Roles of outer membrane protein W (OmpW) on survival, morphology, and biofilm formation under NaCl stresses in *Cronobacter sakazakii*

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ABSTRACT

Cronobacter sakazakii is an important foodborne pathogen associated with rare but severe infections through consumption of powdered infant formula. Tolerance to osmotic stress in *Cronobacter* has been described. However, the detailed factors involved in tolerance to osmotic stress in *C. sakazakii* are poorly understood. In this study, roles of outer membrane protein W (OmpW) on survival rates, morphologic changes of cells, and biofilm formation in *C. sakazakii* under different NaCl concentrations between wild type (WT) and OmpW mutant (Δ OmpW) were determined. The survival rates of Δ OmpW in Luria-Bertani medium with 3.5% or 5.5% NaCl were reduced significantly, and morphological injury of Δ OmpW was significantly increased compared with survival and morphology of WT. Compared with biofilm formation of the WT strain, biofilms in Δ OmpW were significantly increased in Luria-Bertani with 3.5% or 5.5% NaCl using crystal violet staining assay after 48 and 72 h of incubation. Detection of biofilms using confocal laser scanning microscopy and scanning electron microscopy further confirmed the changes of biofilm formation under different NaCl stresses. This study demonstrates that OmpW contributes to survival of cells in planktonic mode under NaCl stresses, and biofilm formation is increased in Δ OmpW in response to NaCl stress.

Key words: *Cronobacter sakazakii*, outer membrane protein (OmpW), NaCl stress

INTRODUCTION

Cronobacter (formerly known as *Enterobacter sakazakii*) is an opportunistic foodborne pathogen causing

rare but severe infections in newborns (Healy et al., 2012). *Cronobacter* spp. are widely distributed in food and environments including food-processing environments, water, soil, and clinical samples (Muyltjens et al., 1983; Gurtler et al., 2005; Xu et al., 2014; Ye et al., 2014a). A positive correlation between severe infections in newborns and powdered infant formula has also been suggested (Muyltjens et al., 1983, 1988; Biering et al., 1989; van Acker et al., 2001). To date, the genus of *Cronobacter* consists of 7 species: *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muyltjensii*, *C. condiment*, *C. universalis*, and *C. dublinensis* (Joseph et al., 2012a,b).

Recently, tolerance or resistance of *Cronobacter* species to environmental stressors such as osmotic resistance were summarized by Gurtler et al. (2005). Breeuwer et al. (2003) demonstrated that *Cronobacter* cells were more resistant to osmotic stress than *Escherichia coli*, *Salmonella* spp., and other strains of *Enterobacteriaceae*. Increasing the compatible solute concentrations in medium was effective in increasing osmotic resistance of *E. coli* and *Salmonella* (Kempf and Bremer, 1998). Growth of 18 of *Cronobacter* strains from dried edible macrofungi samples in 1.0, 2.5, 4.0, and 5.5% NaCl indicated that *Cronobacter* has unusual ability to survive under NaCl stress (Ye et al., 2014b). To understand the mechanisms involved in osmotic stress response, 53 proteins were identified using 2-dimensional gel electrophoresis coupled with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS (Riedel and Lehner, 2007).

Outer membrane protein W (OmpW) is an important outer membrane protein involved in response to osmotic stress or salt regulation in *Photobacterium damsela* (Wu et al., 2006), *Vibrio parahaemolyticus* (Xu et al., 2004), *Aeromonas hydrophila* (Maiti et al., 2009), and *Vibrio cholerae* (Nandi, et al., 2005). However, little attention has been focused on the role of OmpW in tolerance to osmotic stress in *Cronobacter* species.

In this study, we determined the survival rates and morphologic changes between *C. sakazakii* wild type

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(WT) and OmpW mutant ($\Delta OmpW$) strains under different NaCl stresses. In addition, we assessed the biofilm formation of strains under NaCl stress using crystal violet staining (CVS) assay, scanning electron microscopy, and confocal laser scanning microscopy (CLSM).

MATERIALS AND METHODS

Growth and Survival of *C. sakazakii* Under NaCl Stress

Cronobacter sakazakii GDMCC1409C (WT) and its OmpW mutant ($\Delta OmpW$) were from Guangdong Microbiology Culture Center (GDMCC; Guangzhou, China). Two *C. sakazakii* strains were incubated into normal Luria-Bertani (LB) medium (control), LB with 1.5% NaCl, LB with 3.5% NaCl, and LB with 5.5% NaCl for 8 h. The number of *C. sakazakii* under different media was counted using a colony counting method. Survival rates were calculated as the number of *C. sakazakii* cells in the control sample divided by the number of cells in different NaCl samples. Each experiment was done in triplicate.

