Journal of Microbiology (2014) Vol. 52, No. 10, pp. 849–855
DOI 10.1007/s12275-014-4369-5

σ^B Affects Biofilm Formation under the Dual Stress Conditions Imposed by Adding Salt and Low Temperature in *Listeria monocytogenes*

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(Received Jun 25, 2014 / Revised Aug 19, 2014 / Accepted Aug 26, 2014)

The food-borne pathogenic bacteria *Listeria monocytogenes* can form biofilms on various surfaces including food-processing equipment. Biofilms offer survival benefits to the organisms entrapped against environmental insults. Moreover, the σ^B transcription factor of *L. monocytogenes* plays an important role in its survival under various stress conditions. In this study, we evaluated whether σ^B contributes to biofilm formation when *L. monocytogenes* is grown under various temperatures and media. When the wild-type strain was grown under static biofilm culture below ambient temperature (15°C) for 72 h, the difference in viable cell number (in both planktonic and biofilm cells) between the wild-type and Δ*sigB* mutant increased by adding NaCl to BHI broth (9% salt BHI > 6% salt BHI > BHI, w/v), and the specific activity of β-galactosidase was highly induced in the wild-type strain grown in 6% salt containing BHI broth. Furthermore, we measured surface-adhered biofilm forming ability using the crystal violet staining method. The wild-type strain formed a four times larger biofilm than that of the Δ*sigB* mutant in 6% salt-BHI medium at 15°C over a 72 h incubation and also showed the highest level of β-galactosidase specific activity. However, both the wild-type and Δ*sigB* mutant *L. monocytogenes* were defective for forming a biofilm in 9% salt-BHI medium at 15°C. Our results suggest that σ^B plays an enhanced role in surface-adhered biofilm formation when *L. monocytogenes* encounters dual stress conditions, such as 6% NaCl and low temperature.

Keywords: *Listeria monocytogenes*, biofilm, σ^B, low temperature, salt stress

Introduction

Listeria monocytogenes is a Gram-positive food-borne pathogen that causes listeriosis with high mortality rates among susceptible human populations. This bacterium is widely distributed in the environment and can grow in a wide variety of foods, including food stored in the refrigerator, minimally processed, or ready-to-eat foods. Moreover, *L. monocytogenes* can adapt and survive in harsh environments such as high salt concentrations (3 M NaCl) (Cole *et al.*, 1990; Becker *et al.*, 1998; Sleator *et al.*, 2001), low pH (pH 2.5) (Wiedmann *et al.*, 1998), broad pH range (4.5–9.0) (Davis *et al.*, 1996), a wide temperature range (-0.4–45°C) (Walker *et al.*, 1990), and carbon starvation (Ferreira *et al.*, 2001). In addition, *L. monocytogenes* can form a biofilm on food processing equipment, on stainless steel, and buna-n rubber and subsequently disperses to contaminate food products (Wong, 1998; Tompkin, 2002).

Biofilms are structured communities of microorganisms enveloped with a self-produced biopolymer known as extracellular polymeric substance (Costerton *et al.*, 1999). Biofilm-forming bacteria have several survival benefits under a variety of environmental and energy insults. The functions of bacterial biofilms are adhesion on abiotic and biotic surfaces, aggregation of bacterial cells to develop high cell densities, a protective barrier conferring disinfectant resistance, and a nutrient source by providing carbon, nitrogen, and phosphorus for utilization by the biofilm cells (Norwood and Gilmour, 1999; Pan *et al.*, 2006). Therefore, the biofilm forming ability of *L. monocytogenes* constitutes the principal threat in various foods and in the food processing environment.

The general stress transcription factor σ^B largely contributes to the resistance properties to various environment and energy stressors in some Gram-positive bacteria. σ^B is important for survival of *L. monocytogenes* during food processing and storage. Example proteins include GadB, a product that controls expression of glutamate decarboxylase acid stress resistance; LtrC, low temperature requirement C protein; and OpuCA, which is similar to the glycine betaine-carnitine-choline ABC transporter for osmotic stress resistance (Fraser *et al.*, 2003; Kazmierczak *et al.*, 2003; Chan *et al.*, 2007; Hain *et al.*, 2008; Shin *et al.*, 2010b). Thus, the σ^B null mutant shows reduced resistance to acid, salt, temperature, and carbon starvation stressors.

In this study, we determined whether σ^B of *L. monocytogenes* contributes to biofilm formation under various stress conditions. Wild-type *L. monocytogenes* and its isogenic Δ*sigB* mutant were grown under various temperatures and in different media to investigate the role of σ^B in biofilm production. Our results demonstrated that σ^B plays a pivotal role in the production of biofilm when *L. monocytogenes* encounters a dual stress condition, such as salt and tem-

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perature stressors.

