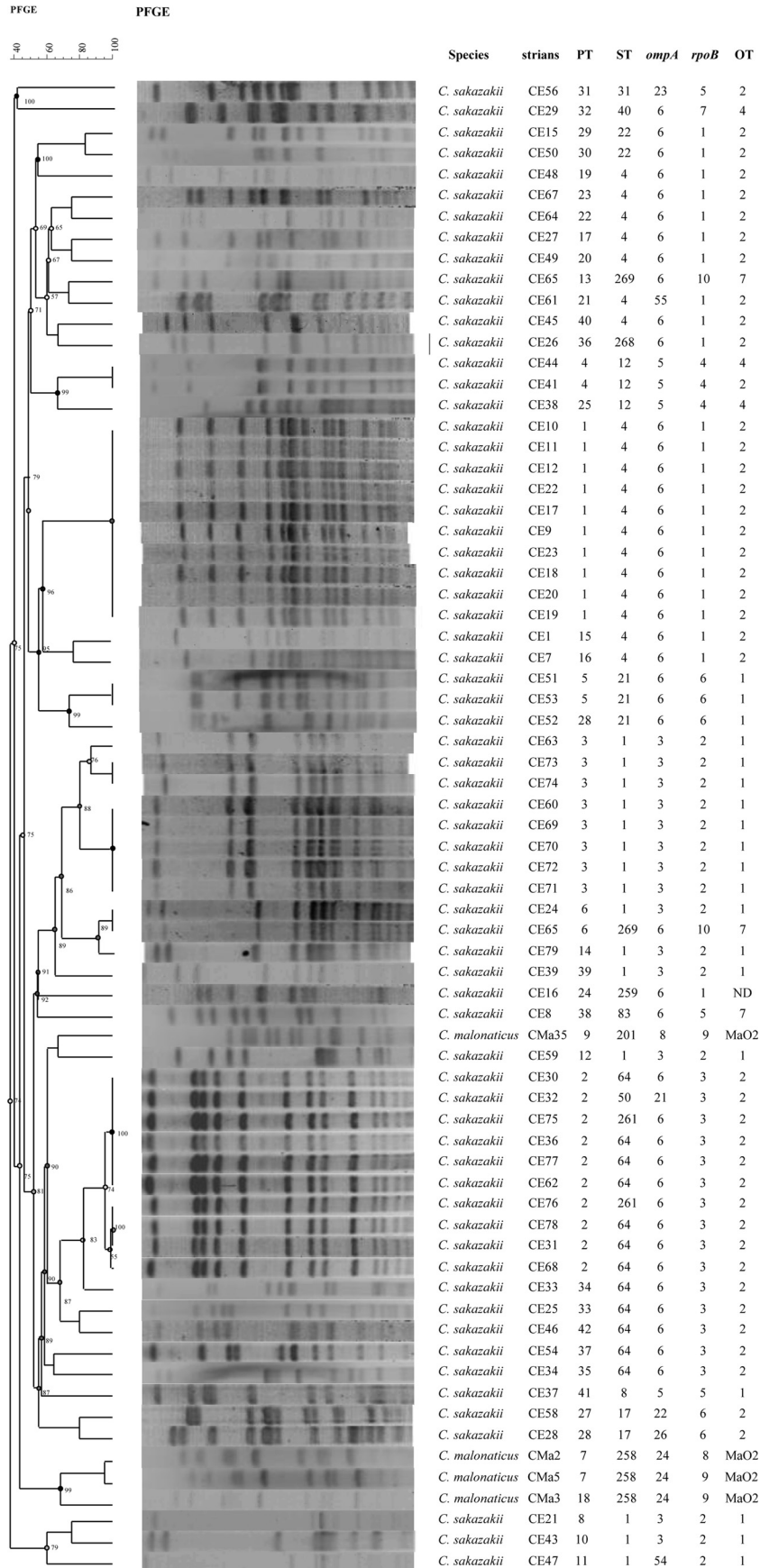| Species	strians	PT	ST	ompA	rpoB	OT
C. sakazakii	CE56	31	31	23	5	2
C. sakazakii	CE29	32	40	6	7	4
C. sakazakii	CE15	29	22	6	1	2
C. sakazakii	CE50	30	22	6	1	2
C. sakazakii	CE48	19	4	6	1	2
C. sakazakii	CE67	23	4	6	1	2
C. sakazakii	CE64	22	4	6	1	2
C. sakazakii	CE27	17	4	6	1	2
C. sakazakii	CE49	20	4	6	1	2
C. sakazakii	CE65	13	269	6	10	7
C. sakazakii	CE61	21	4	55	1	2
C. sakazakii	CE45	40	4	6	1	2
C. sakazakii	CE26	36	268	6	1	2
C. sakazakii	CE44	4	12	5	4	4
C. sakazakii	CE41	4	12	5	4	2
C. sakazakii	CE38	25	12	5	4	4
C. sakazakii	CE10	1	4	6	1	2
C. sakazakii	CE11	1	4	6	1	2
C. sakazakii	CE12	1	4	6	1	2
C. sakazakii	CE22	1	4	6	1	2
C. sakazakii	CE17	1	4	6	1	2
C. sakazakii	CE9	1	4	6	1	2
C. sakazakii	CE23	1	4	6	1	2
C. sakazakii	CE18	1	4	6	1	2
C. sakazakii	CE20	1	4	6	1	2
C. sakazakii	CE19	1	4	6	1	2
C. sakazakii	CE1	15	4	6	1	2
C. sakazakii	CE7	16	4	6	1	2
C. sakazakii	CE51	5	21	6	6	1
C. sakazakii	CE53	5	21	6	6	1
C. sakazakii	CE52	28	21	6	6	1
C. sakazakii	CE63	3	1	3	2	1
C. sakazakii	CE73	3	1	3	2	1
C. sakazakii	CE74	3	1	3	2	1
C. sakazakii	CE60	3	1	3	2	1
C. sakazakii	CE69	3	1	3	2	1
C. sakazakii	CE70	3	1	3	2	1
C. sakazakii	CE72	3	1	3	2	1
C. sakazakii	CE71	3	1	3	2	1
C. sakazakii	CE24	6	1	3	2	1
C. sakazakii	CE65	6	269	6	10	7
C. sakazakii	CE79	14	1	3	2	1
C. sakazakii	CE39	39	1	3	2	1
C. sakazakii	CE16	24	259	6	1	ND
C. sakazakii	CE8	38	83	6	5	7
C. malonaticus	CMa35	9	201	8	9	MaO2
C. sakazakii	CE59	12	1	3	2	1
C. sakazakii	CE30	2	64	6	3	2
C. sakazakii	CE32	2	50	21	3	2
C. sakazakii	CE75	2	261	6	3	2
C. sakazakii	CE36	2	64	6	3	2
C. sakazakii	CE77	2	64	6	3	2
C. sakazakii	CE62	2	64	6	3	2
C. sakazakii	CE76	2	261	6	3	2
C. sakazakii	CE78	2	64	6	3	2
C. sakazakii	CE31	2	64	6	3	2
C. sakazakii	CE68	2	64	6	3	2
C. sakazakii	CE33	34	64	6	3	2
C. sakazakii	CE25	33	64	6	3	2
C. sakazakii	CE46	42	64	6	3	2
C. sakazakii	CE54	37	64	6	3	2
C. sakazakii	CE34	35	64	6	3	2
C. sakazakii	CE37	41	8	5	5	1
C. sakazakii	CE58	27	17	22	6	2
C. sakazakii	CE28	28	17	26	6	2
C. malonaticus	CMa2	7	258	24	8	MaO2
C. malonaticus	CMa5	7	258	24	9	MaO2
C. malonaticus	CMa3	18	258	24	9	MaO2
C. sakazakii	CE21	8	1	3	2	1
C. sakazakii	CE43	10	1	3	2	1
C. sakazakii	CE47	11	1	54	2	1