Morphologic Changes of *C. sakazakii* Cells Under Different Osmotic Stresses

Cronobacter sakazakii GDMCC1409 was incubated in LB, LB + 1.5%NaCl, LB + 3.5%NaCl, and LB + 5.5%NaCl, and the cells were harvested to detect morphologic changes using scanning electron microscopy (Hitachi, Tokyo, Japan). The treatment procedure was performed according to description by Wang et al. (2013).

Biofilm Formation of *C. sakazakii*

For detection of biofilms by CVS assay, 2 *C. sakazakii* strains were inoculated into LB, LB + 1.5% NaCl, LB + 3.5% NaCl, and LB + 5.5% NaCl and incubated at 37°C for 10 to 12 h with constant shaking. Twenty microliters of overnight culture [optical density (OD) at 600 nm = 0.5] was 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 OD values of each well were measured at 590 nm. Each experiment was done in triplicate.

For scanning electron microscopy, glass coverslips (Jingan, Shanghai, China) were placed into the 48-well plates containing 1.98 mL of LB, LB + 1.5% NaCl, LB + 3.5%NaCl, and LB + 5.5%NaCl, respectively. Then, *C. sakazakii* overnight culture (20 μ L) was mixed with the LB with different NaCl concentrations in 48-well plates (Baiyan, Shanghai, China) for incubation at 37°C for 24, 48, and 72 h. During the biofilm formation period, old medium was replaced with fresh LB at 24-h intervals. The glass coverslips from different incubation times were prepared for scanning electron microscopy as described previously by Wang et al. (2013).

For CLSM, glass coverslips were prepared in the same manner as for scanning electron microscopy detection, and bacterial biofilms on glass slips were stained by using the LIVE/DEAD BacLight bacterial viability kit according to instructions (Invitrogen/Thermo Fisher Scientific, Shanghai, China), and were then observed by CLSM (Zeiss, Berlin, Germany).

Statistics Analysis

Survival rates and biofilm-forming ability using CVS between WT and $\Delta ompW$ under osmotic stresses were assessed in triplicate and analyzed using OriginPro 8.5.1 (OriginLab Corp., Northampton, MA).

RESULTS AND DISCUSSION

As important foodborne pathogens, *Cronobacter* spp. have unusual ability to survive under different environ-

Figure 1. Survival rates of *Cronobacter sakazakii* wild type (WT) and outer membrane protein W mutant ($\Delta OmpW$) under different NaCl stresses. Error bars indicate standard deviations. Asterisks indicate difference between WT and mutant at * $P < 0.05$ and ** $P < 0.01$.

Table 1. Biofilm formation (measured as optical density at 590 nm; means $\pm$ SD) of wild type (WT) and outer membrane protein W mutant ($\Delta OmpW$) under NaCl stress and incubated for 24, 48, and 72 h

Treatment	24 h		48 h		72 h	
	WT	$\Delta OmpW$	WT	$\Delta OmpW$	WT	$\Delta OmpW$
Luria Bertani (LB; control)	0.459 $\pm$ 0.074 ^a	0.640 $\pm$ 0.004 ^b	0.814 $\pm$ 0.044 ^a	1.082 $\pm$ 0.142 ^b	0.554 $\pm$ 0.055 ^a	0.735 $\pm$ 0.090 ^b
LB + 1.5% NaCl	0.403 $\pm$ 0.035 ^a	0.583 $\pm$ 0.067 ^b	0.681 $\pm$ 0.023 ^A	0.916 $\pm$ 0.045 ^B	0.481 $\pm$ 0.025 ^a	0.682 $\pm$ 0.074 ^b
LB + 3.5% NaCl	0.355 $\pm$ 0.009 ^a	0.389 $\pm$ 0.009 ^b	0.494 $\pm$ 0.023 ^A	0.624 $\pm$ 0.015 ^B	0.376 $\pm$ 0.009 ^A	0.421 $\pm$ 0.012 ^B
LB + 5.5% NaCl	0.285 $\pm$ 0.002 ^a	0.300 $\pm$ 0.009 ^b	0.337 $\pm$ 0.006 ^A	0.356 $\pm$ 0.002 ^B	0.291 $\pm$ 0.012 ^A	0.332 $\pm$ 0.006 ^B

^{a,b}Means with different lowercase letters indicate significant differences between WT and $\Delta OmpW$ at the same time point ($P < 0.05$).
^{A,B}Means with different uppercase letters indicate significant differences between WT and $\Delta OmpW$ at the same time point ($P < 0.01$).