Materials and Methods

Strains and culture conditions

Two *L. monocytogenes* strains, wild-type strain 10403S (serotype 1/2a) and an isogenic Δ*sigB* mutant, were used. These strains were obtained from Dr. Martin Wiedmann (Cornell University, USA). *L. monocytogenes* cells were maintained on brain-heart infusion (BHI) (BD, USA) agar or broth, at 37°C. The wild-type and Δ*sigB* mutant *L. monocytogenes* strains carrying a single-copy, σ^B-dependent *opuCA-lacZ* reporter gene fusion were used to determine σ^B activity, which were constructed in our previous study (Shin *et al.*, 2010a). For static biofilm cultures, overnight-grown cultures were adjusted to OD_{600} = 1 in BHI broth, diluted 100 times in BHI broth, and 3 ml of each diluted bacterial solution was cultured in six-well polystyrene cell culture plates (SPL Life Sciences, Korea) at various temperatures (room temperature, 15, 25, 37, and 42°C) in BHI broth and 6% or 9% salt containing BHI broth for 24, 48, and 72 h without shaking.

Viable cell counts

Total cell numbers from both the biofilm-forming and planktonic cells of the wild-type and Δ*sigB* mutant *L. monocytogenes* grown in static biofilm cultures at various temperatures and media were harvested by scraping. Each culture solution was vortexed to disperse the aggregated cells, and then all cells were obtained by centrifugation at 5,000 × *g* for 10 min. After washing the cells with sterile saline (0.85% NaCl, pH 7), they were dispersed in 1 ml saline. The suspensions were then serially diluted in saline, and appropriate dilutions were plated onto BHI agar. The plates were incubated at 37°C for 2 days, and the surviving *L. monocytogenes* were counted as colony forming units. Unattached cells (planktonic cells) were removed to measure viable cells from the surface-attached biofilms of wild-type and Δ*sigB* mutant *L. monocytogenes*. After washing the biofilms in six-well plates, they were detached by scraping, pelleted by centrifugation at 5,000 × *g* for 10 min, and dispersed in 1 ml saline. Viable cells were measured in the same manner as total cells counts. The number of viable cells was determined in at least two independent experiments using two replicates each.

β-Galactosidase assay

A β-galactosidase assay was performed as described by Miller (1972) with a modification for *L. monocytogenes* to measure the σ^B activity indirectly. Wild-type and Δ*sigB* mutant *L. monocytogenes* carrying the fusion reporter genes were grown under static biofilm culture at various temperatures and with various media. After 24, 48, and 72 h of static biofilm culture, all cells (biofilm and planktonic cells) or surface-attached biofilm cells were harvested. Each cell was collected by centrifugation for 1 min at 6,000 × *g* at room temperature. Then, the cells were washed with Z buffer (Miller, 1972), permeabilized by vigorous vortexing for 30 sec in the presence of SDS and chloroform, and incubated at 28°C with *o*-nitrophenyl β-D-galactopyranoside as the substrate. The reaction was stopped by adding 0.5 ml of 1 M Na_2CO_3, and the samples were centrifuged to remove cellular interference. Absorbance was read at 420 nm, and protein concentrations were determined using the Protein Assay reagent (Bio-Rad, USA). Specific activity was defined as $\Delta A_{420\ nm} \times 1000\ min^{-1}\ mg^{-1}$ protein. For all tests, cells were grown and assayed in at least two independent experiment using two replicates each, and representative results are shown.

