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Differentiation of *Enterobacter sakazakii* from Closely Related *Enterobacter* and *Citrobacter* Species Using Fatty Acid Profiles

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Capillary gas chromatography with flame ionization detection (GC-FID) was used to determine the cellular fatty acid (CFA) profiles of 134 *Enterobacter sakazakii* strains, and these were compared to the CFA profiles of other closely related *Enterobacter* and *Citrobacter* species. For GC-FID analysis, whole cell fatty acid methyl esters (FAMEs) from cells cultured on brain heart infusion (BHI) agar at 37 °C for 24 h were obtained by saponification, methylation, and extraction into hexane/methyl *tert*-butyl ether. A database for *E. sakazakii* was prepared using fatty acid profiles from the 134 strains. Major fatty acids of *E. sakazakii* strains evaluated in this study were straight-chain 12:0, 14:0, and 16:0, unsaturated 18:1 ω7c, and 17:0 ωcyclo 7−8. Principal component analysis (PCA) based on CFA profiles for *E. sakazakii* strains shows separation of *E. sakazakii* subgroups A and B. The CFA profiles for *E. sakazakii* and *Enterobacter cloacae* show that there are several fatty acids, 14:0, 17:0 ωcyclo 7−8, 18:1 ω7c, and summed 16:1 ω6c/16:1 ω7c, that differ significantly between these two species. A PCA model based on CFA profiles for *E. sakazakii* strains clearly shows separation of *E. sakazakii* from closely related *Enterobacter* and *Citrobacter* species. Analysis of FAMEs from *E. sakazakii* strains grown on BHI agar by a rapid GC-FID method can provide a sensitive procedure for the identification of this organism, and this analytical method provides a confirmatory procedure for the differentiation of *E. sakazakii* strains from closely related *Enterobacter* and *Citrobacter* species.

KEYWORDS: Gas chromatography; fatty acids; *Enterobacter sakazakii*

INTRODUCTION

Enterobacter sakazakii is a Gram-negative, rod-shaped pathogen that has been implicated in foodborne diseases. In 1929, Pangalos reported that a yellow-pigmented coliform was the cause of septicemia in an infant (*1*). Subsequently, Urmenyi and Franklin (1961) reported two cases of terminal neonatal meningitis that occurred in 1958 during an outbreak in England, and Joker et al. (1965) in Denmark reported an infection caused by *E. sakazakii* in a child who survived meningitis but developed severe mental and neurological impairment (*1*−*3*). In the later reports, the pathogen was described as a yellow-pigmented *Enterobacter cloacae*. It was not until 1977 that *E. sakazakii* was distinguished from *E. cloacae* (*4*). *E. sakazakii* has emerged as a rare cause of life-threatening neonatal meningitis, necrotizing enterocolitis, and septicemia (*5*). The bacterium has been detected in dry infant milk formula products and, with improper handling, has been implicated in several clinical investigations. Clark et al. (*6*) in 1990 were the first to correlate *E. sakazakii* isolated from two neonatal patients and dried infant formula by epidemiologic typing. *E. sakazakii* infections are rarely reported but have been implicated in causing illness in neonates and children aged 3 days to 4 years with at least 76 cases of infections and 19 deaths in infants and children (*7*, *8*). The organism has been detected in either prepared formula, the environment in which it was prepared, or unopened products. The first report in the United States of *E. sakazakii* infection associated with infant formula was in 2001, prompting recall of a commercial product (*9*). A letter was sent by the U.S. Food and Drug Administration (FDA) informing health care professionals that *E. sakazakii* infections are associated with the use of milk-based powdered infant formulas (*10*).

In this study, a rapid GC-FID method was used to determine if it could identify *E. sakazakii* strains from food, the environ-

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Table 1. Bacterial Agents Analyzed by GC