Figure 2. Morphological changes of *Cronobacter sakazakii* wild type (WT) and outer membrane protein W mutant ($\Delta OmpW$) under different NaCl stresses using scanning electron microscopy.

mental stresses (Gurtler et al., 2005). In addition, the capsule biofilms formed by *Cronobacter* spp. contribute to survival under environmental stresses (Iversen and Forsythe, 2003). Using a transposon mutagenesis meth-

od, 29 transposon mutants in *C. sakazakii* DPD6529 with impaired growth were determined (Alvarez-Ordóñez et al., 2014). In the current study, we determined the role of OmpW in response to osmotic stress. As

Figure 3. Biofilm formation of *Cronobacter sakazakii* wild type (WT) and outer membrane protein W mutant ($\Delta OmpW$) under different NaCl stresses and incubation for 24, 48, and 72 h using scanning electron microscopy.

shown in Figure 1, survival rates of WT and $\Delta OmpW$ under different NaCl concentrations (1.5, 3.5, and 5.5% NaCl) were $8.06 \pm 0.59\%$ and 7.29 ± 0.315 , $1.64 \pm 0.30\%$ and $0.19 \pm 0.02\%$, and $0.57 \pm 0.19\%$ and $0.14 \pm 0.02\%$, respectively, compared with those in the control (LB medium). In addition, under 3.5% and 5.5% NaCl stress, survival rates of WT were significantly increased

($P < 0.05$) compared with those of $\Delta ompW$. Ye et al. (2014b) noted that the growth of different *Cronobacter* strains from dry edible macrofungi samples under the same NaCl concentrations was distinct. In *Aeromonas hydrophila*, expression of OmpW protein was found to be salt-dependent (Maiti et al., 2009). In *Photobacterium damsela*, OmpW increased the ability of *P.*

Figure 4. Biofilm formation of *Cronobacter sakazakii* wild type (WT) and outer membrane protein W mutant ($\Delta OmpW$) under different NaCl stresses and incubation for 24, 48, and 72 h using confocal laser scanning microscopy. Color version available online.

damselae to tolerate salts. (Wu et al., 2006). Proteomic analysis in *Vibrio parahaemolyticus* indicated that expression of OmpW in 3.5% NaCl was higher than that in 0.85% NaCl (Xu et al., 2004). Expression of OmpW in *Vibrio cholerae* was found to depend environmental factors and might be linked to the adaptive response of the organism under stress conditions (Nandi et al., 2005). With increasing NaCl concentrations, morphological injuries, including cell disruption and shrinkage, were observed among the WT and $\Delta ompW$ strains in the current study, as shown in Figure 2, and injuries of cells were more predominant in $\Delta ompW$ than in WT, suggesting that OmpW contributes to survival of *C. sakazakii* cells under NaCl stress.

Bacterial biofilms formed on surfaces of food processing environments and equipment might be critical factors in persistent contamination by foodborne pathogens (Cappitelli et al., 2014). In the current study, biofilm formation (assessed using CVS assay) by $\Delta ompW$ was significantly increased compared with that by WT under NaCl stress (Table 1). After 24 h, no significant quantitative changes in biofilm were observed, whereas biofilms were significantly reduced after 48 and 72 h compared with control groups. As shown in Figure 3, after 24 h of incubation in LB medium, the 2 strains could not form biofilms and cells were loosely adhered to the glass coverslips. After 48 h of incubation, biofilms with mature and spatial structure were observed in the 2 strains, and disassembly of biofilms occurred after 72 h of incubation. In particular, biofilms in $\Delta ompW$ were more compact and complete compared with those of the WT strain under the same salt concentrations, suggesting the $\Delta ompW$ mutant strain could adapt to NaCl stress by enhancing biofilm formation. As shown in Figure 4, mature and compact biofilms of WT and $\Delta ompW$ were formed after 48 h of incubation at 37°C; after 72 h, dead cells were obvious and were more predominant under 5.5% NaCl compared with other groups. In addition, the biofilms of $\Delta ompW$ were more compact than those of the WT strain. Outer membrane proteins A and X are required for invasion of *C. sakazakii* into biotic cells (Kim et al., 2010). Furthermore, the outer membrane proteins such as OmpC and TolB were found to be involved in biofilm formation in *C. sakazakii* (Li et al., 2015). Ye et al. (2016) demonstrated that incubation conditions, including temperature, time, and NaCl concentration, play important roles in biofilm formation in *Cronobacter* strains. In *Pseudoalteromonas* sp. D41, OmpW mutant formed reduced and altered biofilms (Ritter et al., 2012). Likewise, calcium-induced biofilm was associated with OmpW expression in *Pseudoalteromonas* sp. 1398 (Patrauchan et al., 2005). Proteomic analysis in *Acinetobacter baumannii* showed

that expression of putative OmpW in biofilm mode was significantly reduced compared with that in planktonic mode (Shin et al., 2009). These results suggest that roles of OmpW under NaCl stress depend on the species or environmental conditions. The detailed mechanism of OmpW on biofilm formation in *C. sakazakii* remains to be revealed.

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