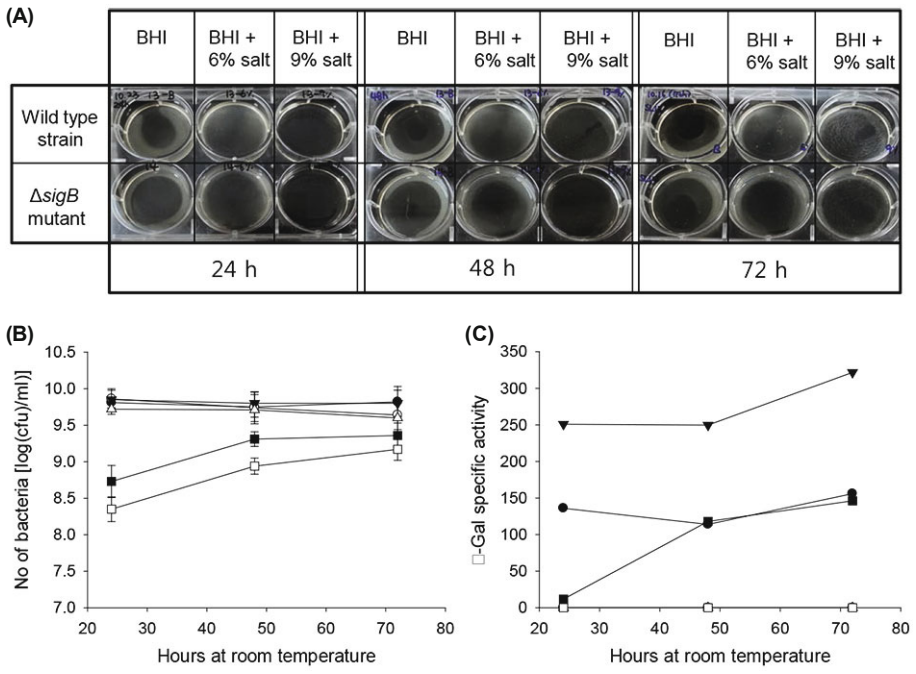

Fig. 1. Characteristics of biofilm culture of wild-type and Δ*sigB* mutant *L. monocytogenes* when cells were grown under salt stress conditions at room temperature (~24°C). (A) Both strains were cultured in six-well polystyrene plates using BHI broth and 6% or 9% salt containing BHI broth for 24, 48, and 72 h. (B) Viable cells were measured after biofilm culture for 24, 48, and 72 h under salt stress conditions. (C) σ^B activity was measured indirectly by a β-galactosidase (β-gal) accumulation assay using single-copy *opuCA-lacZ* reporter fusions under salt stress conditions. (●) wt-BHI, (○) Δ*sigB*-BHI, (▼) wt-6% salt, (△) Δ*sigB*-6% salt, (■) wt-9% salt, (□) Δ*sigB*-9% salt.

Biofilm assay

Surface-attached biofilm formation was assayed in 10 cm diameter Petri dishes using the crystal violet (CV) staining method described by Djordjevic *et al.* (2002) with a modification. Briefly, static biofilm cultures of wild-type and the Δ*sigB* mutant were grown in 10 ml BHI broth or salt-containing BHI broth for 24, 48, 72 h at specified temperatures. To quantify biofilm formation, planktonic cells were removed by pipetting, and the biofilms were washed with 10 ml saline to remove loosely attached cells. The biofilms were stained with 5 ml of an aqueous 0.1% (w/v) CV solution for 30 min. After staining, the Petri dishes were washed with saline and then air dried. The CV bound to the biofilms was fully solubilized with 10 ml 95% ethyl alcohol, and 200 µl was transferred to a polystyrene 96-well plate. The OD_{590nm} was then measured using a microplate reader (Bio-Rad 680). For all testes, cells were grown and assayed in at least two independent experiment using two replicates each.

Results

Static biofilm growth of the wild-type and Δ*sigB* mutant *L. monocytogenes* in salt-containing BHI broth at room temperature

The wild-type and Δ*sigB* mutant *L. monocytogenes* were grown under static biofilm culture mode in six-well polystyrene plates in BHI broth and 6% or 9% NaCl containing BHI broth at room temperature (~24°C) for 24, 48, and 72 h. As shown in Fig. 1A, a growth difference between the wild-type, and Δ*sigB* mutant cells were clearly observed at 72 h when salt was added to the medium. We measured the total number of viable cells (biofilm-forming cells and planktonic cells) from the biofilm cultured cells. Viable cell numbers were similar when both strains were grown in BHI or in BHI-6% salt medium at the three time points (Fig. 1B). When both strains were grown in BHI-9% salt, the numbers of viable cells decreased compared with those obtained in BHI and BHI-6% salt medium, but the difference in viable cell numbers between the wild-type and Δ*sigB* mutant was significant (Fig. 1B). Specific activity of β-galactosidase at the three time points was the highest when wild-type *L. monocytogenes* was grown in BHI-6% salt medium (Fig. 1C). The difference in β-galactosidase specific activity between the wild-type cells grown in BHI and BHI-6% salt medium was about two-fold at each time point (Fig. 1C). Wild-type cells grown in BHI-9% salt showed basal of β-galactosidase specific activity at 24 h, but activity increased drastically at 48 h when the cells started to grow (Fig. 1C). The specific activity of β-galactosidase was not observed in the Δ*sigB* mutant *L. monocytogenes* in any of the media tested (Fig. 1C).

Viable cell counts in the wild-type and Δ*sigB* mutant *L. monocytogenes* grown under static biofilm conditions at various temperatures

The wild-type and Δ*sigB* mutant *L. monocytogenes* were grown under static biofilm culture conditions in six-well polystyrene plates in BHI broth and 6% or 9% NaCl containing BHI broth at 15, 25, 37, and 42°C for 24, 48, and 72 h. After the biofilm culture, all cells (biofilm-forming cells and planktonic cells) were harvested and counted on the BHI plates. The number of viable cells decreased by adding NaCl to the medium at 15°C (Fig. 2A). The largest difference in

