Classification and fatty acid composition analysis of *Cronobacter* spp. isolated from powdered infant formula in China

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Abstract This study aimed to classify a collection of *Enterobacter sakazakii* (*E. sakazakii*) strains previously identified from powdered infant formula (PIF) to species level by *recN* gene sequencing and biochemical testing to determine the distribution of *Cronobacter* species in China and investigate the strain diversity by cellular fatty acid (CFA) analysis. Of 24 *E. sakazakii* isolates, 23 were identified as *C. sakazakii* and one was *C. malonaticus*. The 23 *C. sakazakii* isolates showed the same CFA profiles. The *C. malonaticus* isolate was discriminated from the *C. sakazakii* isolates by the significant difference in the amounts of C_{12:0}, C_{14:0}, and C_{17:0 cyclo} acids. These results showed that *C. sakazakii* and *C. malonaticus* were the common *Cronobacter* species distributed in PIF in China and that the isolates of the two species exhibited different CFA profiles. These findings are of value for epidemiological investigations and provide an alternative method for confirming various *Cronobacter* spp.

Keywords: *Cronobacter*, powdered infant formula, classification, cellular fatty acids

Introduction

Enterobacter sakazakii (*E. sakazakii*) is an important pathogen that is commonly found in powdered infant formula (PIF) and causes severe illnesses in neonates (1–4). Recent taxonomic work shows that the *E. sakazakii* strains have been defined as a new genus (*Cronobacter*) and that they comprises seven species: *C. sakazakii*, *C. malonaticus*, *C. muytjensii*, *C. dublinensis*, *C. turicensis*, *C. universalis*, and *C. condimenti* (5–6). Therefore, all isolates of the *E. sakazakii*-*Cronobacter* complex should be identified precisely to species level and be characterized in more detail. However, the current methods and systems used for *Cronobacter* identification fail identification at species level, and the species often share overlapping phenotypic characteristics (7). Thus, reclassification of isolates, especially, the discrimination between *C. sakazakii* and *C. malonaticus*, is difficult (8). Currently, several molecular typing approaches, including ribotyping, 16S rRNA gene sequencing, pulsed-field gel electrophoresis, and multiple-locus variable-number tandem-repeat analysis combined with biochemical differentiation tests (7–11), have been applied to characterize and discriminate *Cronobacter* isolates.

Except for phenotypic and genotypic approaches, chemotaxonomic methods such as those involving cellular fatty acid (CFA) have been developed and possesses highly discriminatory properties for bacterial

identification. CFA analysis by using gas chromatography (Sherlock Microbial Identification System (MIS); MIDI Inc., Newark, DE, USA) is a widely available method that has been successfully used to characterize numerous bacterial pathogens (12,13). This approach has already been used to differentiate *E. sakazakii* from the closely related species of *Enterobacter* (14,15). The taxonomic relationship among members of *Cronobacter* genus continues to be validated, however, this method has not yet been tested on the seven *Cronobacter* species.

This study aimed to classify a collection of *Cronobacter* strains that were previously identified as *E. sakazakii* from PIF samples to species level by phylogenetic analysis based on *recN* gene sequencing and biochemical testing to determine the distribution of *Cronobacter* species in China and then investigate the strain diversity by CFA analysis.

Materials and Methods

Bacterial strains In this study, we studied a collection of 24 isolates originating from 530 PIF samples purchased from 54 different types of markets in 12 provinces (20 cities) in China from January to December 2011. The isolates were previously identified as *E. sakazakii*

Fig. 1. Phylogenetic tree based on *recN* gene sequence.

by using the ISO/TS22964 standard method for the detection of *E. sakazakii* in PIF (16), with slight modification. In brief, 100 g of each sample was incubated into 900 mL modified lauryl sulfate tryptone broth (Huankai, Guangzhou, China) at 44°C for 24 h, of which 0.5 mL was added to 5 mL brain-heart infusion broth (Huankai) and cultured for another 4 h at 37°C. Moreover, 10 µL of selective enrichment was then streaked onto a chromogenic medium, Druggan Forsythe 2 (DFI) agar (Huankai, Guangzhou, China). Presumptive positive isolates producing blue-green colonies were identified using the API20E test (bioMérieux, Marcy l'étoile, France). Then, the isolates were further confirmed by duplex PCR combining ITS-SG with *ompA* gene (17).

