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Genetic relatedness of *Cronobacter* spp. (*Enterobacter sakazakii*) isolated from dried food products in Indonesia

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Abstract

Cronobacter spp. (formerly known as *Enterobacter sakazakii*) is an opportunistic pathogen and has been reported to cause fatal illnesses, such as meningitis and necrotizing enteritis, especially to high risk-infants through consumption of powdered infant formula (PIF). The aim of this study was to analyze the genetic relatedness of *Cronobacter* spp. isolated from several dried food products (PIF, weaning food and corn starch) commercially marketed in Bogor district of Indonesia. Eleven isolates were characterized using PCR assay based on their partial sequence of 16S rRNA gene to confirm the identity of the isolates. Based on the phylogenetic analysis of the partial sequence of 16S rRNA gene, it is concluded that all isolates from Indonesia were located in same cluster and had lineage under *C. sakazakii* ATCC 29544 and *C. malonaticus*. Isolates DES b7a and DES b7b which were obtained from the same package of weaning food had a very close genetic relatedness and were possibly of the same species. Similar result was also observed on isolates of YR w1 and YR w3 which were isolated from weaning food produced by a different manufacturer.

Introduction

Cronobacter spp. (formerly known as *Enterobacter sakazakii*) are classified as severe pathogens to a restricted population. The bacteria have been reported to cause several cases of fatality and diseases in premature babies, immuno-compromised newborn infants, and newborn infants up to few weeks of age. Meningitis and necrotizing enterocolitis (NEC) diseases caused by *C. sakazakii* in newborn infant have been linked to consumption of powdered infant formula (PIF) (van Acker *et al*., 2001). The origin of this opportunistic pathogen is not known clearly; however, *Cronobacter* spp. can be found in environment, and has been isolated from a variety of foods such as UHT milk, cheese, meat, vegetables, grains, grain sorghum, rice, herbs, spices, fermented bread, fermented drinks, tofu, and sour tea (Muytjens *et al*., 1988; Skaldal *et al*., 1993; Gassem, 1999; Iversen and Forsythe, 2003; Iversen *et al*., 2004). Other studies also reported that *Cronobacter* spp. and *Enterobacter* species can be isolated from the environment such as soil, PIF manufacturers, chocolate factories and households, and also from animals such as rats and flies (Shaker *et al*., 2007).

Reclassification of *E. sakazakii* into *Cronobacter* spp. was based on phenotypic characterization and independent molecular methods including f-AFLP (fluorescence amplified fragment length polymorphisms), automated ribotyping, full-length 16S rRNA gene sequencing and DNA-DNA hybridization. According to the scheme, this genus can be differentiated into at least six genomospecies, i.e. *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii*, *C. dublinensis*, and *C. genomospecies* I (Iversen *et al*., 2007; 2008). In Indonesia, the presence of these bacteria in powdered infant formula and weaning food was previously reported by Estuningsih *et al*. (2006) and Meutia *et al*. (2009). Meutia *et al*. (2008) confirmed the identity of *Cronobacter* isolates using PCR assay based on 16S rRNA gene and concluded that the isolates were located in different cluster from *Cronobacter* species isolated from other countries. This study aimed to further investigate the genetic relatedness and/or diversity of *Cronobacter* strains isolated from several dried food products

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(PIF, weaning food and corn starch) commercially marketed in Bogor district of Indonesia, and whether they are related to *Cronobacter* strains worldwide.

Material and Methods

Bacterial strains

All *Cronobacter* strains (Table 1) were maintained on slant agar of Tryptone Soy Agar (Oxoid, UK) at 37°C. For stock cultures, the bacteria were preserved in beads by mixing with glycerol (Merck) as cryoprotectant and stored at -20°C.