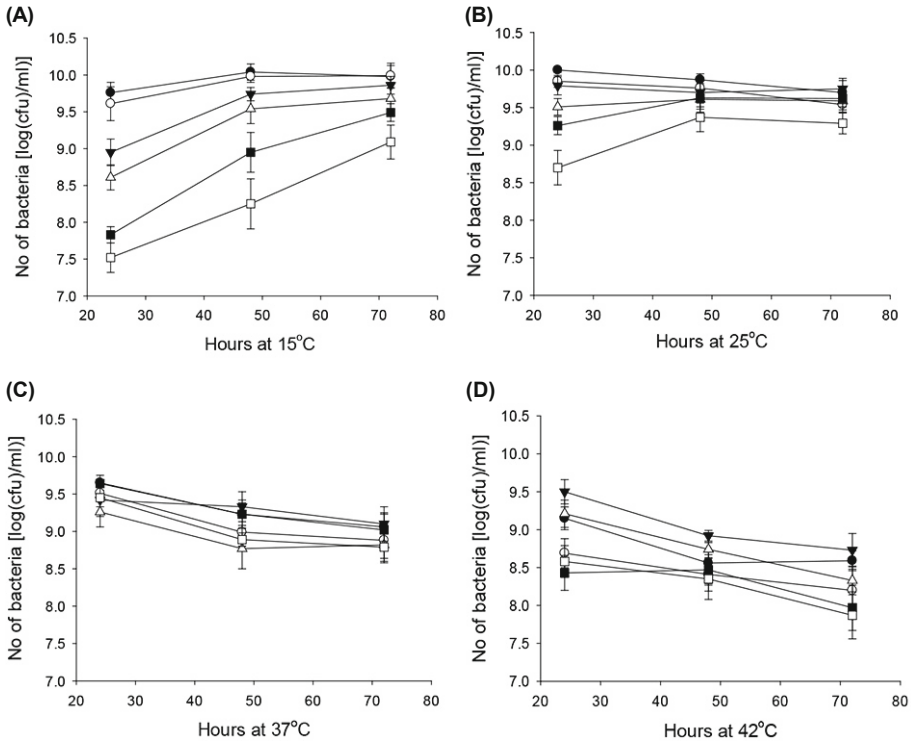

Fig. 2. Viable cell counts using the wild-type and Δ*sigB* mutant *L. monocytogenes* cultured in biofilm mode at different salt concentrations and temperatures. (A) 15°C, (B) 25°C, (C) 37°C, (D) 42°C. (●) wt-BHI, (○) Δ*sigB*-BHI, (▼) wt-6% salt, (△) Δ*sigB*-6% salt, (■) wt-9% salt, (□) Δ*sigB*-9% salt.

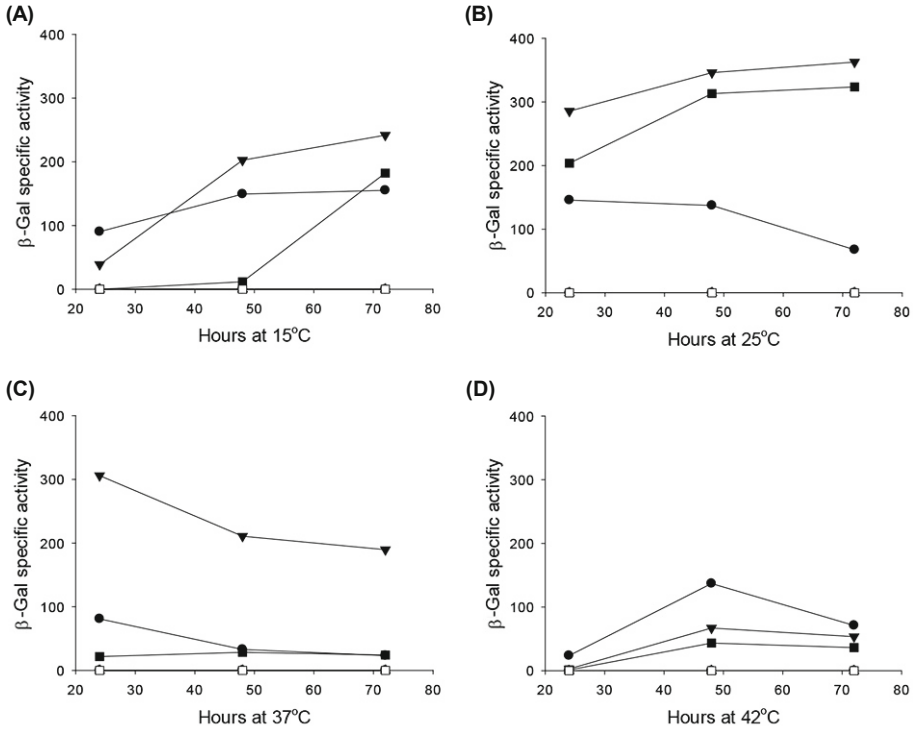

Fig. 3. Measurement of σ^B activity indirectly by a β-galactosidase (β-gal) accumulation assay using the wild-type and Δ*sigB* mutant *L. monocytogenes* carrying the reporter gene fusion cultured under biofilm mode at different salt concentrations and temperatures. (A) 15°C, (B) 25°C, (C) 37°C, (D) 42°C. (●) wt-BHI, (○) Δ*sigB*-BHI, (▼) wt-6% salt, (△) Δ*sigB*-6% salt, (■) wt-9% salt, (□) Δ*sigB*-9% salt.

viable cell numbers was observed between the wild type and Δ*sigB* mutant *L. monocytogenes* in BHI-9% NaCl medium, followed by cells grown in BHI 6% media and those in BHI at 15°C (Fig. 2A). Unlike cells grown at 15°C, the number of viable cells of the wild-type and Δ*sigB* mutant *L. monocytogenes* grown at 25, 37, and 42°C decreased during the 72 h incubation, except cells grown in BHI-9% broth at 25°C (Figs. 2B–2D).