***recN* sequencing and phylogenetic analysis** The *recN* gene sequence was determined using forward primer Es-L (5'-CTGGCACAATTAACCAT CAGYAA-3') and reverse primer Es-R (5'-TGGGTAACGCACATCACCTGA GT-3') described by El-Sharoud *et al.* (7). Sequencing was performed

by the Beijing Genomics Institute (BGI)-Shenzhen, Shenzhen, China. The phylogenetic tree was created using MEGA 5.10 (mode: nucleotide; maximum composite likelihood) that tested bootstrap consensus (replications: 1000, random seed: 64238, cut-off value condensed tree: 95%).

Biochemical testing All 24 *Cronobacter* isolates were differentiated using important biochemical tests for the differentiation of *Cronobacter* spp., including indole production, malonate utilization, and acid production from carbohydrate (dulcitol) (11). In brief, strains were incubated in malonate broth (Huankai) to determine malonate utilization and incubated in dulcitol broth (Huankai) to test acid production from dulcitol. Indole production was measured by the addition of Kovacs' indole reagent (Huankai) after growth in peptone water (Huankai) without indole. All broths were incubated at 37°C for 24 h.

CFA analysis Strains were cultivated on plates of tryptic soy agar (BD, Sparks, MD, USA) at 37°C for 8 h. Lysis of cells, saponification, methylation of fatty acids, and extraction of fatty acid methyl esters were performed according to the manufacturer's instruction (13). Subsequently, fatty acid methyl esters were analyzed in an Agilent 7890A gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) by using the CLIN6 method of the MIS. Operating conditions were as follows: initial temperature was 170°C, which was increased at a rate of 5°C/min to 260°C, increased to 310°C at 40°C/min, and held for 1.5 min.

Statistical analysis All data were expressed as means±standard deviation. The differences between groups were determined by analysis of variance followed by one-way least significant difference tests. Statistical differences were considered significant at $p<0.05$. SPSS software version 21.0 was used for all analyses (SPSS Inc., Chicago, IL, USA).

Results and Discussion

Sequences of the *recN* genes of the 24 isolates and related sequences from gene bank were used to construct the phylogenetic tree, as shown in Fig. 1. *recN* gene sequencing revealed that isolate 07CES199 was affiliated to *C. malonaticus*, showing more than 98% sequence similarity to the type strain of *C. malonaticus* 18702. The remaining 23 isolates were clustered with the type strains of *C. sakazakii*.

Biochemical tests were performed to distinguish the phenotypes of the *Cronobacter* isolates and confirm the speciation. Biochemical tests further confirmed that isolate 07CES199 was *C. malonaticus*, and the remaining 23 isolates were *C. sakazakii* (Table 1).

The identification obtained by *recN* gene sequencing was in good agreement with biochemical differentiation tests. Among the 24 isolates, two *Cronobacter* species, namely, *C. sakazakii* and *C. malonaticus*, were identified. These species were recognized as the common species distributed in the contaminated PIF in China. Moreover, 96.0% were *C. sakazakii*, and 4.0% were *C. malonaticus*, confirming the results found by Mullane *et al.* (18) who stated that a higher number of *C. sakazakii* strains is distributed in PIF products.

The major fatty acids in 24 *Cronobacter* isolates were straight-chain saturated (12:0, 14:0, and 16:0), unsaturated (16:1 and 18:1), and 17:0 *cyclo*. Virtually, the same CFA profiles were observed in the 23 isolates of *C. sakazakii*, and the summary of fatty acid percentages is shown in Table 2. The isolate 07CES199 (Fig. 2) shared the main characteristics in fatty acids with the 23 isolates of *C. sakazakii*. However, significant differences of fatty acid percentages in $C_{12:0}$, $C_{14:0}$ ($p<0.01$), and $C_{17:0 \text{ cyclo}}$ acids ($p<0.05$) were detected when compared with *C. sakazakii* 07CES458 (Fig. 3) (Table 3). CFA analysis confirmed the views that isolate 07CES199 differed from the remaining 23 isolates, as was in agreement with the *recN* sequencing

