Genes involved in tolerance to osmotic stress by random mutagenesis in *Cronobacter malonaticus*

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ABSTRACT

Cronobacter malonaticus is one of the opportunistic food-borne pathogens in powdered infant formula and has unusual abilities to survive under environmental stresses such as osmotic conditions. However, the genes involved in osmotic stress have received little attention in *C. malonaticus*. Here, genes involved in osmotic stress were determined in *C. malonaticus* using a transposon mutagenesis approach. According to the growth of mutants ($n = 215$) under 5.0% NaCl concentration, the survival of 5 mutants under osmotic stress was significantly decreased compared with that of the wild type strain. Five mutating sites, including potassium efflux protein KefA, inner membrane protein YqjF, peptidylprolyl isomerase, Cys-tRNA(Pro)/Cys-tRNA(Cys) deacylase, and oligogalacturonate lyase were successfully identified. In addition, the biofilm formation of 5 mutants was determined using crystal violet staining, scanning electron microscopy, and confocal laser scanning microscopy, and the biofilms of 5 mutants significantly decreased within 72 h compared with that of wild type strain. This is the first report to determine the genes involved in osmotic tolerance in *C. malonaticus*. The findings provided valuable information for deep understanding of the mechanism of survival of *C. malonaticus* under osmotic stress, and a possible relationship between biofilm formation and tolerance to osmotic stress was also demonstrated in *C. malonaticus*.

Key words: *Cronobacter malonaticus*, random mutagenesis, osmotic stress, biofilm formation

INTRODUCTION

Cronobacter is a recently described genus, including *Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter turicensis*, *Cronobacter muytjensii*, *Cronobacter dublinensis*, *Cronobacter universalis*, and *Cronobacter condimenti* (Caubilla-Barron et al., 2007). *Cronobacter* species are associated with necrotizing enterocolitis, bacteremia, and meningitis in infants, with mortality rates of up to 40 to 80% (Healy et al., 2010). Although *Cronobacter* has been isolated from food, soil, food processing environments, and clinical samples, powdered infant formula is considered to be the major transmission path of *Cronobacter* infections (Norberg et al., 2012; Ye et al., 2014).

Cronobacter has shown unusual abilities to survive under environmental stresses. Alvarez-Ordóñez et al. (2012) demonstrated that *Cronobacter* spp. showed stronger growth ability to survive under osmotic conditions than other *Enterobacteriaceae*. In addition, the heterogeneity about growth under osmotic stress among *C. sakazakii* strains was also observed. Transposon mutagenesis was used to reveal genes involved in osmotic stress and drying in *C. sakazakii*, and 29 genes were found to be response to osmotic stress (Alvarez-Ordóñez et al., 2014). Riedel and Lehner (2007) identified differentially expressed proteins involved in osmotic stress using a comparative proteomics method. Genomic analysis showed that *C. malonaticus* is 1 of 3 *Cronobacter* species causing severe infections (Kucerova et al., 2010). Furthermore, the most reported cases indicated that *C. malonaticus* infected adults more than infants (Ogrodzki and Forsythe, 2017). Although only 1 case of meningitis from *C. malonaticus* has been documented in neonate, powdered infant formula is also an important point of origin for *C. malonaticus* (Hariri et al., 2013; Ogrodzki and Forsythe, 2017). Consequently, the potential risks of *C. malonaticus* to neonates should be great of concern. A high osmotic pressure encountered by *Cronobacter* during spray drying in the powdered formula manufacturing process affects its

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persistence in powdered infant formula (Amalaradjou and Venkitanarayanan, 2011). However, the factors involved in tolerance to osmotic stress in *C. malonaticus* have received little attention.

In our study, a transposon mutagenesis approach was used to construct a random mutagenesis library. Mutants showing significant growth differences compared with wild type strains (**WT**) in osmotic stress (5.0% NaCl concentration) were screened, and the insertion sites were also identified by PCR amplification. Finally, the biofilm formation between mutants and WT were determined using crystal violet staining (**CVS**), scanning electron microscopy, and confocal laser scanning microscopy (**CLSM**).