Measurement of σ^B activity in the wild-type and Δ*sigB* mutant *L. monocytogenes* grown under static biofilm conditions at various temperatures

The specific activity of β-galactosidase in wild-type *L. monocytogenes* grown in BHI-6% salt medium increased about five-fold at 48 h compared to the 24 h incubation at 15°C. The specific activity of cells grown in BHI-9% salt medium increased drastically at 72 h compared to that at 48 h. However, the wild-type strain grown in BHI medium without salt observed similar specific activity for 72 h at 15°C (Fig. 3A). In contrast to the specific activity observed at 15°C in BHI-salt medium, β-galactosidase specific activity increased slightly only in BHI-salt media at 25°C (Fig. 3B), but the activities decreased at 25 and 37°C (Figs. 3B and 3C). β-galactosidase specific activity temporarily increased at 48 h when the wild-type strains were grown at 42°C (Fig. 3D). The specific activity of β-galactosidase was not observed in the Δ*sigB* mutant *L. monocytogenes* grown in any of the media or temperatures tested.

Biofilm formation in the wild-type and Δ*sigB* mutant *L. monocytogenes* at 15 and at 25°C

When the wild-type and Δ*sigB* mutant *L. monocytogenes* were grown in salt containing BHI media under static biofilm conditions at temperatures <37°C, the difference in the num-

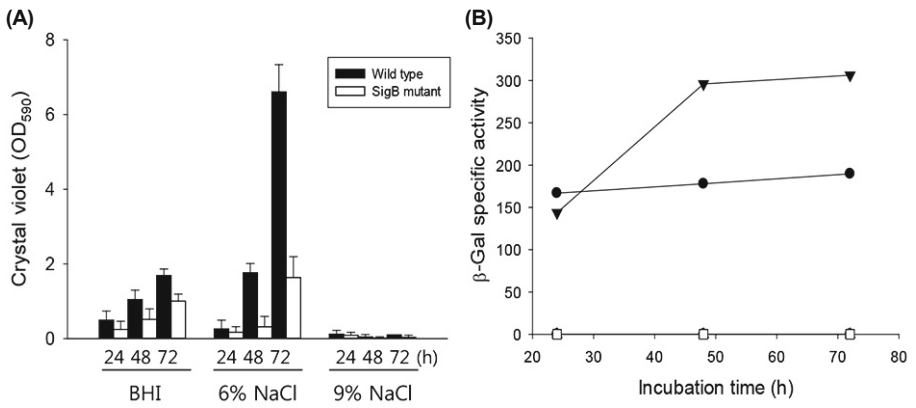

Fig. 4. Characteristics of the wild-type and Δ*sigB* mutant *L. monocytogenes* attached on polystyrene Petri dishes at 15°C in the biofilm mode of growth. (A) The level of surface-attached biofilms was quantified using crystal violet assay at 15°C after 24, 48, and 72 h of biofilm culture in BHI and 6% or 9%-BHI broth. (B) σ^B activity was measured using attached cells grown in biofilm mode at 15°C. (●) wt-BHI, (○) Δ*sigB*-BHI, (▼) wt-6% salt, (△) Δ*sigB*-6% salt, (■) wt-9% salt, (□) Δ*sigB*-9% salt.

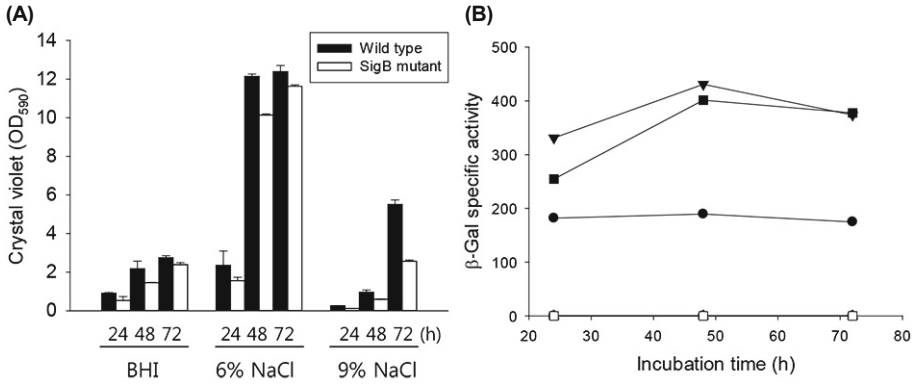

Fig. 5. Characteristics of the wild-type and Δ*sigB* mutant *L. monocytogenes* attached on polystyrene Petri dishes at 25°C in the biofilm mode of growth. (A) The level of surface-attached biofilms was quantified using crystal violet assay at 15°C after 24, 48, and 72 h of biofilm culture in BHI and 6% or 9%-BHI broth. (B) σ^B activity was measured using attached cells grown in biofilm mode at 25°C. (●) wt-BHI, (○) Δ*sigB*-BHI, (▼) wt-6% salt, (△) Δ*sigB*-6% salt, (■) wt-9% salt, (□) Δ*sigB*-9% salt.

