Short communication: Roles of outer membrane protein W on survival, cellular morphology, and biofilm formation of *Cronobacter sakazakii* in response to oxidative stress

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ABSTRACT

Cronobacter species are a group of opportunistic food-borne pathogens that cause rare but severe infections in neonates. Tolerance to environmental stress in *Cronobacter* is known; however, factors involved in oxidative stress are undefined. In this study, *Cronobacter sakazakii* survival, cellular morphology, and biofilm formation in response to oxidative stress were evaluated between the wild type (WT) and an outer membrane protein W (*OmpW*) mutant. The survival rates of $\Delta OmpW$ strain after treatment with 1.0 and 1.5 mM hydrogen peroxide were significantly reduced compared with those of WT. Morphological changes, including cell membrane damage and cell fragmentation, in $\Delta OmpW$ were more predominant than those in WT. By crystal violet staining, we also observed increased biomass in $\Delta OmpW$ biofilms as compared with WT following treatment with 0.5 and 1.0 mM H₂O₂. Biofilms using scanning electron microscopy and confocal laser scanning microscopy further confirmed the structural changes of biofilms between WT and $\Delta OmpW$ in response to oxidative stress. The current findings show that *OmpW* contributed to survival of planktonic cells under oxidative stress and the deletion of *OmpW* facilitated the biofilm formation in *C. sakazakii* to adapt to oxidative stress.

Key words: *Cronobacter sakazakii*, outer membrane protein W (*OmpW*), oxidative stress, biofilm formation

Short Communication

Cronobacter species are important food-borne pathogens that cause rare but severe infections in newborns and adults (Holý and Forsythe, 2014; Ye et al., 2014). Powdered infant formula is the main transmission ve-

hicle of *Cronobacter* infections in newborns (Biering et al., 1989; van Acker et al., 2001), and tolerance or resistance to environmental stress in *Cronobacter* species have been reviewed (Gurtler et al., 2005).

Hydrogen peroxide has been widely applied to inactivate *Escherichia coli* O157, *Listeria monocytogenes*, and *Salmonella* (Huang and Chen, 2011; Back et al., 2014; Jiang et al., 2017), because no harmful residues remain following treatment in food engineering applications. Alvarez-Ordóñez et al., (2012) reported significant heterogeneity in RpoS activity and stress tolerance among natural isolates of *Cronobacter sakazakii*. Effects of genes, including *crtX*, *crtE*, and *crtY*, involved in yellow pigmentation of *C. sakazakii* ES5 on oxidative stress have been described (Johler et al., 2010). Under conditions of stress, *E. coli* and related bacteria increase the expression of RpoS, and RpoS-dependent gene expression leads to general stress resistance of cells (Battesti et al., 2011). In *E. coli*, regulators including OxyR, SoxRS, and RpoS were involved in the resistance to oxidative stress (Chiang and Schellhorn, 2012). An RNA chaperone, hfq, has been found to contribute to the tolerance to oxidative stress in *C. sakazakii* ATCC29544 (Kim et al., 2015). Expression of *OmpW* is regulated by environmental factors such as oxidative stress in *Salmonella* and *E. coli* (Gil et al., 2007, 2009; Asakura et al., 2008; Morales et al., 2012).

We evaluated oxidative stress comparing the wild type (WT) *C. sakazakii* GDMCC1409C1 and an outer membrane protein W (*OmpW*) mutant. Construction of the *OmpW* mutant was previously described in Ling et al. (2018). In brief, 2 fragments flanking the targeted genes were amplified independently by PCR using primers containing the attB sequences (outside primers, W5-O: 5'-GGGGACAAGTTTGTACAAAAAAGCAG-GCTACACTCCTTGGTTGGCAGAT-3' and W3-O: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGT-GCCTGCCTGAGCATTTCCTT-3') and linking sequences (inside primers, G5-I: 5'-GGTCCGGGTTC-GCTATCTATACGATAAGTT TTTTCATTCC-3' and

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G3-I: 5'-ATAGATAGCGAACCCGGACCCGGGC-TACCGCTTCTGAGTT-3') with genomic DNA as the template. The fragments were then joined together by fusion PCR using primers W5-O and W3-O as described (Ling et al., 2018). The resulting fusion homology arm was then purified and transformed into plasmid PHGM01 for recombination. Thereafter, the correct destination vector for mutagenesis was transferred by conjugation into *C. sakazakii*. The positive mutant was confirmed by sequencing the mutated regions.

To determine the survival of *C. sakazakii* cells, the enrichment cultures of WT and $\Delta OmpW$ were incubated in sterile Luria-Bertani broth (Luqiao, Beijing, China) with 0.5, 1.0, and 1.5 mM H_2O_2 (Sinopharm Chemical Reagent Co. Ltd., Shanghai, China) at 37°C for 1.0 h in the dark. *Cronobacter sakazakii* cells under oxidative stress were enumerated by plate counts on tryptic soy agar (Luqiao). Bacterial survival rates were calculated as the number of hydrogen peroxide-exposed cells divided by the initial cell number multiplied by 100%. Oxidative stress survival was evaluated by 3 independent experiments. Based on normal distribution of data and homogeneity of variance, significant analysis of survival rates were performed using the Student's *t*-test statistical method with OriginPro 8.5.1 software (OriginLab, Northampton, MA).