FIG 2 Dendrogram based on XbaI-mediated PFGE profiles of 71 *Cronobacter* spp. The tree was drawn using UPGMA and the Dice coefficient with 1.5% tolerance.

FIG 3 Neighbor-joining tree of O-antigen sequences from 18 *Cronobacter* isolates. The tree was structured by MEGA, version 6, with 1,000 bootstrap replicates. The 18 *Cronobacter* isolates represent 18 different STs of *Cronobacter* spp.

longed to ST4, ST22, ST250, ST260, ST268, and ST269, overlapped with some of those in *ompA6*.

O-antigen serotype analysis of *Cronobacter* sp. isolates. A neighbor-joining tree of the *C. sakazakii* and *C. malonaticus* serotypes is shown in Fig. 3. According to the different sequence sizes of target genes, all strains were divided into five serotypes. Each serotype was composed of a number of STs (Fig. 3; Tables 1 and 2). The largest number of strains belonged to *C. sakazakii* serotype O2 (39/70 [55.71%]) and included eight *C. sakazakii* sequence types: ST4, ST17, ST21, ST31, ST50, ST64, ST261, and ST268. *C. sakazakii* serotype O1 (20/70 [28.57%]) contained *C. sakazakii* ST1, ST8, ST21, and ST260. *C. sakazakii* serotype O4 (4/70 [5.71%]) was composed of *C. sakazakii* strains belonging to ST12 and ST40. *C. sakazakii* serotype O7 was composed of three strains, which belonged to ST83 and ST269 (two strains). Finally, *C. malonaticus* serotype O2 (4/70 [5.71%]) contained *C. malonaticus* ST201 and ST258. No sequence types were found in more than one O-antigen serotype. Furthermore, *C. sakazakii* serotype O2 (55.71%) and *C. sakazakii* serotype O1 (28.57%) were the dominant serotypes isolated from PIF and an infant formula production factory.