ber of total viable cells was significant between the two strains, and β-galactosidase specific activity in the wild-type strain was highly induced. Therefore, to test if σ^B affected biofilm formation when *L. monocytogenes* was grown under salt stress below 37°C, we performed the surface-attached biofilm assay using CV staining. The wild-type strain grown in BHI-6% salt medium at 15°C formed a robust biofilm compared to that of the Δ*sigB* mutant (Fig. 4A). Although the wild-type strain formed biofilm about two-fold larger than that of the Δ*sigB* mutant in BHI medium at 15°C, the biofilm forming ability was much lower than that formed in BHI-6% salt medium at 72 h. The wild-type and Δ*sigB* mutant *L. monocytogenes* grown BHI-9% salt did not form a biofilm the 72 h incubation at 15°C. Moreover, the specific activity of β-galactosidase of the attached wild-type biofilm cells in BHI-6% salt medium was higher than that of wild-type strain grown in BHI broth at 48 and 72 h (Fig. 4B). In contrast, no large difference in biofilm forming ability was observed between the wild-type and Δ*sigB* mutant *L. monocytogenes* grown in BHI-6% salt medium at 25°C (Fig. 5). The wild-type *L. monocytogenes* grown in BHI-9% salt medium formed a biofilm at 25°C during 72 h unlike the cells grown at 15°C (Fig. 4A vs. Fig. 5A), and the specific activity of β-galactosidase was also induced (Fig. 4B vs. Fig. 5B).

Discussion

In this study we demonstrated that the general stress transcription factor σ^B played a pivotal role in *L. monocytogenes* biofilm formation. Our results show that robust biofilms were formed when wild-type *L. monocytogenes* simultaneously encountered salt (6% NaCl) and low temperature (15°C) stressors. Furthermore, σ^B activity was largely induced in the biofilm-forming *L. monocytogenes*.

The role of stress response-related genes involved in *L. monocytogenes* biofilm formation has not been fully characterized. The authors of the first study to identify the role of σ^B in biofilm formation reported that σ^B is not essential for initial surface attachment of *L. monocytogenes* (Schwab *et al.*, 2005). In contrast, another investigator showed that σ^B expression was specifically induced in static and continuous-flow biofilms when cells were grown at 20°C for 48 h in BHI media (van der Veen and Abee, 2010). The role of positive regulatory factor A (PrfA) in formation of the *L. monocytogenes* biofilm has been studied recently. PrfA is a master virulence gene regulator in *L. monocytogenes* and σ^B contributes to *prfA* transcription (Schwab *et al.*, 2005a). The virulence regulator PrfA promotes extracellular biofilm formation in *L. monocytogenes* (Lemon *et al.*, 2010; Zhou *et al.*, 2011). Therefore, our results extend the role of *L. monocytogenes* σ^B in biofilm forming ability.

We measured σ^B activity in the wild-type and the Δ*sigB* mutant *L. monocytogenes* using a β-galactosidase accumulation assay under dual stress conditions to substantiate the functional link between σ^B and biofilm production. As shown in Fig. 4A, the wild-type strain grown in BHI-6% salt medium at 15°C formed a robust biofilm compared to that of the Δ*sigB* mutant. The wild-type strain formed a biofilm at 48 h of incubation that was about seven-fold larger than that after 24 h of incubation, and σ^B activity was also induced rapidly at 48 h. In addition, the biofilm at 72 h of incubation was about four-fold greater than that at 48 h, and σ^B activity was maintained at a high, constant level in the wild-type strain (Fig. 4B). The wild-type and Δ*sigB* mutant cells encountered multiple stressors of cold and salt shock as well as energy stress such as carbon starvation. These multiple stressors triggered σ^B activation, and the activated σ^B largely contributed to biofilm formation in the wild-type strain. We quantified biofilm production under conditions that did not activate σ^B to further substantiate the role of σ^B in biofilm production. For example, neither strain grown in BHI-9% salt medium formed a biofilm after the 72 h incubation at 15°C, and σ^B was not activated (Fig. 4A). Although fewer viable cells were observed in BHI-9% medium than those grown in BHI or BHI-6% salt medium at 15°C, viable cells of both strains increased gradually (Fig. 2A). However, neither strain formed a biofilm under the more severe salt stress conditions compared to that in 6% salt at 15°C (Fig. 4A). These results indicate that *L. monocytogenes* undergo planktonic growth rather than surface attachment when cells encounter 9% salt and low temperature stressors (15°C). Additionally, we found that σ^B activity was more highly induced in biofilm-forming cells than that in planktonic cells. As shown in Fig. 2A, the largest difference in viable cell numbers (biofilm-forming and planktonic cells) was observed between the wild-type and Δ*sigB* mutant *L. monocytogenes* in BHI-9% NaCl medium at 15°C. However, the difference in σ^B activity between the strains was much greater in BHI-6% NaCl at 15°C. As mentioned above, cells grown in BHI-9%

NaCl medium at 15°C underwent mostly planktonic growth, and the difference in σ^B activity between the strains was smaller than that grown in BHI-6% salt medium because the cells grown in BHI-9% salt medium at 15°C did not form a biofilm unlike the cells grown in BHI-6% salt medium at 15°C (Fig. 2A vs. Fig. 3A vs. Fig. 4A).