MATERIALS AND METHODS

Construction of a Random Mutagenesis Library

We used a transposon mutagenesis approach to produce mutants. In brief, *Escherichia coli* WM3064 [pBSL180 with kanamycin (**Km**) and ampicillin (**Amp**) resistance] with deficiency of 2,6-diaminopimelic acid. *Cronobacter malonaticus* YE-001 identified using multilocus sequence typing (Baldwin et al., 2009) is sensitive to Km and Amp. *Escherichia coli* WM3064 and *C. malonaticus* YE-001 were used as the donor and receptor, respectively. The donor and receptor were inoculated into Luria Bertani (**LB**; Huankai, Guangzhou, China) broth at 37°C until reaching the exponential phase. The donor and receptor (3:1) were then mixed and doped onto filter membrane at 37°C for incubation of 20 h. Finally, the filter membrane was washed using sterile LB broth, and streaked onto chromogenic *Enterobacter sakazakii* agar (kanamycin) and chromogenic *Enterobacter sakazakii* agar (ampicillin kanamycin) at 37°C for 12 h. The true mutants can grow on chromogenic *Enterobacter sakazakii* agar (kanamycin) and cannot grow on chromogenic *Enterobacter sakazakii* agar (ampicillin).

Screening of Mutants of Significant Differences Under Osmotic Stress

For testing bacterial growth under osmotic conditions, overnight culture (optical density at 600 nm = 0.8, vol/vol, 1%) was inoculated into LB broth containing 5.0% NaCl at 37°C for 8 h. Growth of mutants (n = 215) and WT were measured spectrophotometrically in 96-well culture plates by determining the optical density at 600 nm. Mutants having significant growth differences compared with WT were subject to further study.

Mapping of Transposon Insertion Sites

The transposon insertion sites were identified by PCR described by Chen et al. (2010) and Merrell et al. (2002) with modification. In brief, chromosomal DNA was extracted using Bacteria DNA Extraction Kit (Tiangen, Beijing, China). The forward primer (5'-GGCCACGCGTCGACTAGTCAGACTACG-GCTAGCTG-3') and the reverse primer (5'-CATCC-GGGGTCAGCACCGTTTC-3') were used. The first PCR amplification was performed for 5 min at 94°C; followed by 6 cycles of 94°C for 30 s, 30°C for 30 s, 72°C for 90 s; then 30 cycles of 94°C for 30 s, 45°C for 30 s, 72°C for 2 min; and finally 10 min at 72°C. The PCR products were purified for the second PCR amplification, the forward primer was 5'-GGCCAC-GTCGACTAGTCA-3', whereas the reverse primer was 5'-GGATCCACTAGTTCTAGAGCGGC-3'. The thermal conditions for the second amplification were 5 min at 94°C; 30 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 2 min; and 10 min at 72°C. The second PCR products were purified for being sequenced using as a primer (5'-GGATCCACTAGTTCTAGAGCGGC-3'). Insertion sites or mutating sites were identified by sequences alignment on NCBI.

Quantitative Analysis of Biofilm Formation

For CVS detection, *C. malonaticus* strains were inoculated into LB containing 5.0% NaCl for incubation at 37°C for 8 h with constant shaking. Twenty microliters of overnight culture (optical density at 590 nm = 0.5) were inoculated into 48-well polystyrene plates containing 1.98 mL of sterile LB broth and incubated at 37°C for 24, 48, and 72 h. The plates were rinsed 3 times with deionized water and the adherent bacteria cells were stained with 1% crystal violet for 30 min. After being rinsed 3 times with deionized water, the crystal violet was liberated by 30% acetic acid following a 10-min incubation. The optical density values of biofilm in each well were measured at 590 nm and each experiment was done in triplicate. The statistics analysis about biofilm-forming abilities among mutants and WT using Originpro 8.5.1 (OriginLab Corp., Northampton, MA).

For scanning electron microscopy, glass coverslips (Jingan, Shanghai, China) were immersed in tryptic soy broth and 20 µL of *C. malonaticus* (WT and 5 mutants) were transferred to 48-well plates (Baiyan, Shanghai, China) containing 1.98 mL of LB with 5.0% NaCl for incubation at 37°C for 24, 48, and 72 h respectively. During this biofilm-formation period, the glass coverslips from different incubation times were prepared and detected by scanning electron microscopy (Hitachi,

Tokyo, Japan) as described previously by Wang et al. (2013). For CLSM, the glass coverslips were prepared the same as scanning electron microscopy detection, and bacterial biofilm formation on glass slips was stained with LIVE/DEAD BacLight bacterial viability Kit according to instructions (Invitrogen, Carlsbad, CA), and were subsequently observed by CLSM (Zeiss, Berlin, Germany).