Microbial source tracking. The powdered infant formula production environment and finished products of a production factory had been monitored continuously from 2009 to 2012. Twenty-six *C. sakazakii* isolates and one *C. malonaticus* isolate were recovered; details are given in Table 2. The isolates were recovered from raw material, a U valve tube, powder lumps after spray drying, powder lumps on a fluidized bed, a fixed bed, and finished products. It is noteworthy that eight *C. sakazakii* PT2 (ST64) strains and seven *C. sakazakii* PT3 (ST1) strains were isolated over a 3-month period from both processing equipment and finished

products. Three *C. sakazakii* ST4 strains were recovered from the PIF finished product but not from environmental samples. In total, one-third (9/27) of *C. sakazakii* isolates belonged to ST1. *C. sakazakii* ST17, ST50, ST261, and ST269 and *C. malonaticus* ST258 were not detected in the finished products.

DISCUSSION

It is well known that *Cronobacter* spp. can cause severe infections in neonates and infants through ingestion of contaminated infant formula (3, 5, 9, 35). China is a major market for PIF, and considerable attention is given to its safety. However, our current knowledge of this organism in PIF and the manufacturing environment in China is limited. Thus, it is necessary to improve our understanding of the diversity of this genus and possible routes of PIF contamination.

Initial identification of these strains using phenotyping (API 20E) gave a presumptive identification of *Enterobacter sakazakii*. However, the use of API 20E for identification of this organism is very limited, because the corresponding database has not been updated with the *Cronobacter* genus. Furthermore, 16S rRNA gene sequence analysis could not determine whether the strains were *C. sakazakii* or *C. malonaticus*. This issue is well known and is due to errors in GenBank and microheterogeneities within the multiple copies of 16S rRNA in the *Cronobacter* genome (24). *ompA* and *rpoB* sequence analyses were able to correctly identify the species of the isolates but did not distinguish between isolates within the species as much as MLST or PFGE (Fig. 2). The lack of reliable power to distinguish between *C. sakazakii* and *C. malonaticus* by 16S rRNA gene sequencing has been reported previously and has led to the adoption of *fusA* DNA sequencing as a

suitable alternative (5, 20). This also has the advantage of being 1 of the 7 loci used in the MLST scheme. Consequently, all *Cronobacter* spp. can be identified using the *fusA* allele and then fully typed using the remaining 6 loci of the MLST scheme (Fig. 1) (5, 20). The phylogenetic tree of concatenated sequences (3,036 bp) from the seven housekeeping genes reflects the whole-genome phylogeny of the *Cronobacter* genus (4). To date, the curated *Cronobacter* MLST databases have >1,000 strains, >350 defined 7-locus STs, and >100 searchable whole genomes as well as meta-data (http://pubmlst.org/cronobacter/). They also contain the profiles for other typing schemes, such as the *ompA* and *rpoB* schemes.

PFGE is a well-established means of profiling bacterial strains for epidemiological purposes but is not used for species identification. In this study, PFGE distinguished between strains within the same sequence type. For example, *C. sakazakii* ST4 strains were divided into 10 pulsotypes. This is a very important observation given the life-threatening meningitis infections associated with this sequence type and the application of PFGE in epidemiological investigations, as well as in source tracking in a PIF production facility. However, as reported by other researchers, not all strains can be analyzed by PFGE, and the method cannot be used to identify *Cronobacter* isolates (2, 11). Therefore, a stepwise analysis by MLST followed by PFGE may be suitable for comprehensive profiling of *Cronobacter* isolates.

