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Identification and Classification of *Cronobacter* spp. Isolated from Powdered Food in Korea

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***Cronobacter* is a major foodborne pathogen in powdered infant formula and can lead to serious developmental after-effect and death to infants. The contamination of *Cronobacter* may be a high risk for the powdered foods. To isolate and identify *Cronobacter* from the powdered foods such as powdered infant formula and *Saengsik* in Korea, a conventional culture method, rapid identification system, PCR, and 16S rDNA sequencing were performed. As the results of isolation, seven *Cronobacter* spp. were isolated from seven out of 102 powdered infant formulas and 41 *Cronobacter* were isolated from 41 out of 86 *Saengsik*s. Forty-eight *Cronobacter* isolates were identified to be *C. sakazakii* and *C. dublenisis* by 16S rDNA sequence analysis. Most of the isolates were *C. sakazakii* and 13% of the isolates were *C. dublinesis*. One fourth of the *C. sakazakii* isolates showed different biochemical characteristics of negative nitrate reduction and nonmotility activities compared with the other strains reported previously.**

Keywords: *Cronobacter*, identification, classification, Korean powdered foods

Cronobacter has been previously known as *Enterobacter sakazakii. Cronobacter* are motile peritrichous, Gram-negative, rod-shaped, non-spore-forming bacteria. This genus contains the species of *C. sakazakii*, *C. malonaticus*, *C. muytjensii*, *C. dublinensis, C. turicensis,* and *Cronobacter* genomospecies I by Iversen's classification based on the biogroups [10]. *Cronobacter* are divided into 16 biogroups according to biochemical characteristics [6, 11, 21]. The phenotypic characteristics of *Cronobacter* from other common Enterobacteriaceae genera [7, 13] are differentiated. Moreover, 16S rDNA analysis divided *Cronobacter* strains into four clusters [8]. *Cronobacter* is recognized worldwide as an emerging foodborne pathogen. Infectious diseases by *Cronobacter* are very important causes of meningitis, bacteremia, necrotizing enterocolitis, and septicemia in infants and neonates [14, 23, 28, 30]. *Cronobacter* is distributed and frequently contaminated in the environment [21], plant materials [15], powdered infant formulas [1, 2], cereal foods [3], fermented beverages [7], fruits, and vegetables [15]. In particular, contamination on powdered infant formula occurs more easily because it is a nonsterilized product. Contamination is caused by poor handling and added raw ingredients during processing, drying, and packaging [5, 17, 21]. In fact, according to report from Jung and Park [15], 20% of powdered infant formulas have been suggested to be contaminated with *Cronobacter* in Korea. Muytjens *et al*. [21] analyzed 141 powdered infant formula samples from 35 countries and reported that 14.2% of the samples were contaminated by *Cronobacter*. Other investigators also reported that 5~20% of samples were contaminated with *Cronobacter* in powdered infant formulas or related products [3, 10, 21, 24–27]. Therefore, *Cronobacter* was isolated from powdered infant formulas and *Saengsik*s and identified by PCR. *Saengsik* consists of raw grains, fruits, and vegetables pulverized after freeze-drying. *Cronobacter* isolates in Korea have been classified by biochemical properties into biogroups [6, 11] and by systematic homology of 16S rRNA sequence into genotypes of Cluster I~IV [8, 12].

Cronobacter was isolated according to the modified BAM of the U.S. FDA. To isolate *Cronobacter* from 102 powdered infant formulas and 86 *Saengsik*, samples 25-g samples were aseptically taken from each product and homogenized in a stomacher after mixing with 225 ml of buffered peptone water (Difco Laboratory, Detroit, MI, U.S.A.). The buffered peptone watersample mixture was incubated at 37°C, overnight, as a pre-enrichment step. After incubation, a 10-ml aliquot was transferred to 90 ml

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Table 1. Oligonucleotide primer pairs for detecting *Cronobacter* spp.

