Genetic relatedness of local *Cronobacter sakazakii* based on invasion gene *ompA*

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**Abstract.** *Cronobacter sakazakii* is a foodborne pathogen, one of which due to the possession of invasion gene *ompA*. Ten *C. sakazakii* previously isolated from dried foods in Indonesia were found to own the *ompA* gene. This study aimed to evaluate the genetic relationship among these local isolates and their similarity with *C. sakazakii* references based on the *ompA* gene. DNA isolation was carried out using Chelex, the *ompA* gene was amplified with PCR followed by agarose gel electrophoresis and sequencing. The sequences were subjected to contig analysis using Bioedit software, and the results were analyzed for homology by the BLAST on NCBI website. The phylogenetic tree was constructed using MEGA v.6 with the maximum-likelihood algorithm and 1000 replication bootstrap. The result shows that *C. sakazakii* FWHb6 and FWHd16 were in one cluster with *C. sakazakii* ATCC 29544 known to invade the human intestine. In general, the *ompA* gene of the local isolates had a close genetic relationship with *C. sakazakii* references. This study suggests that local *C. sakazakii* possess similar invasion to other *C. sakazakii*. This finding can contribute to *Cronobacter* classification.

1. Introduction

*Enterobacter sakazakii* based on DNA-DNA hybridization was subsequently proposed to be re-classified into a new genus *Cronobacter*, composed of six distinct species: *Cronobacter sakazakii*, *C. malonaticus*, *C. muytjensii*, *C. dublinensis*, and *C. genomospecies* (*C. universalis*) [1]. *Cronobacter sakazakii* is a foodborne pathogen bacterium that can infect healthy adults and poses a risk to infant and immuno-compromised individuals [2,3]. This bacterium has been associated with outbreaks of meningitis, septicemia, and necrotizing enterocolitis (NEC) [4]. Even when infants survive *Cronobacter* infection, they often experience serious sequelae, including brain abscesses, developmental delay, and impairment of sight and hearing [5]. Despite the serious perils associated with *C. sakazakii*, little is know at the molecular level about the mechanism of its pathogenicity and virulence factors.

As an oral pathogen causing a systemic infection, *C. sakazakii* must translocate from the intestinal lumen into the blood circulation. The genus *Cronobacter* is capable of actively invading various epithelial and endothelial cells of human and animal origin [6, 7, 8]. Kim and Loessner [6] reported that the active invasion of human intestinal Caco-2 cells by *C. sakazakii* requires *de novo* bacterial protein synthesis and the host cell cytoskeleton and that the invasion efficiency of *C. sakazakii* was enhanced in the absence of cellular tight junctions. With regard to the virulence determinants related to
Cronobacter penetration of the host cells, Mohan Nair and Venkitanarayanan [7] and Singamsetty et al [8] reported that outer membrane protein A (ompA) of Cronobacter plays an important role in the invasion of human intestinal epithelial INT-407 cells and human brain microvascular endothelial cells (HBMECs); invasion was dependent on both microfilaments and microtubules in INT-407 cells but only on microtubule condensation in HBMECs.

Early years, Kim et al [9] reported about invasion factors responsible for the intestinal translocation of C. sakazakii, they constructed outer membrane protein X (ompX) and A (ompA) deletion mutants using the lambda Red recombination system. The ompX and ompA deletion mutants showed significantly reduced invasion of human enterocyte-like epithelial Caco-2 and human intestinal epithelial INT-407 cells, and significantly fewer mutant cells were recovered from the livers and spleens of rat pups. Outer membrane protein A (ompA) and X (ompX) are involved in basolateral invasion by C. sakazakii.

Cronobacter sakazakii has been isolated from various dried food samples in Indonesia, such as MP-ASI [10,11], powder infant formula [12], cornstarch, chocolate powder [13], sugar, flour, and pepper powder [11]. Ten local C. sakazakii were found to own the ompA gene. The role of ompA gene on local isolate is unknown. Here, we report genetic relatedness of local C. sakazakii based on invasion gene ompA with C. sakazakii references. We also show that locals C. sakazakii have a close genetic relationship with C. sakazakii references.