RESULTS AND DISCUSSION

Cronobacter spp. is an important food-borne pathogens associated with severe but rare infections in neonates. *Cronobacter malonaticus* predominates in adult infection cases, and the contaminated level of *C. malonaticus* in powdered infant formula is also high (Hariri et al., 2013; Ogrodzki and Forsythe, 2017). From our mutant library, 5 mutants were successfully screened, and the growth of 5 mutants under osmotic stress was significantly ($P < 0.01$) decreased compared with those of WT, as shown in Figure 1. Furthermore, using arbitrary PCR detection, the transposon insertion sites were successfully identified and shown in Table 1. These mutating genes encode inner membrane protein YqjF, potassium efflux protein KefA, Peptidylprolyl isomerase (PPIase), Cys-tRNA(Pro)/Cys-tRNA(Cys) deacylase, and oligogalacturonate lyase. *Cronobacter* spp. has unusual abilities to survival under different environmental stresses. A total of 29 genes in *C. sakazakii* were involved in osmotic stress belonging to various clusters of orthologous groups functional categories by using a transposon mutagenesis method (Alvarez-Ordóñez et al., 2014). In another paper, proteins upregulated in response to osmotic stress fell into DNA/RNA stabilizing enzymes, molecular chaperones,

Figure 1. *Cronobacter malonaticus* growth of mutants and wild type strains in Luria Bertani broth with 5%NaCl. OD_{600nm} = optical density at 600 nm. Error bars represent SD. ** $P < 0.01$, *** $P < 0.001$.

and oxidative-related proteins through the comparative proteomics analysis (Riedel and Lehner, 2007).

The inner membrane protein YqjF is a conserved inner membrane protein of unknown function. In our study, the deletion of *YqjF* caused impaired growth rates under osmotic stress in *C. malonaticus*. Functions of other inner membrane proteins were preliminarily studied previously (Baba et al., 2006; Mamat et al., 2008; Anderson et al., 2017). Baba et al. (2006) stated that *yhjD* is a nonessential gene in WT *E. coli*. In another study, single AA substitutions in *yhjD* could confer viability to 3-deoxy-D-manno-oct-2-ulosic acid-depleted *E. coli* (Mamat et al., 2008). In *E. coli*, the

Table 1. Mapping of transposon insertion sites in *Cronobacter malonaticus* YE01

Mutant ¹	Gene encoded proteins (organism)	Relevant features	Locus tag
Mutant 11	Inner membrane protein YqjF (<i>Cronobacter malonaticus</i> CMCC 45402)	Structure of cell inner membrane	P262_05196 ²
Mutant 17	Potassium efflux protein KefA (<i>Cronobacter malonaticus</i> CMCC 45402)	Transport potassium	P262_04184 ³
Mutant 35	Peptidylprolyl isomerase (<i>Cronobacter malonaticus</i> CMCC 45402)	Catalyze the isomerization of peptide bonds N-terminal of prolines	P262_04121 ⁴
Mutant 56	Cys-tRNA(pro)/cys-tRNA(cys) deacylase (<i>Cronobacter malonaticus</i> CMCC 45402)	Can hydrolyze misacylated cys-tRNA(pro) and cys-tRNA(cys)	P262_04972 ⁵
Mutant 71	Oligogalacturonate lyase (<i>Cronobacter malonaticus</i> CMCC 45402)	Catalyze the conversion of digalacturonate into monogalacturonate	P262_RS03690 ⁶

¹Mutants all from the current study.

²<https://www.ncbi.nlm.nih.gov/protein/564116232>.

³<https://www.ncbi.nlm.nih.gov/protein/AHB71343.1>.

⁴<https://www.ncbi.nlm.nih.gov/protein/564116355>.

⁵<https://www.ncbi.nlm.nih.gov/protein/AHB71899.1>.

⁶<https://www.ncbi.nlm.nih.gov/protein/564116801>.

synthesis of inner membrane protein YhiM is necessary for growth at high temperatures and low osmolarity (Anderson et al., 2017).

Potassium efflux protein KefA has received little attention on its detailed function. Other potassium efflux proteins, such as bacterial KefC protein, were originally considered to be the K^+ channel (Booth et al., 2003). Another study indicated that KefC functioned as a K^+/H^+ antiporter mode of action (Fujisawa et al., 2007). A previous study showed that increasing acute hyperosmotic NaCl stress causes a dose-dependent K^+ leak from the cell, resulting in a substantial decrease in cytosolic K^+ content and a concurrent accumulation of Na^+ in the cell (Shabala et al., 2009; Roosild et al., 2009; Aranda-Sicilia et al., 2012). In our study, the deletion of potassium transporter protein KefA caused decreasing survival of *C. malonaticus* under osmotic stress, which might result from loss of K^+ channel under high NaCl concentrations and interruption of intracellular and extracellular ion balance.

The function of PPIase is to catalyze the isomerization of peptide bonds N-terminal of prolines, and PPIase plays an important role in protein folding or trafficking (Schmidpeter et al., 2015; Kang et al., 2017; Liu

et al., 2017). The PPIase may be involved in the salt adaptation of *Shewanella* sp. WP3 (Li et al., 2006). In the present study, PPIase also contributed to survival of *C. malonaticus* under osmotic stress. We inferred that the structure or functions of some proteins may be interfered due to the absence of PPIase gene in mutant strain, which significantly affected the metabolic procession and growth under osmotic stress.