Table 1. Biochemical identification profiles of *Cronobacter* isolates

Isolate	Species	Dulcitol	Indole	Malonate
07CES007	<i>C. sakazakii</i>	-	-	-
07CES012	<i>C. sakazakii</i>	-	-	-
07CES107	<i>C. sakazakii</i>	-	-	-
07CES199	<i>C. malonaticus</i>	-	-	+
07CES249	<i>C. sakazakii</i>	-	-	-
07CES431	<i>C. sakazakii</i>	-	-	-
07CES458	<i>C. sakazakii</i>	-	-	-
07CES460	<i>C. sakazakii</i>	-	-	-
07CES465	<i>C. sakazakii</i>	-	-	-
07CES466	<i>C. sakazakii</i>	-	-	-
07CES467	<i>C. sakazakii</i>	-	-	-
07CES468	<i>C. sakazakii</i>	-	-	-
07CES469	<i>C. sakazakii</i>	-	-	-
07CES470	<i>C. sakazakii</i>	-	-	-
07CES474	<i>C. sakazakii</i>	-	-	-
07CES476	<i>C. sakazakii</i>	-	-	-
07CES487	<i>C. sakazakii</i>	-	-	-
07CES491	<i>C. sakazakii</i>	-	-	-
07CES492	<i>C. sakazakii</i>	-	-	-
07CES493	<i>C. sakazakii</i>	-	-	-
07CES494	<i>C. sakazakii</i>	-	-	-
07CES495	<i>C. sakazakii</i>	-	-	-
07CES496	<i>C. sakazakii</i>	-	-	-
07CES501	<i>C. sakazakii</i>	-	-	-

Table 2. Summary of fatty acid percentages for 23 isolates of *C. sakazakii*

Cellular fatty acid	Fatty acid percentages ¹⁾
12:0	1.35±0.07
14:0	9.26±0.23
16:1 ω5c	0.14±0.02
16:0	32.29±0.97
17:0 <i>cyclo</i>	0.76±0.32
17:0	0.10±0.01
18:0	0.55±0.20
Summed 16:1 <i>iso</i> l/14:0 3OH	8.30±0.39
Summed 16:1 ω7c/16:1 ω6c	22.54±1.45
Summed 18:1 ω7c	24.68±1.16
Similarity index	0.92±0.04

¹⁾Values are means±standard deviation ($n=23$).

and biochemical testing results.

In this study, the percentages and unique fatty acids in the 23 strains of *C. sakazakii* are similar to previous reports with *E. sakazakii* (14,15). However, to the best of our knowledge, the CFA profiles for various *Cronobacter* species have never been reported. A study by Hoffmann *et al.* (14) also reported that two isolates (2328, 2329) out of 30 *E. sakazakii* isolates present different CFA profiles. The CFA profiles of the two isolates (2328, 2329) were identical with those of the *C. malonaticus* 07CES199 isolate in our study. Therefore, the CFA profiles may serve as a characteristic feature for discrimination between *C. sakazakii* and *C. malonaticus*.

In conclusion, *C. sakazakii* and *C. malonaticus* were the common

Fig. 2. Cellular fatty acid chromatogram for a food strain of *C. malonaticus* (isolate 07CES199).

Fig. 3. Cellular fatty acid chromatogram for a food strain of *C. sakazakii* (isolate 07CES458).

Cronobacter species distributed in the contaminated PIF in China. The recovered *C. sakazakii* and *C. malonaticus* isolates exhibited

Table 3. Comparison of cellular fatty acid profiles for *C. sakazakii* and *C. malonaticus* isolates

Cellular fatty acid	<i>C. sakazakii</i> (isolate 07CES458)	<i>C. malonaticus</i> (isolate 07CES199)
12:0	1.28±0.06 ¹⁾	3.13±0.17**
14:0	9.22±1.19	7.21±0.15**
16:1 ω 5c	0.14±0.02	0.09±0.08
16:0	31.64±1.55	32.78±0.98
17:0 cyclo	0.62±0.26	1.32±0.21*
18:0	0.48±0.06	0.45±0.01
Summed 16:1 iso l/14:0 3OH	8.24±0.13	8.68±0.30
Summed 16:1 ω 7c/16:1 ω 6c	22.54±1.05	21.61±0.72
Summed 18:1 ω 7c	25.73±1.09	24.60±0.13

¹⁾Values are means±standard deviation ($n=3$). * $p<0.05$, ** $p<0.01$ vs. *C. sakazakii* 07CES458.

different CFA profiles. This study was the first to report on the CFA profiles of various *Cronobacter* species, namely, *C. sakazakii* and *C. malonaticus*. These findings may lead to a greater understanding of prevalence, strain types, and characteristics of *Cronobacter* spp. in PIF in China and provide an alternative method for confirming various *Cronobacter* spp.

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Disclosure The authors declare no conflict of interest.

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