Extraction of bacterial DNA

Total DNA for PCR assay was prepared using phenol chloroform extraction method (Brown, 1992). *Cronobacter* isolates grown in NB (Oxoid, UK) for 48 h was centrifuged at 18000 rpm for 3 mins. Cell pellets were resuspended in 200 µl of TE buffer (pH 8.0), added with 50 µl of SDS 10% (Promega, USA) and mixed well. Ten µl of proteinase-K (10 mg/ml) (Fermentas, Canada) was added and the mixture was incubated at 37°C for 1 h. Following incubation, 80 µl of CTAB/NaCl (Merck) was added and incubated at 65°C for 20 mins. A mixture of phenol : chloroform : isoamyl alcohol (25:24:1) was then added with 1:1 ratio and homogenized by vortex for 2 mins. The mixture was centrifuged at 13,500 rpm for 10 mins and the top phase was transferred into a new tube, added with a mixture of chloroform: isoamyl alcohol (24:1) with the same volume. The resulting mixture was centrifuged at 13500 rpm for 10 mins and the top layer was then transferred to new tubes. It was subsequently added with 0.1 volume of Na-acetate 3M (pH 5.2) and isopropanol with same volume. Tubes were incubated at -20°C for 1 h and DNA was precipitated by centrifugation at 13500 rpm for 10 mins. To the DNA precipitate, 500 µl of ethanol 70% was added and centrifugation was done at 13,500 rpm for 10 mins. The DNA pellets was dried and resuspended in 50 µl of TE buffer.

PCR Assay

For amplification of 16S rRNA gene, a reaction mixture (total volume 50 µl) was made containing 25 µl of PCR master mix (0.05 U/µl Taq DNA Polymerase, 4 mM $MgCl_2$, 0.4 mM of each dNTP) (Fermentas, Canada), 2 µl of 2 µM (each) primers 16SUNI-L/Saka-2b or ESA-1/16SUNI-R (Alpha DNA, Notre-Dame St. W., Montreal, Quebec), nuclease free water, and template DNA was made. Volume of template DNA and nuclease free water varied depending on the DNA concentration obtained. Primers 16SUNI-L (AGA GTT TGA TCA TGG CTC AG) and 16SUNI-R (GTG TGA CGG GCG GTG TGT AC) (Kuhnert *et al*., 1996) correspond to bases 8–28 and bases 1392–1411of the 16S rRNA gene sequence of *Escherichia coli* (NCBI accession no. J01859), respectively. Primers Saka-2b (TCC CGC ATC TCT GCA GGA) and ESA-1 AAT CCT GCA GAG ATG CG) (Hassan *et al*., 2007) correspond to bases 1006–1023 and 1004–1020 of the 16S rRNA gene sequence of *E. coli*, respectively. PCR assay using set of primer 16SUNI-L/Saka-2b generates an amplicon of 977 bp, while that using ESA-1/16SUNI-R generates 408 bp of amplicon. The amplification reactions were performed using PCR Applied Biosystem 2720 Thermal Cycler (Foster City, California). The PCR conditions using primer set of 16SUNI-L/Saka-2b and ESA-1/16SUNI-R were: 4 min at 94°C, 30 cycles (94°C, 50 s; 52°C, 50 s; 72°C, 60 s) and 4 min at 94°C, 30 cycles (94°C, 50 s; 57°C, 50 s; 72°C, 50 s), respectively. Cycling was completed by a final elongation step at 72°C for 4 min. The PCR products were analyzed on 1.5% agarose gel (Merck, Germany) in 1x Tris acetate-EDTA buffer (pH 8.0). The gel was stained for 30 min in 1x TAE buffer containing 0.5 µg/ml ethidium bromides (Amersham bioscience, Sweden) and visualized under UV Gel Doc XR (Bio Rad).

Table 1. *Cronobacter* isolates used in this study

Strain code	Isolation source	Accession numbers of 16S rRNA sequence
DES b7a	Weaning food, Bogor, Indonesia	-
DES b7b	Weaning food, Bogor, Indonesia	-
DES b10	Weaning food, Bogor, Indonesia	JF800181
YR w1	Weaning food, Bogor, Indonesia	JF800184
YR w3	Weaning food, Bogor, Indonesia	JF800185
YR k1b	Weaning food, Bogor, Indonesia	JF800186
YR k2a	Weaning food, Bogor, Indonesia	JF800187
YR t2a	PIF, Bogor, Indonesia	JF800182
YR c3a	PIF, Bogor, Indonesia	JF800183
DES c7	Corn starch, Bogor, Indonesia	JF800180
DES c13	Corn starch, Bogor, Indonesia	JF800179
ATCC 29544	Child's throat, USA[a]	EF088379
ATCC 51329	Not known[a]	AY752937
E825	Breast abscess, USA[a]	EF059881
z3032	Meningitis neonates, Swiss[a]	EF059891
E799	Milk powder manufacturing facility, Ireland[a]	EF059879
6a	Weaning food, Indonesia[b]	AY624069
10a	Weaning food, Indonesia[b]	AY624071
39a	Weaning food, Indonesia[b]	AY624070
39d	Weaning food, Indonesia[b]	AY624073