In previous studies, ST4, ST1, ST40, ST9, and ST3 were shown to be the main *C. sakazakii* sequence types isolated from PIF and milk powder production factories in several countries (5, 21). In this study, ST4, ST1, and ST64 were recovered from PIF and an infant formula production factory. Although the genetic basis of *Cronobacter* virulence has yet to be established for different clinical presentations, certain *Cronobacter* sequence types have been found to be associated with particular infections: *C. sakazakii* CC4 is strongly associated with cases of neonatal meningitis, *C. sakazakii* ST12 with necrotizing enterocolitis, and *C. malonaticus* CC7 with adult infections (5). Among the 19 sequence types isolated in this study, *C. sakazakii* ST268 differed from ST4 at only one locus (*gltB*, position 144, C or T, respectively) and is therefore within *C. sakazakii* CC4. *C. malonaticus* ST201 is in CC7, since it differed by only one locus (*gltB*, position 256, C or T, respectively) from the ST7 profile. Therefore, MLST not only identified and genotyped isolates but also reflected the potential clinical significance of neonatal infection and enabled accurate source tracking and/or attribution.

The K_a/K_s values (0 to 0.0471) showed that the 7 MLST alleles were in phase-stabilizing or purifying selection, which is consistent with the characteristics of housekeeping genes. The strong clonality within the *C. sakazakii* and *C. malonaticus* species, as given by the stability of the clonal group equivalents using 7-locus MLST, 54-locus ribosomal MLST, and 1,865-locus core genome MLST (5), should be noted.

Although *ompA* and *rpoB* sequence analyses have been used for identification purposes, neither method gave greater discrimination between strains than either MLST or PFGE. Typing of strains according to their O antigen was commonly used for bacterial pathogens such as *Salmonella* spp., *Escherichia coli*, and *Listeria monocytogenes* before DNA-sequencing methods became more accessible. Though of interest due to the considerable knowledge of the O antigen in other members of the *Enterobacteriaceae*, O-antigen serotyping of *Cronobacter* spp. has revealed only 17 distinguishing profiles across the seven species in the genus (33). The method also requires the species of the isolates to be identified before serotyping, due to the overlap of serotypes across different *Cronobacter* species (33, 34). *C. sakazakii* serotype O2 is the primary serotype of isolates from powdered infant formula from Chinese retail markets, and this serotype often corresponds with *C. sakazakii* ST4, which causes neonatal meningitis (15, 21). However, *C. sakazakii* serotype O2 is also found to comprise several sequence types that are not yet of such clinical importance. In this study, these sequence types were ST17, ST21, ST31, ST50, ST64, ST261, and ST268. This broad range of sequence types is also shown in the *Cronobacter* MLST databases, where 28 sequence types are given as *C. sakazakii* serotype O2. The O antigen does not follow the phylogeny of the *Cronobacter* genus; the DNA sequence for the same serotype occurs in more than one *Cronobacter* species, and even in *E. coli* O29 and O103 (34).

Tracking the sources of *Cronobacter* strains can reveal the possible intrinsic contamination routes in the production of PIF, enabling the reduction of contamination with *Cronobacter* spp. Craven et al. investigated the *Cronobacter* contamination of five Australian milk powder factories by PFGE. They suggested that *Cronobacter* strains are spread in the milk powder production environment by the movements of air, milk powder dust particles, and personnel (11). Sonbol et al. further studied the *Cronobacter* strains from the study of Craven et al. by MLST profiling. They found that *C. sakazakii* ST4 was present in tanker bays, factory roofs, the milk powder processing environment, and the outside grounds of five milk powder factories in a 1-year period (22). In a survey of PIF and follow-up formulas (FUF), it was reported that 49 of 399 samples were contaminated with *Cronobacter* spp. (36). The authors speculated that nutrient addition during PIF and FUF production increased the risk of intrinsic product contamination. However, in our results, the strains isolated from the raw materials and nutrients were not found in the finished products. Instead, the spray-drying, fluidized-bed, and packing areas were regarded as the major contamination sites. These results indicate that these areas of the plant should be considered the higher-risk processing areas and should be subjected to enhanced surveillance activity.

Dust particles in the air of a manufacturing plant can be a vector of *Cronobacter* dispersal, and higher concentrations are found during bagging and the final packing of the PIF (37, 38). Thus, during PIF production, especially in the spray-drying, fluidized-bed, and packing areas, once the air is contaminated with *Cronobacter* spp., the strains may have an opportunity to contaminate the final product. To reduce contamination by *Cronobacter* spp. in the production of PIF, some measures should be taken, such as keeping the air humidity low, reducing the number of dust particles in the air, cleaning production equipment frequently, and treating waste powder effectively. In addition, the population of airborne microorganisms was closely related to the climate and was higher in the winter than in the summer (38). Finally, it should be noted that contamination of powdered infant formula can also occur due to extrinsic contamination from the preparation equipment and personnel (3, 19).

This study has improved our understanding of the genetic diversity of *Cronobacter* spp. isolated from PIF and the production environment of PIF in China and has provided guidance for reducing *Cronobacter* contamination in the production of PIF.

Fei et al.

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