Gene	Primer	Sequence (5'–3')	Reference
ompA	OmpA1	GGATTTAACCGTTTCC	Mohan Mair and Venkitanarayanan [19]
	OmpA2	CGCCAGCGATGTTAGAAGA	
16S rRNA	Saka-1	ACAGGGAGCCAGCTTGCTGC	Hassan *et al.* [9]
	Saka-2a	TGCTGCGGTTATTAACCAC	
tRNA–23s rRNA intergenic space	FSFor	ATCTCAAAAMTGACTGTAAAGTCACGTT	Derzelle *et al.* [4]
	EsRevB	CCGAARAAGTMTTCGKGCTGC	

of EE (*Enterobacter sakazakii* enrichment broth; Difco Laboratory, Detroit, MI, U.S.A.) broth, and after a further overnight incubation at 37°C, the broth was streaked on violet red bile glucose agar (VRBGA; Oxoid Ltd., Hampshire, England). The presumptive colonies were picked up to chromogenic *Enterobacter sakazakii* agar (Oxoid Ltd., Hampshire, England). Suspicious colonies were restreaked on tryptic soy agar (Difco Laboratory, Detroit, MI, U.S.A.) and incubations were performed at 37°C for 18 h in the aerobic incubator (Difco Laboratory, Detroit, MI, U.S.A.). To detect *Cronobacter*, PCR was carried out for its identification. For preparation of the PCR template, 1 ml of the overnight culture in TSB was centrifuged at 10,000 ×*g* at 4°C for 5 min and washed two times with the sterile distilled water. DNA was isolated with a DNA tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The primers, shown in Table 1, were targeted for the *ompA* gene, 16S rRNA, and tRNA–23s rRNA intergenic region, and synthesized commercially (Bioneer, Daejon, Korea). The PCR products were analyzed using 1% TAE buffer with 0.5 mg/ml of ethidium bromide. The gel was visualized and photographed under a UV transilluminator (Seolin, Suwon, Korea) after electrophoresis at 5 V/cm. The isolates showed each PCR product, which was the same as *Cronobacter* ATCC51329 (Fig. 1). As the result of the PCR analysis, *Cronobacter* was isolated from seven out of 102 powdered infant formulas, and from 41 out of 86 *Saengsiks*. Iversen *et al*. [11] isolated *Cronobacter* from two out of 82 powdered infant formulas, five out of 49 infant weaning foods, and 40 out of 122 spices. Shaker *et al*. [25] reported that *Cronobacter* was isolated from two of eight follow-up formulas based on wheat. Restaino *et al*. [24] isolated *Cronobacter* from two out of six dried infant formulas, and Chap *et al*. [3] isolated it from three out of 89 follow on formulas and 24 out of 170 other infant foods. Moreover, Park *et al*. (unpublished data) reported *Cronobacter* isolated from 10 out of 175 powdered infant formulas. In this study, *Cronobacter* was detected in seven samples out of 102 powdered infant formulas. Thus, we confirmed a similar contamination ratio of *Cronobacter* from powdered infant formulas. In particular, we isolated 41 *Cronobacter* spp. from 41 (45%) out of 86 *Saengsik* samples and Kim *et al*. [16] reported 17 (47%) *Cronobacter* isolates from *Sunsik*. *Saengsik* is very similar to *Sunsik* by