[a] Iversen *et al*., (2007); [b] Hassan *et al*., (2007)

DNA Sequencing

DNA sequencing of all PCR products was performed by Macrogen Inc, Korea. Both 977 and 408 bp amplicons were sequenced using 3730XL DNA sequencer with capillary electrophoresis system.

Data Analysis

Partial sequences of 16S rRNA gene of *Cronobacter* isolates both for 977 bp and 408 bp were analyzed using BLAST program (*http://blast.ncbi.nlm.nih.gov*) to help identify the members of gene families and to calculate the statistical significance of

matches. They were then aligned and compared with the several sequences of 16S rRNA gene deposited in GenBank by Iversen *et al*. (2007) and Hassan e*t al*. (2007) using ClustalW program of the MEGA4 program package to construct a phylogenetic tree.

Results

PCR assay

Eleven *Cronobacter* isolated from Indonesia were analyzed by PCR using two sets of primer pairs, i.e. 16SUNI-L/Saka-2b and ESA-1/16SUNI-R and gave PCR products with a size of 950 bp (segment 1) and 400 bp (segment 2), respectively (Figures not shown).

Percent similarity of the 16S rRNA gene

Using BLAST (Basic Local Alignment Search Tool) program, the partial sequences of 16S rRNA gene of eleven *Cronobacter* isolates were compared with the highly similar DNA sequences of *Enterobacteriaceae* in the GenBank database. The result showed that the sequences of both segment 1 and segment 2 of the 16S rRNA gene of the *Cronobacter* isolates had high similarity level with sequences of 16S rRNA gene of *Cronobacter* strains deposited in the GenBank; thus all isolates analyzed were confirmed as *Cronobacter* spp.

Partial sequences of *Cronobacter* 16S rRNA gene segment 1 and segment 2 were further analyzed by comparing them to partial sequences of *Cronobacter* 16S rRNA gene deposited in GenBank by Iversen *et al*. (2007) and Hassan *et al*. (2007). Partial sequence of *Cronobacter* 16S rRNA gene deposited in GenBank by Hassan *et al*. (2007) were also originated from *Cronobacter* strains isolated from Indonesia by Estuningsih *et al*. (2006).

Segment 1 and segment 2 of the partial sequence of 16S rRNA gene of *Cronobacter* strains analyzed showed a high level of similarity with the reference *Cronobacter* strains. Percent sequence similarity of the segment 1 of *Cronobacter* strains analyzed and reference *Cronobacter* strains varied between 91 to 99%; while that for segment 2 was in the range of 93 to 100% (Table 2). The partial sequences of 16S rRNA gene of both segment 1 and segment 2 among the *Cronobacter* strains were also analyzed. Segment 1 of the partial sequence of 16S rRNA gene among the *Cronobacte*r strains analyzed had 91 to 99% similarity (Table 3); while segment 2 had 96 to 98% of similarity (Table 4).

Phylogeny of Cronobacter species

The relationships between *Cronobacter* isolates used in this study were depicted in a dendogram using neighbour-joining method from MEGA4 (Molecular Evolutionary Genetic Analysis) package program. The statistical significance of the branch points was evaluated by bootstrap analysis using 1000 replicates. The phylogenetic tree was rooted to *Pantoea agglomerans* ATCC 33243 which was the out-group of *Cronobacter*, and the scale bar represented the percentage of sequence divergence as measured by the length of horizontal lines connecting any two strains (Figure 1).

Based on the partial sequences of 16S rRNA gene segment 1, the phylogenetic tree of *Cronobacter* isolates formed four clusters. All Indonesian *Cronobacter* isolates from this study had the same cluster with *C. sakazakii* ATCC 29544 and *C. malonaticus* strain E825. This cluster consisted of two sub-clusters with 75% of bootstrap values at the branch points. Three other clusters consisted of *C. turicensis* strain z3032, *C. dublinensis* strain E799, and *C. muytjensii* ATCC 51329 which formed the second, third and fourth cluster, respectively.