From Figure 1A, we found that survival rates of $\Delta OmpW$ after treatment with 1.0 and 1.5 mM H_2O_2 were significantly reduced ($P < 0.05$) compared with those of wild type. The findings might be related to reduction of OmpW expression or changes in the spatial structure of OmpW under oxidative stress. In enterohemorrhagic *E. coli* O157:H7, Asakura et al., (2008) demonstrated that the high level of *OmpW* expression sensitized bacteria to oxidative stress. In *Salmonella enterica* serovar Typhimurium, *OmpW* functions as an efflux channel for toxic compounds generated during oxidative stress (Gil et al., 2009). Gil et al., (2007) also demonstrated that *OmpW* mediated efflux and resistance to paraquat in *Salmonella* Typhimurium and expression of *OmpW* was inactivated by a superoxide. Morales et al., (2012) found that expression of *OmpW* in *Salmonella* Typhimurium was negatively regulated on exposure to H_2O_2 and hypochlorous acid by response regulator AcrA, suggesting a negative correlation between expression of *OmpW* and survival of cells.

To determine the cellular morphological changes of *C. sakazakii* under oxidative stress, the cells were harvested from the overnight culture. Cells were then treated with 0, 0.5, 1.0, and 1.5 mM H_2O_2 for 1 h at 37°C with 180 rpm/min. Scanning electron microscopy was used to observe morphological changes, as described by Wang

Figure 1. Survival and cellular morphology of *Cronobacter sakazakii* wild type (WT) and $\Delta OmpW$ under oxidative stress. (A) Survival rates were counted as the number of the hydrogen peroxide-exposed cells divided by the initial cell number multiplied by 100%; data = means $\pm$ SD. * $P < 0.05$. (B) Morphological changes of *C. sakazakii* WT and $\Delta OmpW$ under 0, 0.5, 1.0, and 1.5 mM H_2O_2 using scanning electron microscopy.

et al. (2013) with little modification. From Figure 1B we see that increasing concentrations of H_2O_2 caused cellular deformation, leakage of intracellular materials, and damage to the cell membrane or cell wall. In addition, morphological injury was more predominant in $\Delta OmpW$, suggesting OmpW might function as an efflux channel of toxic element (Gil et al., 2009). In *E. coli*, the porin OmpW participates in the efflux of ethidium multidrug resistance protein E-specific substrates across the outer membrane (Beketskaia et al., 2014). The *OmpW* expression in *Vibrio cholerae* was modulated by temperature, salinity, and availability of nutrients or oxygen to adapt to stress (Nandi et al., 2005). In *Vibrio alginolyticus*, OmpW expression is associated with the tolerance of high sodium concen-

trations, whereas OmpV does not confer tolerance to sodium (Xu et al., 2005).

Bacterial biofilms on surfaces in processing facilities and equipment are the critical factor for cross-contamination in food samples. To determine the biofilm formation under oxidative stress (0, 0.5, 1.0, and 1.5 mM H_2O_2), crystal violet staining, scanning electron microscopy, and confocal laser scanning microscopy were used as previously described (Ye et al., 2016) with little modification. In Supplemental Table S1 (<https://doi.org/10.3168/jds.2018-14643>), the biofilms of $\Delta OmpW$ significantly increased at 24, 48, and 72 h compared with the biofilms of WT under 0.5 and 1.0 mM H_2O_2 . The highest biomasses were formed after 48 h incubation for 2 strains. To determine the biofilms, the vision

Figure 2. Biofilm formation of *Cronobacter sakazakii* wild type (WT) and $\Delta OmpW$ under oxidative stress using scanning electron microscopy at 24, 48, and 72 h.

Figure 3. Biofilm formation of *Cronobacter sakazakii* wild type (WT) and $\Delta OmpW$ under oxidative stress using confocal laser scanning microscopy at 24, 48, and 72 h.

standing for overall biofilm from 5 locations was chosen. From Figure 2, mature biofilms were formed at 48 h and disassembly of biofilms appeared at 72 h. In addition, the biofilm formation was significantly reduced with the increasing concentrations of H_2O_2 . From Figure 3, the biofilm disassembles at 72 h and dead cells were more predominant compared with biofilms at 48 h. The *OmpW*, *OmpA*, and a type IV pilus were induced in biofilms, and their deletion decreased biofilm formation in *Pseudoalteromonas* sp. strain D41 (Ritter et al., 2012). Likewise, *OmpW* expression showed increased abundance in the calcium-induced biofilm in *Pseudoalteromonas* sp. 1398 (Patrauchan et al., 2005). In *C. sakazakii*, the outer membrane proteins such as *OmpC* and *TolB* were potentially involved in biofilm formation using the 2-dimensional electrophoresis method (Ye et al., 2016). Outer membrane protein A (*OmpA*)

and X (*OmpX*) were required for invasion and adhesion of *Cronobacter sakazakii* to Caco-2 and INT-407 cells (Kim et al., 2010). In *C. sakazakii* ATCC 29544, *FliC*, *FlhA*, and *FliG* contribute to the autoaggregation of cells (Hoeflinger and Miller, 2017). In *V. cholerae*, matrix proteins including *RbmA*, *Bap1*, and *RbmC* play important roles in biofilm formation (Fong and Yildiz, 2015). In addition, the lectins *LecA* and *LecB*, and the *Psl*-binding matrix protein *CdrA* in *Pseudomonas aeruginosa* have also been reviewed to be involved in the biofilm formation (Fong and Yildiz, 2015).

Here, the roles of *OmpW* in *C. sakazakii* under oxidative stress were determined. In the future, the complement of $\Delta OmpW$ will be constructed to further confirm the roles of *OmpW* under other environmental stresses, including desiccation, acid, osmotic, and oxidative stress.

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