microorganism	designation	source of strain	ATCC no.
Enterobacter sakazakii	613	food	
	1424	clinical	29544
	1425	unknown	51329
	1426–1428	environmental	
	1429	clinical	
	1430	unknown	
	1431	clinical	
	1432	unknown	
	1433	clinical	
	1434	unknown	
	1435	food	
	1445	clinical	
	1447	clinical	
	1449	clinical	
	1508–1509	environmental	
	1511–1517	environmental	
	1519–1520	environmental	
	1523–1531	environmental	
	1533–1534	environmental	
	1536	food	
	1537	clinical	
	1538	food	
	1542	clinical	
	1543	unknown	
	1545	unknown	
	1547–1561	unknown	
	1563–1565	clinical	
	1566	environmental	
	1568–1569	food	
	1570	clinical	
	1571	food	
	1573	food	
	1578	food	
	1584	clinical	29004
	1585–1586	unknown	2868
	1587–1591	nonclinical[a]	
	1593–1595	nonclinical	
	1596	environmental	
	1597–1598	nonclinical	
	1600	nonclinical	
	1602–1605	nonclinical	
	1632	unknown	
	1638	unknown	
	1640	unknown	
	1642–1645	unknown	
	1662–1665	food	
	1666	environmental	
	1668–1687	food	
	1891–1894	unknown	
	2148–2157	clinical	
Enterobacter sakazakii that tested as *Enterobacter cloacae*	1532	environmental	
	1544	environmental	
	1667	environmental	
	1895	unknown	
Enterobacter cloacae	1437	food	
	1646	unknown	
	2140	unknown	35030
	2141	clinical	13047
	2142	clinical	49141
Enterobacter agglomerans	2134	food	49008
Enterobacter intermedius	2135	environmental	33110
Citrobacter freundii	2136	unknown	43864
Enterobacter amnigenus	2137	unknown	33731
Enterobacter aerogenes	2138	clinical	13048
Enterobacter asburiae	2139	clinical	35956
Enterobacter gergoviae	2143	clinical	33028
Enterobacter hormaechei	2144	clinical	49163
Citrobacter koseri	2145	clinical	25408
Enterobacter dissolvens	2146	food	23373
Enterobacter cancerogenus	2147	clinical	35314

[a] Nonclinical is an unknown food or environmental source.

Table 2. Comparison of Cellular Fatty Acid Profiles for *E. sakazakii* and *E. cloacae*[a]

cellular fatty acid	*E. sakazakii* (%)	*E. cloacae* (%)	*P* value
12:0	1.58 ± 0.53	2.66 ± 1.34	<0.001
13:0	0.00 ± 0.00	0.35 ± 0.31	<0.001
unknown 13.951	0.04 ± 0.14	0.20 ± 0.30	0.003
14:0	9.61 ± 0.78	7.82 ± 1.61	<0.001
unknown 14.502	0.82 ± 0.19	0.94 ± 0.18	0.052
14:0 2OH	0.00 ± 0.00	0.25 ± 0.50	<0.001
15:0	0.09 ± 0.17	1.03 ± 1.38	<0.001
16:1 ω5c	0.04 ± 0.06	0.07 ± 0.07	0.085
16:0	31.21 ± 1.85	30.55 ± 2.54	0.310
15:0 3 OH	0.00 ± 0.00	0.07 ± 0.09	<0.001
17:1 ω8c	0.00 ± 0.04	0.14 ± 0.26	<0.001
17:0 ω cyclo 7–8	3.74 ± 1.59	11.70 ± 5.20	<0.001
17:0	0.18 ± 0.22	2.05 ± 1.55	<0.001
18:1 ω7c	24.02 ± 2.09	16.30 ± 2.44	<0.001
18:0	0.45 ± 0.14	0.50 ± 0.10	0.216
19:0 cyclo ω8c	0.49 ± 0.36	1.60 ± 1.30	<0.001
19:0 iso	0.00 ± 0.00	0.08 ± 0.17	<0.001
19:0	0.02 ± 0.05	0.08 ± 0.09	0.001
summed 13:0 3OH/15:1 iso	0.00 ± 0.00	0.21 ± 0.42	<0.001
summed 14:0 3OH/16:1 iso	9.01 ± 0.95	8.67 ± 0.86	0.299
summed 16:1 ω6c/16:1 ω7c	18.63 ± 2.19	14.46 ± 6.15	<0.001
similarity index	0.802 ± 0.118	0.743 ± 0.104	0.150
n	134	9	

[a] Values are the mean ± SD.

ment, and clinical samples, and if it can differentiate *E. sakazakii strains* from closely related *Enterobacter* and *Citrobacter* species.

MATERIALS AND METHODS

Bacterial Agents and Growth Conditions. *E. sakazakii* strains from an FDA collection and other closely related *Enterobacter* and *Citrobacter* species that were analyzed are listed in **Table 1**. All bacteria were grown on brain−heart infusion (BHI) agar (Difco, Detroit, MI) and were initiated from frozen stocks. The growth medium was prepared with 52 g of BHI agar/L distilled water, pH 7.0. Twenty milliliters of medium was added to each 100 mm Petri plate. All bacteria were incubated at 37 ± 1 °C for 24 h.