2. Materials and methods

2.1. Bacterial isolates studied and culture condition

Ten Cronobacter sakazakii isolates used in this study are listed in table 1. The frozen stocks of C. sakazakii were revived by placing one loop of suspension into the Brain Heart Infusion (BHI) broth followed by 18 h incubation at 35°C. After incubation, one loop was streaked onto a Brilliance Enterobacter sakazakii Agar (BESA) agar plate and confirmed as C. sakazakii as blue-green pigmented colonies on BESA agar medium. The culture was made by inoculating one colony from BESA agar medium into 10 ml BHI broth (Oxoid, UK) and incubating it for 18 h at 35°C to reach ca. 10⁹ CFU/ml.

| Strain | Source | Acc to Gene Bank |
|--------|--------|------------------|
| E1     | MP-ASI | -                |
| E2     | MP-ASI | -                |
| Yrt2b  | Powder infant formula | JF800182.1 |
| Yrw3b  | MP-ASI | JF800185         |
| DESb10c| Baby food | JF800179.1     |
| DESc13c| Corn starch | JF800181.1   |
| DESc7c | Corn starch | JF800180.1   |
| FWHe3d | Cassava starch | -          |
| FWHe6d | Flour | -                |
| FWHe16d| Pepper powder | JX535018.1 |

Note: a[10]; b[12]; c[13]; d[11]

2.2. DNA isolation and amplification of the ompA gene

DNA isolation used Chelex method as described in the previous study [14]. Concentrations and purity of DNA were measured using a Nanodrop. The PCR reaction mixture (20 µl) was made of 10 µl PCR GoTaq Green DNA master mix (Promega, Madison, USA), 1 µl primer forward, 1 µl primer reverse, 1
\( \mu l \) DNA template (100ng/\( \mu l \)), and 7 \( \mu l \) Nuclease Free Water (ThermoFischer Scientific Massachusetts, USA). The primer forward \textit{ompA} (5’TAGACTTTACATCGCCAGGG’3) and the primer reverse \textit{ompA} (3’GAGCTTTACGTTGTCACAG’5) had 1055 bp amplicon. Amplification of DNA carried out using standard PCR (Applied Biosystem Thermal Cycler 2720 Thermal Cycler) with an initial denaturation step at 94°C for 3 min followed by 30 cycles of denaturation step at 95°C for 1 min, annealing step at 50°C for 1 min and extension step at 72°C for 1 min. The amplification was ended with a final extension step at 72°C for 5 min and cooling at 4°C for 1 min. The amplification products were analyzed with electrophoresis on 2% agarose gel and examined under UV light (Bio-Rad Laboratories Pte. Ltd, Singapore) [15]. The sequencing of all PCR products was carried out by 1 Base Pte Ltd, Malaysia using the highest capacity-based genetic analyzer platform.

2.3. Sequences analysis

This step refers to the research previously by Fei et al [16]. The results of \textit{ompA} sequencing were contig analyzed using Bioedit software. Then the results were analyzed for homology using the BLAST on NCBI website. \textit{Cronobacter} references used for homology analysis were \textit{C. sakazakii} ATCC 29544, \textit{C. sakazakii} ATCC BAA-894, and \textit{C. muytjensii} ATCC 51329. The outgroup of phylogenetic tree used was \textit{Escherichia coli} K-12. The \textit{ompA} gene sequences of \textit{Cronobacter} references and outgroups were obtained from GenBank. Construction of the phylogenetic trees for each gene was carried out using MEGA v.6 software with maximum-likelihood algorithm and bootstrap 1000 replications.

3. Results and discussion

3.1. DNA concentration, DNA purity, and PCR result

The results of DNA isolation are pure if the ratio of \( A_{260} \) nm and \( A_{280} \) nm is 1.8-2.0 and has met the requirements needed in the molecular analysis [17]. In this study, DNA purity was in the range between 169 – 1.93. A purity value below 1.8 indicates the presence of contaminants in the form of proteins, while the purity values above 2.0 indicate that there are still contaminants in the form of RNA [18]. Local strains with a purity below 1.8, the possibility of DNA extraction process was washed for a longer time using ethanol so that protein impurities can be lost. The DNA concentration obtained varied, so the dilution process was carried out to homogenize the DNA concentration to 100 ng/\( \mu l \). The results of DNA purity and concentration can be seen in table 2.

| Strain | DNA purity \( (A_{260}/A_{280}) \) | DNA concentration (ng/\( \mu l \)) |
|--------|-------------------------------|---------------------------------|
| E1     | 1.76                          | 649.45                          |
| E2     | 1.69                          | 1079.70                         |
| Yrt2a  | 1.94                          | 1510.85                         |
| Yrw3   | 1.89                          | 732.65                          |
| DESb10 | 1.80                          | 1391.50                         |
| DESc13 | 1.73                          | 2331.85                         |
| DESc7  | 1.93                          | 1377.50                         |
| FWHc3  | 1.74                          | 1840.80                         |
| FWHb6  | 1.82                          | 878.20                          |
| FWHd16 | 1.86                          | 1019.10                         |
| Average| 1.82                          | 1281.16                         |