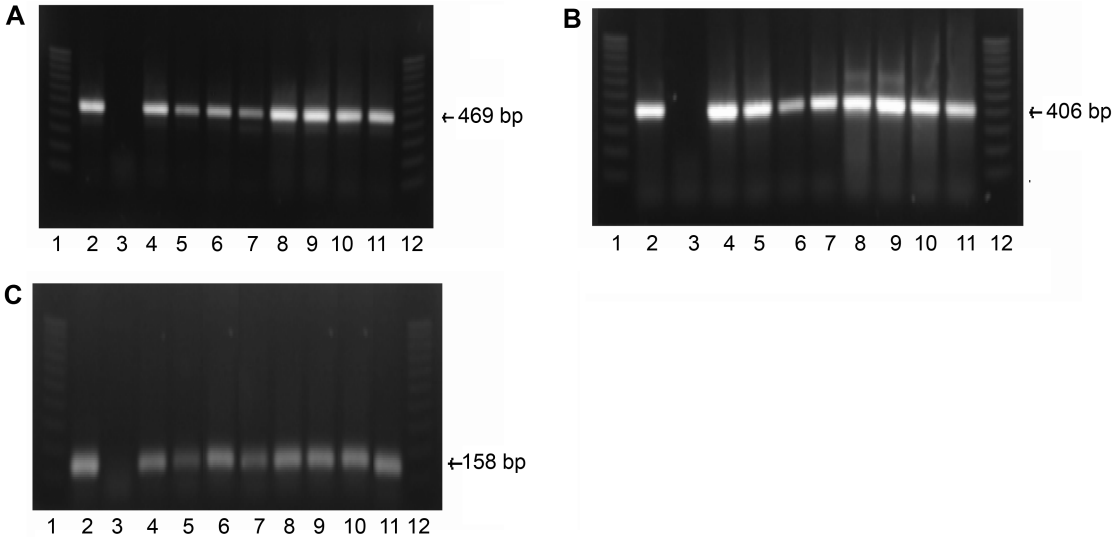

Fig. 1. Detection of *Cronobacter* spp. isolated from powdered foods by PCR.
A. *ompA* gene; **B**. 16S rRNA; **C**. tRNA-23S rRNA intergenic space. Lane; 1, 12 : 100 bp DNA ladder; Lane 2: *C. sakazakii* ATCC 51329; Lane 3: *E. cloacea* KCTC 1949; Lane 4–11: *C. sakazakii* isolated from powdered foods.

Fig. 2. Neighbor-joining tree of *Cronobacter* spp. and related organisms based on partial 16S rDNA sequences.

Cluster I

Biogroup 1: KYU 6, KYU 17, KYU 19, KYU 21, KYU 22, KYU 27, KYU 31, KYU 44, KYU 45. Biogroup 2: KYU 39, KYU 40, KYU 42. Biogroup 3: KYU 18, KYU 23, KYU 34, KYU 46, KYU 49. Biogroup 4: KYU 1, KYU 2, KYU 47, KYU 48. Biogroup 4a: KYU 43. Biogroup 5: KYU 25. Biogroup 5a: KYU 24. Biogroup 8: KYU 41. Biogroup 8a: KYU 51, KYU 53, KYU 54, KYU 59, KYU 60. Biogroup 8b: KYU 58. *New Biogroup 17*: Biogroup 17: KYU 56, KYU 62, KYU 64, KYU 70. Biogroup 17a: KYU 57, KYU 63. Biogroup 17b: KYU 65. Biogroup 17c: KYU 66. Biogroup 17d: KYU 76, KYU 68, KYU 69.

Cluster IV

Biogroup 6: KYU8, KYU 52. Biogroup 12: KYU35, KYU37, KYU38, KYU50.

a simple manufacturing process and food composition such as cereals, vegetables, fruits, and other functional plants. However, *Saengsik* has no heat-treated process for its production. These foods have been taken as a breakfast substitute by many consumers and as health-care foods for healthy or feeble persons [15]. If *Cronobacter* is contaminated in these foods, this pathogen may cause little problem to healthy person, but is very hazardous to the infant and feeble persons. Therefore, it is required to manufacture these food products through more hygienic processes like HACCP and GMP.

These 48 *Cronobacter* isolates were also classified by 16S rDNA sequencing analysis. To analyze the 16S rDNA sequence of about 1.2 kb, *Cronobacter* genomic DNA was used as template for the PCR. The 16S rRNA sequencing reaction was performed in the MJ Research Gradient Cycler using a dye terminator cycle sequencing-ready reaction kit with AmpliTaq DNA polymerase (Applied Biosystems, CA, U.S.A.) following the protocols supplied by the manufacturer. The samples were resuspended in distilled water and subjected to electrophoresis in a sequencer (Applied Biosystems, CA, U.S.A.). All primary readings were edited to remove vector sequences and unreliable data using the program Factura (Perkin Elmer, CT, U.S.A.). Homology searches were performed in a PC using the BLAST. The BLAST programs and the Net blast client are distributed by the NCBI (http://ncbi.nlm.nih.gov). Through the comparisons of the homology, 48 isolates were identified as *Cronobacter*. The 48 *Cronobacter* sp. isolates were grouped into genome clusters by drawing the phylogenetic tree using the DNAstar Lasergene 8.0 software (Fig. 2).