Chemical Procedures and GC Analysis. For gas chromatography with flame ionization detection (GC-FID) analysis, bacterial cells were harvested from the culture plates, and whole cell fatty acid methyl esters (FAMEs) were prepared by saponification, methylation, and extraction into hexane/methyl *tert*-butyl ether. Using a sterile disposable wooden stick, approximately 25 mg of bacterial cells was harvested from the culture plates and placed in sterile 13 × 100 mm tubes. One milliliter of 3.75 N NaOH (1:1, methanol/distilled water) was added to each tube containing the bacteria to saponify the fatty acids. The tubes were heated in a boiling water bath for 5 min, vortexed, heated for an additional 30 min in a boiling water bath, and then cooled in tap water. Two milliliters of 3.25 N HCl (1:1.18, methanol/6 N HCl) was added for methylation of the fatty acids, and the tubes were heated for 10 min at 80 °C. The tubes were cooled, and the FAMEs were extracted by the addition of 1.25 mL of 1:1 hexane/methyl *tert*-butyl ether with gentle tumbling for 10 min. The lower phase was pipetted off, and 3.0 mL of 0.3 N NaOH was added to the organic phase as a base wash and tumbled for an additional 5 min. The organic phase was then removed for GC analysis. The FAMEs were analyzed by GC using the rapid Microbial Identification System (MIS, MIDI Inc., Newark, DE) software (RCLN50) to identify the relative amounts of fatty acids in the bacteria and were expressed as a percentage of the total fatty acids. The GC used was an Agilent 6890 with a flame ionization detector and an Agilent autosampler and injector (Agilent 7683) (Agilent Technologies, Palto Alto, CA). A 25 m (length) × 0.2 mm i.d. × 0.33

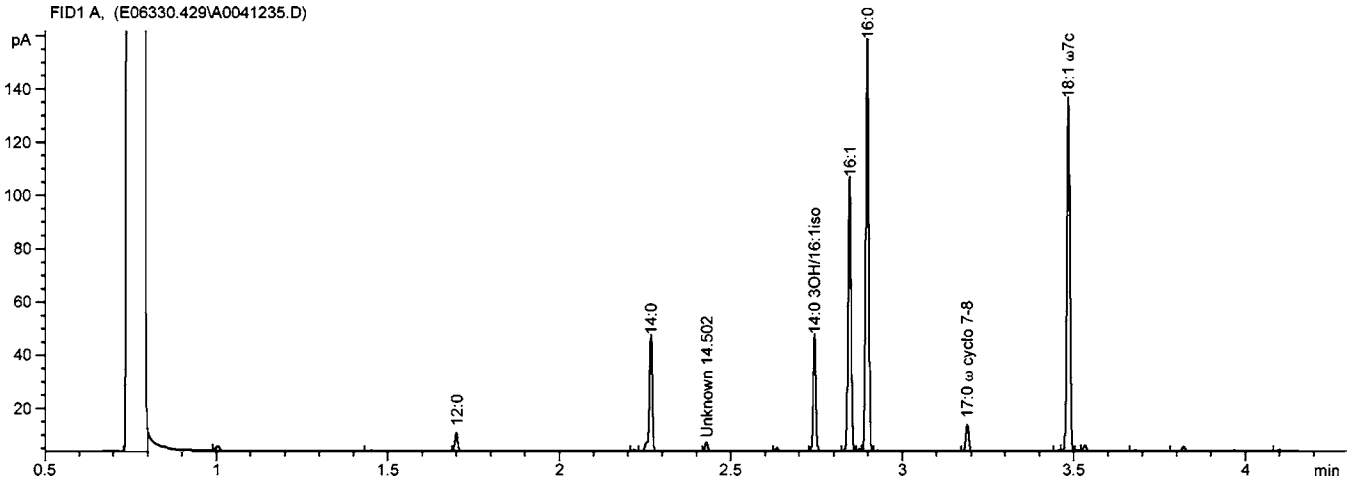

Figure 1. Chromatogram for a clinical strain of *Enterobacter sakazakii* (sample 2157).