The Cys-tRNA(Pro)/Cys-tRNA(Cys) deacylase can hydrolyze misacylated Cys-tRNA(Pro) and Cys-tRNA(Cys). Previous reports showed that the hydrolysis of aminoacyl-tRNA could influence cell growth (Ruan and Soll, 2005; Liu et al., 2015). In our study, Cys-tRNA(Pro)/Cys-tRNA(Cys) deacylase significantly reduced the growth of *C. malonaticus* under osmotic stress. However, information about effects of the gene on the survival in other food-borne pathogens under osmotic stress is not available.

Oligogalacturonate lyase (OGL) is an enzyme of pectate lyases in *Enterobacteriaceae*; OGL in *Yersinia enterocolitica* use a β -elimination mechanism to preferentially catalyze the conversion of saturated and unsaturated digalacturonate into monogalacturonate (Abbott et al., 2010). The present study showed that the

Figure 2. Biofilm formation of *Cronobacter malonaticus* mutants and wild type strains in Luria Bertani broth with 5% NaCl using crystal violet staining. OD_{600nm} = optical density at 600 nm. Error bars represent SD. ** $P < 0.01$, *** $P < 0.001$.

Figure 3. Biofilm formation of *Cronobacter malonaticus* mutants and wild type strains (WT) in Luria Bertani broth with 5% NaCl using scanning electron microscopy.

Figure 4. Biofilm-forming ability of *Cronobacter malonaticus* mutants and wild type strains (WT) in Luria Bertani broth with 5% NaCl using confocal laser scanning microscopy. Color version available online.

deletion of OGL gene had negative effects on survival of *C. malonaticus*; however, the detailed mechanism of OGL on survival in *C. malonaticus* remains to be revealed.

Biofilms are an interface-associated consortia of microorganisms that are typically embedded in an endogenous slimy matrix referred to as extracellular polymeric substance, which enhance resistance to a variety of environmental stresses (Hartmann et al., 2010). Furthermore, the biofilm formation of 5 mutants and WT was determined using CVS, scanning electron microscopy, and CLSM. The mutants and WT formed the highest number of biofilms after 48 h of incubation, as shown in Figure 2. Different biofilm-forming abilities were observed among *C. sakazakii* strains under different conditions (Ye et al., 2016). In addition, we also found that the biofilm-forming abilities in mutants significantly decreased compared with those of WT (Figures 3 and 4). The 5 mutant strains formed weaker biofilms than WT within 72 h of incubation under osmotic stress. In recent years, the biofilm formation in *Cronobacter* spp. under different environmental conditions has been described (Lee et al., 2012; Jung et al., 2013; Ye et al., 2015). Hartmann et al. (2010) demonstrated that cellulose biosynthesis, flagellar structure and biosynthesis, and basic cellular procession were involved in biofilm formation in *C. sakazakii*. Furthermore, k-antigen and colonic acid capsular profiles among *C. sakazakii* and *C. malonaticus* were determined (Ogrodzki and Forsythe, 2015), which showed that capsular type [K2:CA2:Cell⁺] were associated with neonatal meningitis and necrotizing enterocolitis. They also found that K2:CA2:Cell⁺ was found in *C. turicensis*, *C. dublinensis*, and *C. universalis* (Ogrodzki and Forsythe, 2017).

We found that the deletion of inner membrane protein YqjF, potassium efflux protein KefA, PPIase, Cys-tRNA(Pro)/Cys-tRNA(Cys) deacylase, and OGL caused the significant reduction of biofilm formation in *C. malonaticus*. Ye et al. (2016) found that PPIase might be the potential protein contributing to biofilm formation in *C. sakazakii* using a comparative proteomics method. In addition, the lowest amount of biofilm formation in KefA mutant strain (mutant 17) was observed among 5 mutants. In *Bacillus subtilis*, the expression of mstX and the downstream putative K⁺ efflux channel, yugO, is necessary for biofilm formation (Lundberg et al., 2013). Biofilm formation is a very complex processing associated with various factors. Other factors including inner membrane protein YqjD, potassium efflux protein KefA, Cys-tRNA(Pro)/Cys-tRNA(Cys) deacylase, and OGL were found to be associated with biofilm formation in *C. malonaticus*. In addition, the findings also confirmed a positive relationship between survival and biofilm-forming abili-

ties under osmotic stresses in *C. malonaticus*, but the detailed mechanism of these genes on biofilm formation remains to be revealed in the future.

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