Recently, Iversen *et al*. [10] reclassified *Cronobacter* by molecular biological characteristics such as *C. sakazakii* (genome cluster group 1), *C. turicensis* (genome cluster group 2), *C. muytjensii* (genome cluster group 3), and *C. dublinensis* (genome cluster group 4). In addition, the existence of the sixteenth biogroup has been reported, and a correlation between 16S rRNA gene sequence analysis (which separated *E. sakazakii* strains into several genetic groups) and biogroups has been demonstrated. The existence of these divergent groups seems to support the suggestion of Farmer *et al*. [16] that *E. sakazakii* may harbour different species [5, 12, 13]. Molecular biological methods, including f-AFLP, automated ribotyping, 16S rRNA gene sequencing, and DNA–DNA hybridization, were employed to clarify the taxonomic relationship of *Cronobacter.* As the results of classification by 16S rRNA sequencing, 42 *C. sakazakii* isolates (87.5%) belonged to the cluster I and 6 *C. dublinensis* isolates (12.5%) appertained to cluster 4 (Table 2). Iversen *et al*. [11, 12] reported that *C. sakazakii* in cluster I was the major DNA cluster and our results were also similar. The 16S rRNA sequences of *Cronobacter* isolates were deposited in the GenBank database under accession numbers GU227651 (KYU2), GU227652 (KYU59), GU227653 (KYU52), GU227654 (KYU6), GU227655 (KYU17), GU227656 (KYU19), GU227657 (KYU21), GU227658 (KYU22), GU227659 (KYU27), GU227660 (KYU31), GU227661 (KYU44), GU227662 (KYU45), GU227663 (KYU39), GU227664 (KYU40), GU227665 (KYU42), GU227666 (KYU18), GU227667 (KYU23), GU227668 (KYU34), GU227669 (KYU46), GU227670 (KYU49), GU227671 (KYU1), GU227672 (KYU47), GU227673 (KYU48), GU227674 (KYU43), GU227675 (KYU25), GU227676 (KYU24), GU227677 (KYU8), GU227678 (KYU41), GU227679 (KYU51), GU227680 (KYU53), GU227681 (KYU54), GU227682 (KYU60), GU227683 (KYU58), GU227684 (KYU35), GU227685 (KYU37), GU227686 (KYU50), GU227687 (KYU56), GU227688 (KYU62), GU227689 (KYU64), GU227690 (KYU70), GU227691 (KYU57), GU227692 (KYU63), GU227693 (KYU66), GU227694 (KYU76), GU227695 (KYU68), GU227696 (KYU69), GU227697 (KYU65), and GU227698 (KYU38), respectively.

Table 2. Biogroup and genotype classification of *Cronobacter* isolates.

Biogroup	Cluster (*Cronobacter*)	No. of isolates	Percent (%)
1, 2, 4, 5, 3, 4a, 5a, 8, 8a, 8b	I (*C. sakazakii*)	31	64.5
17,17a,17b,17c,17d	I (*C. sakazakii*)	11	23.0
6, 12	IV (*C. dublinesis*)	6	12.5
16	II (*C. turicensis*)	-	-
15	III (*C. muytjensii*)	-	-
		48	100

-: No detection.

Forty-two *C. sakazakii* and six *C. dublinesis* isolates were classified by genotype, as shown by the above results. The biochemical characteristics of *Cronobacter* isolates were compared with the biogroups originally described by Farmer *et al.* [6] and Iversen *et al.* [11]. Biochemical tests for phenotype analysis were Voges–Proskauer, nitrate reduction, ornithine utilization, motility, methyl red, acid production from inositol, acid production from dulcitol, indole production, malonate utilization, and gas formation (Table 3) through the VITEK (BioMérieux, Marcy l'Etoile, France), Microgen^{TM} BIOCHEMICAL ID (Microgen Bioproducts, Camberly, England), and traditional methods. The procedure was followed according to the manufacturer's instructions for VITEK, and *Cronobacter* ATCC51329 was used as a positive control with *E. cloacea* KCTC 1949 as a negative control.