Table 3. Comparison of Cellular Fatty Acid Profiles for Various *Enterobacter* Strains

cellular fatty acid	*E. agglomerans* (2134)	*E. intermedius* (2135)	*C. freundii* (2136)	*E. amnigenus* (2137)	*E. aerogenes* (2138)	*E. asburiae* (2139)
10:0	0	0	0	0	0	0.49
11:0	0	0	0	0	0	0.50
12:0	3.89	3.76	3.75	2.88	0.91	0.52
13:0	0	0.58	0.40	0.69	0.16	0.34
unknown 13.951	0.80	0.55	0.49	0.60	0	0
14:0	5.56	6.12	7.58	0.47	10.34	8.82
unknown 14.502	0.81	1.46	1.04	0.61	0.92	0
14:0 2OH	0	0	0	0	0.70	0
15:1 ω8c	0	0.20	0.12	0.17	0.05	0.12
15:0	0.17	4.92	2.99	4.10	1.92	4.44
16:1 ω5c	0.15	0.24	0.24	0.22	0.12	0.33
16:0	32.62	26.53	28.28	24.73	31.47	28.11
15:0 3 OH	0	0.21	0.15	0.17	0.10	0.24
17:1 ω8c	0	0.37	0.34	0.93	0.24	0.43
17:1 ω cyclo 7–8	5.32	2.96	1.39	5.78	10.71	11.15
17:0	0.13	1.49	1.16	2.62	1.25	3.01
18:1 ω7c	9.03	10.23	13.59	11.32	16.78	14.88
18:0	0.34	0.24	0.21	0.59	0.39	0.36
19:0	0	0	0	0.14	0.09	0.14
19:0 cyclo ω8c	0.08	0	0	0	1.76	0.40
summed 13:0 3OH/15:1 iso	0	0	0	0.91	0	0
summed 14:0 3OH/16:1 iso	10.08	9.12	8.55	8.76	8.80	7.49
summed 16:1 ω6c/16:1 ω7c	30.79	30.83	29.72	29.66	12.88	17.91

cellular fatty acid	*E. gergoviae* (2143)	*E. hormaechei* (2144)	*C. koseri* (2145)	*E. dissolvens* (2146)	*E. cancerogenus* (2147)
10:0	0	0	0	0	0.05
11:0	0	0	0	0.07	0.09
12:0	0.54	1.33	2.05	1.55	0.79
13:0	0	1.08	0.30	1.14	3.79
unknown 13.951	0	0	0	0	0.50
14:0	10.65	8.56	9.72	7.50	5.59
unknown 14.502	0.54	0	0.98	0	0
14:0 2OH	0.61	0	0	0.26	0
15:1 ω8c	0	0.27	0.08	0.29	0.16
15:0	0.12	7.26	2.74	7.22	4.68
16:1 ω5c	0.14	0.19	0.21	0.19	0.14
16:0	36.28	21.72	28.09	23.12	0.26
15:0 3 OH	0	0.34	0.15	0.37	0.26
17:1 ω8c	0	0.91	0.39	1.24	0.58
17:1 ω cyclo 7–8	18.06	7.35	2.73	9.69	14.20
17:0	0	3.21	1.33	4.15	3.46
18:1 ω7c	15.67	17.39	18.10	14.80	12.49
18:0	0.42	0.29	0.24	0.29	0.57
19:0	0.25	0.15	0	0.13	0.17
19:0 cyclo ω8c	2.07	0.26	0	0.26	0.91
summed 13:0 3OH/15:1 iso	0	2.04	0	1.84	1.47
summed 14:0 3OH/16:1 iso	6.15	8.67	7.75	7.40	8.59
summed 15:0 iso 2OH/16:1 ω7c	8.01	18.63	25.05	18.11	14.06