The wild-type strain formed a biofilm about two-fold larger than that of the Δ*sigB* mutant in BHI medium with no salt at 15°C, but the amount of biofilm was much lower than that formed in BHI-6% salt medium at 15°C (Fig. 4A). Although σ^B activity remained at a relatively constant level (167–190) in the wild-type strain (Fig. 4B), it did not contribute largely to biofilm forming ability. Energy stress may cause σ^B activity in the wild-type strain. Taken together, our results suggest that *L. monocytogenes* σ^B is activated to form a biofilm as a survival mechanism in harsh environments such as 6% salt and 15°C.

In addition σ^B plays an important role in biofilm formation in other Gram-positive bacteria. As an example, Staphylococcal biofilm formation is a complicated process and multiple regulatory factors are involved. The SigB transcription factor mediates both *Staphylococcus epidermidis* biofilm formation through RsbU-dependent regulation and *Staphylococcus aureus* biofilm maturation (Knobloch *et al.*, 2004; Lauderdale *et al.*, 2009). The *ica* operon is involved in σ^B-dependent biofilm formation in these bacteria (Knobloch *et al.*, 2004). However, the activation and requirement of genes regulated by σ^B during *L. monocytogenes* biofilm formation is poorly understood. In this study, we used salt and low temperature stressors to evaluate biofilm forming ability between wild-type and Δ*sigB* mutant *L. monocytogenes*. Salt is commonly used in processed food for storage or flavor. *L. monocytogenes* is able to survive various environmental stress conditions including salt stress and σ^B is largely contributes to this survivability. We performed proteomics analysis to identify proteins involved in biofilm formation in the wild-type strain. As results, 12 proteins such as OpuCA, OpuCC, Lmo0913, Lmo2158, Lmo2748, Lmo0722, Lmo1694, Lmo0911, Lmo0794, SigmaB, Lmo1261, and Lmo0669 regulated by σ^B were identified (data not shown). Among them, OpuCA and OpuCC proteins are similar to the glycine betaine-carnitine-choline ABC transporters that contribute to *L. monocytogenes* survival in the lumens of the small intestine and duodenum with increased osmotic pressure (Wemekamp-Kamphuis *et al.*, 2002), or under low temperature conditions (Wemekamp-Kamphuis *et al.*, 2004). Taken together, our results indicate that both σ^B and its regulon members are activated by stress signals to contribute to biofilm formation when *L. monocytogenes* inhabits locations outside of mammalian host cells. Unlikely outside host cells, activation of σ^B is also required for expression of many virulence proteins inside host cells to infect cells.

In conclusion, our results demonstrate that σ^B plays an important role in biofilm formation through stress signals in *L. monocytogenes*. Interestingly, biofilm forming ability increased under dual stress signals such as high salt concentration and low temperature exposure. σ^B activity was also highly induced in biofilm forming cells. These results suggest that σ^B directly regulates expression of genes related to *L. monocytogenes* biofilm formation and eventually σ^B contributes to increased bacterial fitness outside host cells. The function of σ^B-dependent genes (proteins) that contribute to the biofilm formation remain to be elucidated in future studies.

Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT, and Future Planning (grant no. 2012R1A1A3008038).

References

Becker, L.A., Cetin, M.S., Hutkins, R.W., and Benson, A.K. 1998. Identification of the gene encoding the alternative sigma factor σ^B from *Listeria monocytogenes* and its role in osmotolerance. *J. Bacteriol.* **180**, 4547–4554.

Chan, Y.C., Boor, K.J., and Wiedmann, M. 2007. σ^B-dependent and σ^B-independent mechanisms contribute to transcription of *Listeria monocytogenes* cold stress genes during cold shock and cold growth. *Appl. Environ. Microbiol.* **73**, 6019–6029.

Cole, M.B., Jones, M.V., and Holyoak, C. 1990. The effect of pH, salt concentration and temperature on the survival and growth of *Listeria monocytogenes*. *J. Appl. Bacteriol.* **69**, 63–72.

Costerton, J.W., Stewart, P.S., and Greenberg, E.P. 1999. Bacterial biofilms: a common cause of persistent infections. *Science* **284**, 1318–1322.

Davis, M.J., Coote, P.J., and O'Byrne, C.P. 1996. Acid tolerance in *Listeria monocytogenes*: the adaptive acid tolerance response (ATR) and growth-phase-dependent acid resistance. *Microbiology* **142 (Pt 10)**, 2975–2982.

Djordjevic, D., Wiedmann, M., and McLandsborough, L.A. 2002. Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. *Appl. Environ. Microbiol.* **68**, 2950–2958.

Ferreira, A., O'Byrne, C.P., and Boor, K.J. 2001. Role of σ^B in heat, ethanol, acid, and oxidative stress resistance and during carbon starvation in *Listeria monocytogenes*. *Appl. Environ. Microbiol.* **67**, 4454–4457.

Fraser, K.R., Sue, D., Wiedmann, M., Boor, K., and O'Byrne, C.P. 2003. Role of σ^B in regulating the compatible solute uptake systems of *Listeria monocytogenes*: osmotic induction of opuC is σ^B dependent. *Appl. Environ. Microbiol.* **69**, 2015–2022.

Hain, T., Hossain, H., Chatterjee, S.S., Machata, S., Volk, U., Wagner, S., Brors, B., Haas, S., Kuenne, C.T., Billion, A., and *et al.* 2008. Temporal transcriptomic analysis of the *Listeria monocytogenes* EGD-e σ^B regulon. *BMC Microbiol.* **8**, 20.