Table 2. Percent similarity between *Cronobacter* strains used in this study and reference *Cronobacter* strains deposited in GenBank based on 16S rRNA gene segment 1 (977 bp) and segment 2 (408 bp)

Code of *Cronobacter* isolats	*Cronobacter* strains from outside of Indonesia					*Cronobacter* strains from Indonesia (Estuningsih *et al*., 2006)			
	C. sakazakii ATCC 29544	*C. muytjensii* ATCC 51329	*C. malonaticus* E825	*C. dublinensis* E799	*C. turicensis* z3032	*E. sakazakii* 6a	*E. sakazakii* 10a	*E. sakazakii* 39a	*E. sakazakii* 39d
based on 16S rRNA gene segment 1 (977 bp)									
DES b7a	92	91	92	91	92	92	92	92	92
DES b7b	93	91	93	91	92	93	93	93	93
DES b10	97	96	97	96	97	97	98	97	98
DES c7	98	97	98	97	98	98	99	98	99
DES c13	98	97	98	96	97	98	98	98	98
YR t2a	97	95	97	96	96	97	97	97	97
YR c3a	98	97	99	97	98	98	99	98	99
YR w1	99	97	99	97	98	99	99	99	98
YR w3	99	97	99	97	98	99	99	99	99
YR k1b	98	97	98	97	98	98	99	98	99
YR k2a	98	97	98	97	97	98	99	98	99
based on 16S rRNA gene segment 2 (408 bp)									
DES b7a	100	98	100	97	97				
DES b7b	100	98	100	97	97				
DES b10	100	98	100	97	97				
DES c7	100	98	100	97	97				
DES c13	100	98	100	97	97				
YR t2a	100	98	100	97	97				
YE c3a	100	98	100	97	97				

[a] partial sequences of *E. sakazakii* 16S rRNA gene deposited in GenBank by Hassan *et al*. (2007)

■ not analyzed since data from Hassan *et al*. (2007) were not available

Table 3. Percent similarity among *Cronobacter* strains analyzed based on 16S rRNA gene segment 1 (977 bp)

Isolate code of *Cronobacter*	DES b7b	DES b10	DES c7	DES c13	YR t2a	YR c3a	YR w1	YR w3	YR k1b	YR k2a
DES b7a	96	92	92	92	91	92	92	92	92	92
DES b7b		93	93	93	92	93	93	93	92	92
DES b10			98	97	96	98	98	97	97	97
DES c7				98	97	99	98	99	98	98
DES c13					97	98	98	98	98	98
YR t2a						98	97	97	97	96
YR c3a							99	98	98	98
YR w1								99	98	98
YR w3									98	98
YR k1b										98

Discussion

Eleven *Cronobacter* spp. isolates used in this research consisted of two isolates obtained from PIF, seven from weaning food and two from corn

Table 4. Percent similarity among *Cronobacter* strains analyzed based on 16S rRNA gene segment 2 (408 bp)

Isolate code of *Cronobacter*	DES b7b	DES b10	DES c7	DES c13	YR t2a	YR c3a	YR w1	YR w3	YR k1b	YR k2a
DES b7a	100	100	100	100	100	100	98	97	93	97
DES b7b		100	100	100	100	100	98	97	93	97
DES b10			100	100	100	100	98	97	93	97
DES c7				100	100	100	98	97	93	97
DES c13					100	100	98	97	93	97
YR t2a						100	98	97	93	97
YR c3a							98	97	93	97
YR w1								96	92	95
YR w3									95	98
YR k1b										96