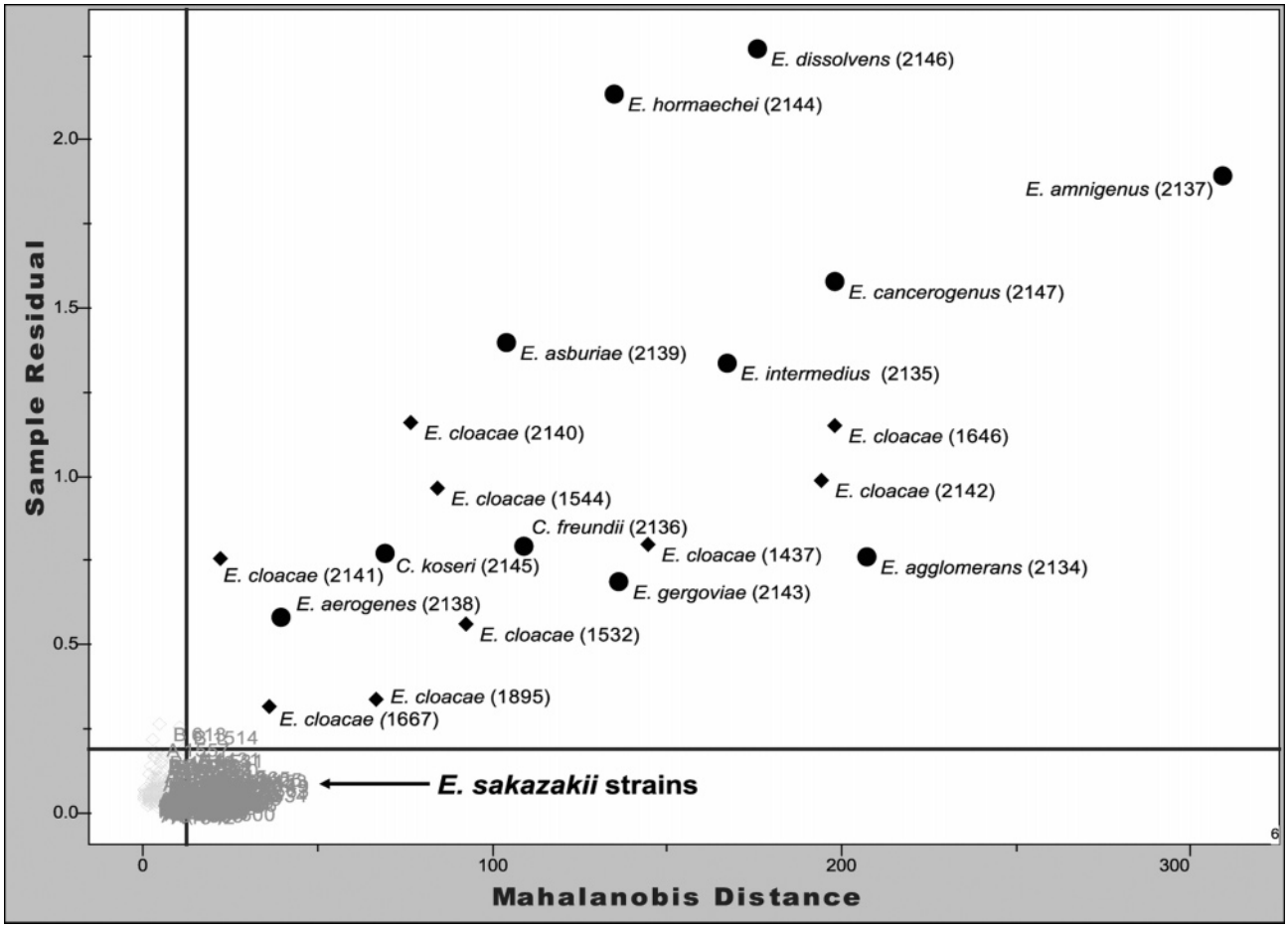

Figure 2. PCA model based on cellular fatty acid profiles for *Enterobacter sakazakii* strains. Outlier detection demonstrates clear separation of *E. sakazakii* from closely related *Enterobacter* and *Citrobacter* species.

μm film thickness, cross-linked 5% phenylmethyl silicone fused silica capillary column (Agilent 19091B-102) was used to separate the fatty acids. Operating conditions were as follows: Initial temperature was 170 °C and was increased at a rate of 28 °C/min to 288 °C and then increased to 310 °C at 60 °C/min and held for 1.25 min. Hydrogen was used as the carrier gas at a constant flow rate of 1.3 mL/min. A calibration analysis was used for the first two injections of every sequence and was automatically reanalyzed after every 11th sample injection using a calibration standard (no. 1300-AA; Microbial ID, Inc., Newark, DE). The similarity index (SI) is a numerical value that expresses how closely the fatty acid composition of an unknown compares with the mean fatty acid composition of the strains used to create the library entry. As each fatty acid varies from the mean percentage, the SI will decrease in proportion to the cumulative variance between the composition of the unknown and the library entry (*11*). Samples with a similarity of 0.500 or higher with a separation of 0.100 between the first and second choices are considered to be good library comparisons.

Statistical Analysis. Differences in fatty acids among bacterial strains were assessed by analysis of variance (ANOVA) (*12*). Values are expressed as means with their standard deviations.

Data analysis and determination of clustering were performed by principal component analysis (PCA) and hierarchical clustering analysis (HCA) using Pirouette 3.11 software (InfoMetrix, Bothell, WA). Each sample represents a single point in the multivariate space defined by the set of independent axes corresponding to each fatty acid concentration.