Kazmierczak, M.J., Mithoe, S.C., Boor, K.J., and Wiedmann, M. 2003. *Listeria monocytogenes* σ^B regulates stress response and virulence functions. *J. Bacteriol* **185**, 5722–5734.

Knobloch, J.K., Jager, S., Horstkotte, M.A., Rohde, H., and Mack, D. 2004. RsbU-dependent regulation of *Staphylococcus epidermidis* biofilm formation is mediated via the alternative sigma factor σ^B by repression of the negative regulator gene *icaR*. *Infect. Immun.* **72**, 3838–3848.

Lauderdale, K.J., Boles, B.R., Cheung, A.L., and Horswill, A.R. 2009. Interconnections between Sigma B, *agr*, and proteolytic activity in *Staphylococcus aureus* biofilm maturation. *Infect. Immun.* **77**, 1623–1635.

Lemon, K.P., Freitag, N.E., and Kolter, R. 2010. The virulence regulator PrfA promotes biofilm formation by *Listeria monocytogenes*. *J. Bacteriol.* **192**, 3969–3976.

Miller, J.H. 1972. Experiments in Molecular Genetics, pp. 352–355.

Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, N.Y., USA.

Miller, J.M. 1972. A new suture retention bridge. *Arch. Surg.* **104**, 853.

Norwood, D.E. and Gilmour, A. 1999. Adherence of *Listeria monocytogenes* strains to stainless steel coupons. *J. Appl. Microbiol.* **86**, 576–582.

Pan, Y., Breidt, F.Jr., and Kathariou, S. 2006. Resistance of *Listeria monocytogenes* biofilms to sanitizing agents in a simulated food processing environment. *Appl. Environ. Microbiol.* **72**, 7711–7717.

Schwab, U., Bowen, B., Nadon, C., Wiedmann, M., and Boor, K.J. 2005a. The *Listeria monocytogenes prfA*P2 promoter is regulated by sigma B in a growth phase dependent manner. *FEMS Microbiol. Lett.* **245**, 329–336.

Schwab, U., Hu, Y., Wiedmann, M., and Boor, K.J. 2005b. Alternative sigma factor σ^B is not essential for *Listeria monocytogenes* surface attachment. *J. Food Prot.* **68**, 311–317.

Shin, J.H., Brody, M.S., and Price, C.W. 2010a. Physical and antibiotic stresses require activation of the RsbU phosphatase to induce the general stress response in *Listeria monocytogenes*. *Microbiology* **156**, 2660–2669.

Shin, J.H., Kim, J., Kim, S.M., Kim, S., Lee, J.C., Ahn, J.M., and Cho, J.Y. 2010b. σ^B-dependent protein induction in *Listeria monocytogenes* during vancomycin stress. *FEMS Microbiol. Lett.* **308**, 94–100.

Sleator, R.D., Wouters, J., Gahan, C.G., Abee, T., and Hill, C. 2001. Analysis of the role of OpuC, an osmolyte transport system, in salt tolerance and virulence potential of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* **67**, 2692–2698.

Tompkin, R.B. 2002. Control of *Listeria monocytogenes* in the food-processing environment. *J. Food Prot.* **65**, 709–725.

van der Veen, S. and Abee, T. 2010. Importance of σ^B for *Listeria monocytogenes* static and continuous-flow biofilm formation and disinfectant resistance. *Appl. Environ. Microbiol.* **76**, 7854–7860.

Walker, S.J., Archer, P., and Banks, J.G. 1990. Growth of *Listeria monocytogenes* at refrigeration temperatures. *J. Appl. Bacteriol.* **68**, 157–162.

Wemekamp-Kamphuis, H.H., Sleator, R.D., Wouters, J.A., Hill, C., and Abee, T. 2004. Molecular and physiological analysis of the role of osmolyte transporters BetL, Gbu, and OpuC in growth of *Listeria monocytogenes* at low temperatures. *Appl. Environ. Microbiol.* **70**, 2912–2918.

Wemekamp-Kamphuis, H.H., Wouters, J.A., Sleator, R.D., Gahan, C.G., Hill, C., and Abee, T. 2002. Multiple deletions of the osmolyte transporters BetL, Gbu, and OpuC of *Listeria monocytogenes* affect virulence and growth at high osmolarity. *Appl. Environ. Microbiol.* **68**, 4710–4716.

Wiedmann, M., Arvik, T.J., Hurley, R.J., and Boor, K.J. 1998. General stress transcription factor σ^B and its role in acid tolerance and virulence of *Listeria monocytogenes*. *J. Bacteriol.* **180**, 3650–3656.

Wong, A.C. 1998. Biofilms in food processing environments. *J. Dairy Sci.* **81**, 2765–2770.

Zhou, Q., Feng, F., Wang, L., Feng, X., Yin, X., and Luo, Q. 2011. Virulence regulator PrfA is essential for biofilm formation in *Listeria monocytogenes* but not in *Listeria innocua*. *Curr. Microbiol.* **63**, 186–192.