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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 (ΔOmpW) were determined. The survival rates of ΔOmpW in Luria-Bertani medium with 3.5% or 5.5% NaCl were reduced significantly, and morphological injury of ΔOmpW was significantly increased compared with survival and morphology of WT. Compared with biofilm formation of the WT strain, biofilms in ΔOmpW 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 ΔOmpW 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 (Muytjens 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 (Muytjens 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. muytjensii, 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 damselae (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 (**Δ*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 (ΔOmpW) 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 μL) 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 ΔompW 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-

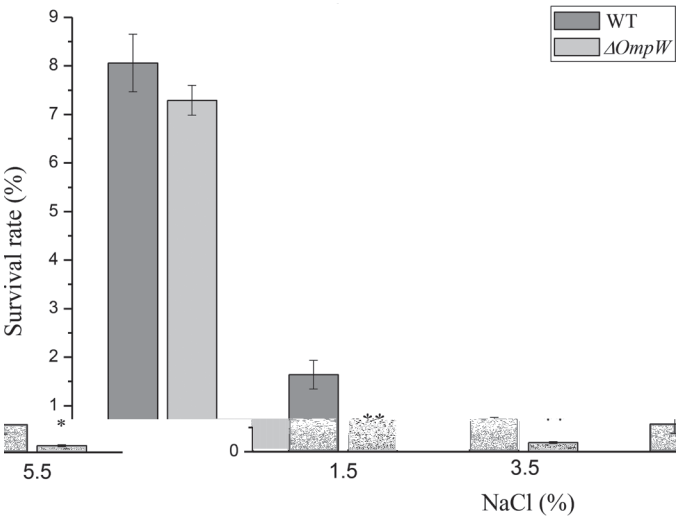

Figure 1. Survival rates of Cronobacter sakazakii wild type (WT) and outer membrane protein W mutant (ΔOmpW) under different NaCl stresses. Error bars indicate standard deviations. Asterisks indicate difference between WT and mutant at *P < 0.05 and **P < 0.01.