Clustering in the data is observed using PCA scores plots in which the data for each sample are projected onto a reduced set of principal components (PCs). The data were scaled by mean centering; PCs were computed using the Nipals method (*Pirouette Manual*).

HCAs were carried out using no data scaling, Euclidian distance metric, and the complete overlap linkage method. Clusters were determined from computed intersample distances and displayed as dendrograms.

RESULTS AND DISCUSSION

GC-FID analysis of chemical components of bacterial cells has provided useful information for the rapid detection and identification of bacteria in clinical and diagnostic bacteriology laboratories and currently has increased significance for both food safety and security (*13*, *14*). In this study, the cellular fatty acid (CFA) profiles of 134 *E. sakazakii* strains were compared to the CFA profiles of other closely related *Enterobacter* and *Citrobacter* species. **Figure 1** is an example of a chromatogram for a clinical strain of *E. sakazakii* (sample 2157).

A database for *E. sakazakii* was prepared using fatty acid profiles from the 134 strains (**Table 1**). The CFA profiles for the 134 strains of *E. sakazakii* are shown in **Table 2**. Major fatty acids are straight-chain saturated (12:0, 14:0, 16:0), unsaturated (18:1 and 16:1), and 17:0 cyclopropane, all indicating a Gram-negative bacteria.

Table 2 compares the CFA profiles for *E. sakazakii* and *E. cloacae*. The CFA profiles for *E. sakazakii* and *E. cloacae* show that there are several fatty acids, 14:0, 17:0 ωcyclo 7−8, 18:1 ω7c, and the summation of 16:1 ω6c/16:1 ω7c, that differ