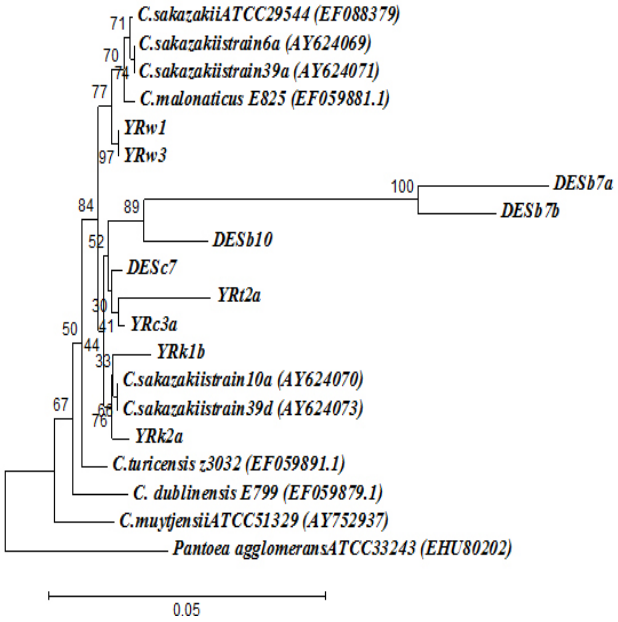

Figure 1. Dendogram of partial sequence of *Cronobacter* 16S rRNA gene using *Neighbor-Joining* method. A neighbor-joining analysis was used with Felsenstein correction (1000 bootstrap replicates). The bar indicates 5% estimated sequence divergence

starch samples from Bogor area. All these Indonesian *Cronobacter* isolates were genetically confirmed using PCR system to amplify their 16S rRNA gene. Sequence of bacterial 16S rRNA gene is frequently used as the target to study bacterial phylogeny and taxonomy because it is ubiquitous in almost bacteria, composed of highly conserved region and variable region, large enough (1500 bp) for informatics purposes, and its function over time has not changed (Janda and Abbot, 2007).

Two bacterial strains can be concluded as the same species when the similarity between their full-length 16S rRNA genes (>1300 bp) ranged between 98.7 to 99% (Stackebrandt and Ebers, 2006; Iversen et al., 2007). Because the amplicon size of the partial sequences of *Cronobacter* 16S rRNA genes analyzed of both segment 1 and segment 2 was less than 1300 bp; they cannot be summed up as the same species with the reference *Cronobacter* strains even though the sequence similarity level was more than 98.7%. However, all Indonesian *Cronobacter* isolates analyzed had closer sequence similarity with *C. sakazakii* ATCC 29544 and *C. malonaticus* strain E825. Here, *C. malonaticus* strain E825 did not form a separate cluster from *C. sakazakii* ATCC 29544 because both these species basically have 99.6% of 16S rRNA gene sequence similarity. In the beginning *C. malonaticus* was classified as a subspecies of *C. sakazakii* (Iversen *et al*., 2007), but the new classification has verified that *C. malonaticus* was identified as a new species of *Cronobacter* belonging to the biogroup 5, 9 and 14 previously described by Farmer *et al*. in 1980 (Iversen *et al*., 2008).

The formation of two sub-clusters on the first cluster showed that there was still genetic diversity between Indonesian *Cronobacter* isolates and *C. sakazakii* ATCC 29544 and *C. malonaticus* E825. However, it could be concluded that all Indonesian *Cronobacter i*solates had a close genetic relatedness although they were originated from different sources.

The phylogenetic tree branch between isolate DES b7a and DES b7b showed 100% bootstrap value; suggesting that both are of the same species although the biochemical test result (Dewanti-Hariyadi *et al*., 2010) identified them as different species. Isolate DES b7a and DES b7b were isolated from the same packaging of weaning food sample. Similar results were also observed on isolate YR w1 and YR w3 which were isolated from a different manufacturer. The two isolates had 96% bootstrap value on the phylogenetic tree branch.

Result of this study suggested that all Indonesian isolates were identified as *Cronobater sakazakii* of the same cluster as *C. sakazakii* which were previously isolated mostly from clinical isolates. Therefore, the isolates have the potential to cause infection and pose risk to public health. However, since isolates obtained from different sources were possibly of the same species, control in food industry and its spread are assumed to be easier.

Conclusions

Based on their 16S rRNA genes, all *Cronobacter* isolates from Indonesia had a close genetic relatedness even though they were originated from different food sources. In addition, they were located in the same cluster with *C. sakazakii* ATCC 29544 and *C. malonaticus* E825. This finding could contribute to the taxonomy reclassification of *Cronobacter*.

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YRc3a. We also to thank the Directorate General of Higher Education, Ministry of Education, Republic of Indonesia for the financial support through Competitive Grant for Graduate Program.

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