Farmer *et al*. [6] described 15 biogroups with 57 *Cronobacter* and Iversen *et al*. [11] added the biogroup 16 according to the new biochemical characteristics. As the result of biogroup analyses, nine *Cronobacter* isolates were included in biogroup 1 and three isolates were

Table 3. Assignment of strains to the biogroups originally defined by Farmer *et al*. [6] and Iversen *et al*. [10–12]

Farmer and Iversen biogroups	Phenotype[a]										Strains
	VP	MR	Nit	Orn	Mot	Ino	Dul	Ind	Mal	Gas	
1	+	-	+	+	+	+	-	-	-	+	KYU6, KYU 17, KYU 19, KYU 21, KYU 22, KYU 27, KYU 31, KYU 44, KYU 45
2	+	-	+	+	+	+	-	+	+	+	KYU 39, KYU 40, KYU 42
3	+	-	+	+	-	+	-	-	-	+	KYU 18, KYU 23, KYU 34, KYU 46, KYU 49
4	+	-	+	-	+	+	-	-	-	+	KYU 1, KYU 2, KYU 47, KYU 48
4a	+	-	+	-	-	+	-	-	-	+	KYU 43
5	+	-	+	+	+	+	-	-	+	+	KYU 25
5a	+	-	+	+	-	+	-	-	+	+	***KYU 24***[b]
6	+	-	+	+	+	+	-	+	-	+	KYU 8, ***KYU 52***
8	+	-	-	+	+	+	-	-	-	+	***KYU 41***
8a	+	-	-	+	+	-	-	-	-	+	KYU 51, ***KYU 53***, KYU 54, KYU 59, ***KYU 60***
8b	+	-	-	+	+	+	-	-	+	+	***KYU 58***
12	+	-	+	+	+	+	-	+	+	+	KYU 35, KYU 37, KYU 38, KYU 50
New biogroup 17	+	-	-	+	-	+	-	-	-	+	KYU 56, KYU 62, KYU 64, KYU 70
New biogroup 17a	+	-	-	+	-	+	-	-	+	+	***KYU 57***, KYU63
New biogroup 17b	+	-	-	+	-	-	-	-	+	+	KYU 65
New biogroup 17c	+	-	-	+	-	-	+	-	-	+	KYU 66
New biogroup 17d	+	-	-	+	-	-	-	-	-	+	KYU 76, KYU 68, KYU 69

[a]VP, Voges–Proskauer; MR, methyl red; Nit, nitrate reduction; Orn, ornithine utilization; Mot, motility at 37°C; Ino, acid production from inositol. Dul, acid production from dulcitol; Ind, indole production; Mal, malonate utilization; Gas, gas production from glucose.
[b]Bold and italic: isolated from infant formula.

included in biogroup 2. In biogroups 3 and 4, each of five isolates were included. Two isolates at biogroup 5 and two isolates at biogroup 6 were included. Seven isolates at biogroup 8 and four isolates at biogroup 12 were included. Specifically, we newly added the biogroup 17 to classify 11 isolates, which showed different characteristics of negative nitrate reduction and nonmotility with the other groups. Moreover, new biogroup 17 was designated for the strains in Cluster I (*C. sakazakii*). We report these phenotype characteristics for the first time, because all biogroups of *Cronobacter* did not show negative nitrate reduction and motility at the same time, even though in the Cluster I, there are many biogroups that have different biochemical characteristics. In particular, one fourth of the isolates shown here have different biochemical characteristics with the biogroups reported. When microbes are present in food, it may have various mechanisms for survival and adaptation. Mutations beneficial to bacterial survival under stress condition can be accumulated. Therefore, some mutated *Cronobacter* species contaminating the desiccated foods such as powdered infant formula can be selected owing to their superior ability to survive under dry condition. Accordingly, we think that biogroups by biochemical property might be newly added at any time.

Therefore, 48 *Cronobacter* spp. isolates were detected, by PCR, from powdered infant formulas and *Saengsik* in Korea. Most of the isolates were *C. sakazakii* of Cluster I and 13% of the isolates were *C. dublenesis* of Cluster IV, by 16S rRNA sequence analysis. One fourth of *C. sakazakii* showed different biochemical characteristics to the other groups reported in Cluster I.

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