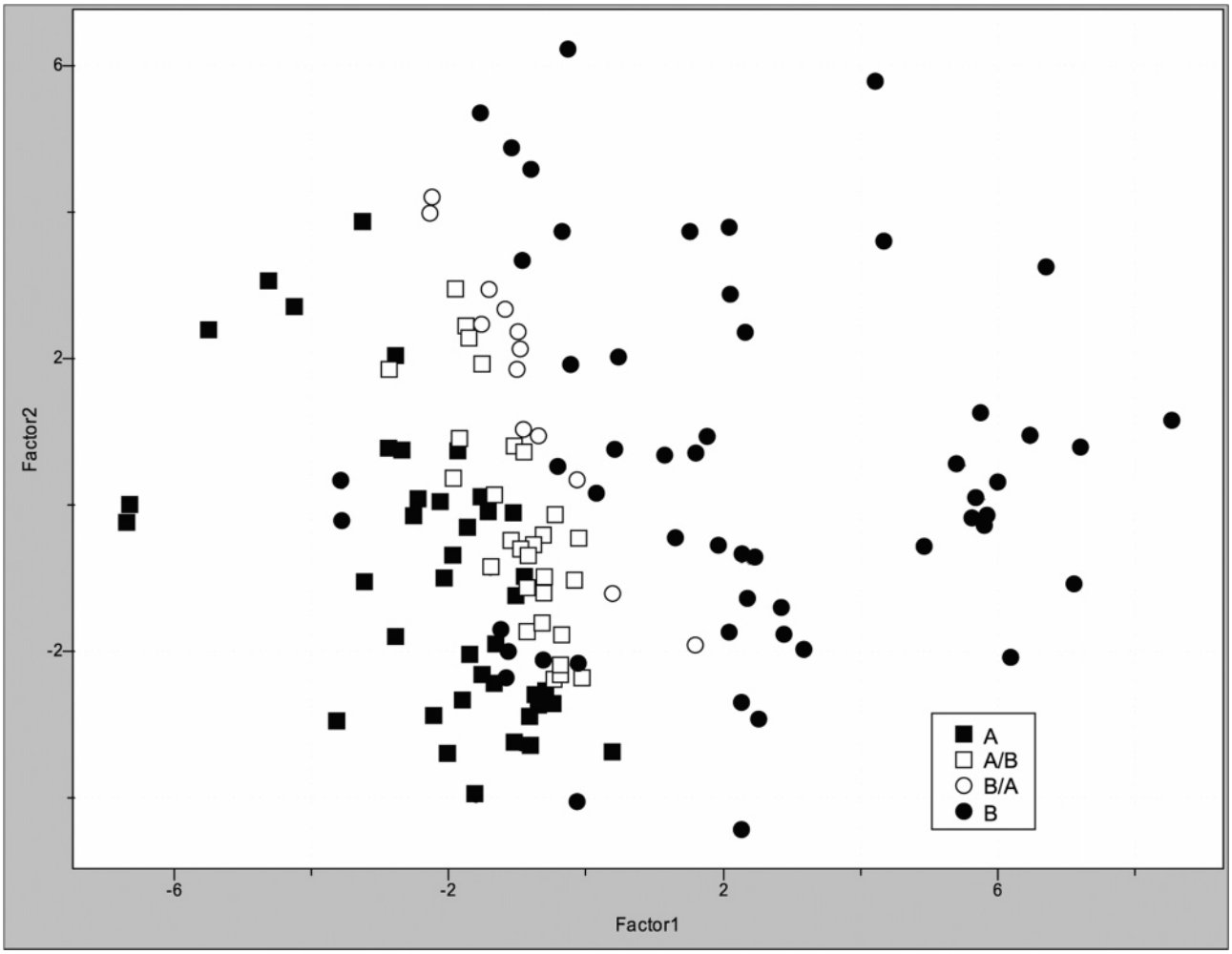

Figure 3. PCA showing a scatter plot of PC1 versus PC2 for *Enterobacter sakazakii* strains classified as A (■), A/B (□), B/A (○), and B (●).

significantly in percentage between these two species (**Table 2**). The mean SI value for the 134 strains of *E. sakazakii* was 0.802 and that for the 9 strains of *E. cloacae* was 0.743 (**Table 2**).

For evaluating fatty acid data for *E. sakazakii* strains, *Enterobacter* species, and *Citrobacter* species PCA was used (**Table 3**). A PCA model based on CFA profiles for *E. sakazakii* strains clearly shows separation of *E. sakazakii* from closely related *Enterobacter* and *Citrobacter* species (**Figure 2**).

A PCA model based on CFA profiles for substrains of *E. sakazakii* was analyzed using a scatter plot of PC1 versus PC2. The subgroups were identified as A and B. Those subgroups with SI values for both A and B and SI value differences of <0.1 were identified as either A/B or B/A, depending on which value was higher. The PCA scatter plot of PC1 versus PC2 for *E. sakazakii* strains classified as A, A/B, B/A, and B is shown in **Figure 3**. A trend indicating class separation is observed on the PC1 axis, but the clusters are not completely separated (**Figure 3**). This was expected as the class boundaries are not distinct and assignments are not exact. However, the trends of A close to A/B and B close to B/A are what would be expected.

A HCA was also performed showing a dendrogram of all *E. sakazakii* strains. The dendrogram shows the separation of the B strains from the A, A/B, and B/A (**Figure 4**). This result is similar to **Figure 2**, indicating that the B category is more widely separated from the others.

Pulsed field gel electrophoresis (PFGE) was also performed on the *E. sakazakii* strains to examine strain to strain relatedness, but no correlation between the PFGE results and the fatty acid profiles was found (*15*). These results suggest that FAME analysis is not as sensitive as PFGE analysis at the strain level, but is a better and faster screening tool at the species level at which it discriminates between closely related species.

The current FDA recommended procedure for the identification of this organism uses standard isolation methods for Enterobacteriacae, with selection of yellow-pigmented organisms, and biochemical identification (*16*). This method is time-consuming and could give false-negative and -positive results (*17*, *18*). A chromogenic agar was developed subsequently by Druggan, Forsythee, and Iversen (DFI agar) in which a chromogenic substrate is used as an indicator of α-glucosidase activity (*19*). Recently, a real-time PCR assay for the specific detection of *E. sakazakii* in infant formula was developed by the FDA using an application of the fluorogenic 5′ nuclease assay (TaqMan) (*20*). To identify the pathogen in powdered milk-based infant formula, a rapid method is being developed that involves pre-enrichment amplification in buffered peptone water for 6 h at 37 °C, centrifugation to concentrate the cell volume, plating on DFI chromogenic agar, overnight incubation at 37 °C, and confirmation with RT-PCR and fatty acid analysis. The real-time PCR and the fatty acid profile assay could be