The amplification products were analyzed with electrophoresis on 2% agarose gel and examined under UV light, the picture can see in figure 1. From the results of this visualization, the quality of products PCR worthy of sequencing. Specific genes detected appear as bands in the agarose gel after staining with EtBr and align with the ladder marker on appropriate length [19]. A negative control is also needed.
to avoid errors in the results that can occur due to contamination from the process before amplification. Negative control indicated by the non-apparently band in agarose gel [20]. In this study, we used no-template DNA as a negative control. Sequencing results are not provided in this paper.

![Figure 1. The visualization of ompA gene amplicons. M: Ladder marker, 1:Yrw3; 2:DESc13; 3:FWHb6; 4:E1; 5:E2; 6:DESc7; 7:DESb10; 8: FWHd16; 9:Yrt2a; 10: FWHc3; 11: Negative control.](image_url)

### 3.2. The relationship of local C. sakazakii and reference C. sakazakii
The homology analysis BLAST results between local C. sakazakii with Cronobacter spp. reference based on data from GenBank ranged from 94% to 95%. Data can be seen in table 3. Most local isolates of C. sakazakii show close genetic relationship with C. sakazakii ATCC 29544 and C. sakazakii ATCC BAA-894. The similarity of local C. sakazakii with C. muytjensii ATCC 51329 based on ompA gene is very reasonable. Iversen et al [21] reported that based on phenotypic and genotypic tests, C. sakazakii ATCC 29544 had a 100% similarity with C. muytjensii ATCC 51329. Outer membrane protein A is one of the most abundant prokaryotic groups of outer membrane proteins, structurally categorized as β-barrel proteins and is known to have 171-N-terminal residue [22].

| Strain | C. sakazakii ATCC 29544 | C. sakazakii ATCC BAA-894 | C. muytjensii ATCC 51329 |
|--------|--------------------------|---------------------------|--------------------------|
| E1     | 94                       | 95                        | 95                       |
| E2     | 95                       | 95                        | 95                       |
| Yrt2a  | 95                       | 95                        | 95                       |
| Yrw3   | 95                       | 95                        | 95                       |
| DESb10 | 95                       | 95                        | 95                       |
| DESc13 | 95                       | 95                        | 95                       |
| DESc7  | 95                       | 95                        | 95                       |
| FWHc3  | 94                       | 94                        | 94                       |
| FWHb6  | 95                       | 95                        | 95                       |
| FWHd16 | 94                       | 94                        | 94                       |

Statistical tests at the branch point of the phylogenetic tree were evaluated using bootstrap analysis with 1000 replicates. The higher the bootstrap value, the better the dendrogram shows the topology of the branches of the phylogenetic tree [23]. The phylogenetic tree of local C. sakazakii can see in figure 2.
In general, based on the phylogenetic tree have two large groups with bootstraps value 99. The genetic relatedness of the isolates from the results of this study based on the phylogenetic tree showed that most of the local isolates from this study had closed genetic relationship with C. sakazakii references.

![Phylogenetic tree of local C. sakazakii based on ompA gene.](image)

Figure 2. Phylogenetic tree of local C. sakazakii based on ompA gene.

Strain local FWHb6 and FWHd16 have bootstraps value 97. These two strain and strain Yrt2a are in one cluster with C. sakazakii ATCC 29544 known to invade the human intestine. It means that these four isolates can be concluded to be very closely related genetically. These might show the similarity in behaviour and function of the ompA gene of local C. sakazakii FWHb6, FWHd16 and Yrt2a with C. sakazakii ATCC 29544. Outer membrane protein plays a role both in stress conditions and plays a major role in adhesion to the mucous membranes of the small intestine and deletion of these genes causing rates of infection [24]. OmpA on Cronobacter spp. is a virulence factor needed for attachment of fibronectin to microvascular endothelial cells of the human brain [25]. Other studies use ompA as the target gene to detect the presence of Cronobacter sakazakii [26, 27].

4. Conclusion
This finding can be used as information for other studies for the classification of locals C. sakazakii based on the ompA gene.

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