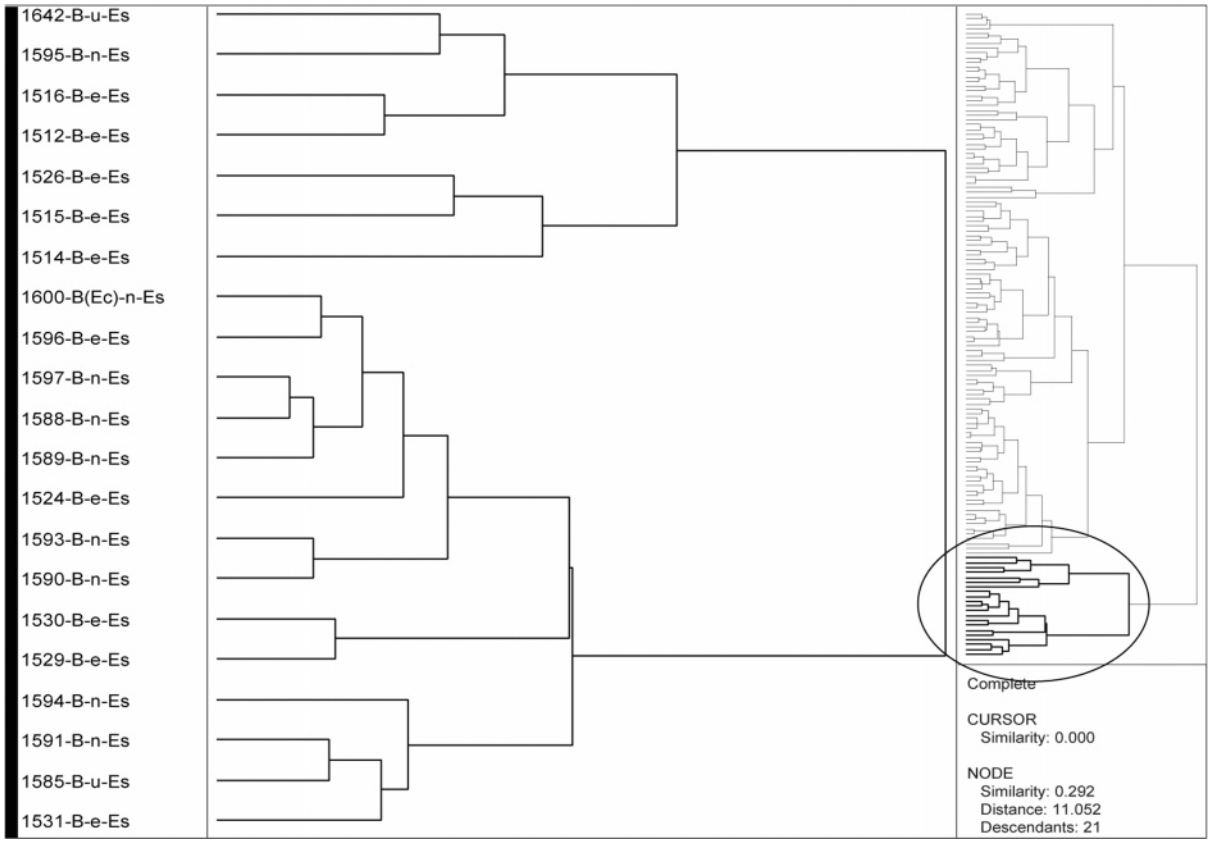

Figure 4. HCA showing a dendrogram of all *Enterobacter sakazakii* strains. The circled area shows clustering of the *E. sakazakii* B strains.

used to rapidly screen infant formula samples for *E. sakazakii* and reduce the time required for the current assay by up to 5 days.

In summary, fatty acids were extracted from whole bacterial cells of *E. sakazakii* strains from a variety of sources, including clinical, food, and environmental and other closely related *Enterobacter* and *Citrobacter* species, and derivatized into methyl esters, and the FAMEs were identified and quantified using GC-FID, which is a rapid and sensitive procedure. The cellular fatty acids can be extracted in approximately 1 h and run on the GC-FID in 5 min. The data in this study show that *E. sakazakii* can be identified after 24 h of growth using BHI agar with an average similarity index of 0.802 for the 134 strains. PCA based on CFA profiles for *E. sakazakii* strains clearly shows separation of *E. sakazakii* from closely related *Enterobacter* and *Citrobacter* species. This study demonstrates the importance of being able to construct a unique library for the identification of *E. sakazakii*, based on type of medium and specific growing conditions. The percentages and unique fatty acids isolated from *E. sakazakii* can provide a sensitive method for identification. This analytical method provides a confirmatory procedure for the differentiation of *E. sakazakii* strains from closely related *Enterobacter* and *Citrobacter* species.

SAFETY

Standard microbiological safety precautions were observed during work with the bacteria strains. Safety glasses, laboratory coats, and disposable gloves were utilized when experimental procedures were performed. The bacteria were completely inactivated using sodium hydroxide that was heated in a boiling water bath before the fatty acid extraction procedure.

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