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Comparison of Environmental Stress Tolerance Between *Lactobacillus fermentum* Strains with High and Low Cell Surface Hydrophobicity

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Abstract Previous studies have suggested a possible correlation between cell surface hydrophobicity (CSH) and stress tolerance in *Bifidobacterium*. In this study, the relationship was examined between CSH and environmental stress tolerance in *Lactobacillus* spp. By measuring the adhesion to hexadecane, 2 *Lactobacillus fermentum* strains- KLB 261 and KLB 231 were found to have high and low CSH, respectively. To measure their tolerance to various stresses, cells were subjected to salt (2 M NaCl), acid (pH 2), H_2O_2 (0.01%, v/v), ethanol (20%, v/v), heat (60°C), and cold (−20°C). Compared with KLB 231, the hydrophobic KLB 261 was found to be much more resistant to the various stresses examined. After being subjected to different stresses for a period of time, KLB 261 and KLB 231 showed 50 and 0% survivability in 2 M NaCl, 108.2 and 0.6% in 0.01%(v/v) H_2O_2, 40.2%(v/v), and 3.7% at 60°C incubation, 4 and 0.6% at −20°C, 12.9 and 0.1% in pH 2, 33.8 and 0.2% in 20%(v/v) ethanol, respectively. Autoaggregation test and morphological observation were also conducted in an attempt to explain these differences. These results suggested that high CSH could strengthen the stress tolerance of lactobacilli.

Keywords: *Lactobacillus*, cell surface hydrophobicity, stress tolerance, autoaggregation, scanning electronic microscope (SEM)

Introduction

The relationship between human lactobacilli microbial flora and health has drawn increasingly more interest because of their beneficial role in maintaining health and their properties that make them promising probiotic organisms (1). The genus *Lactobacillus* encompasses a diverse assemblage of Gram-positive, catalase-negative, non-spore-forming, and rod-shaped organisms. They are facultative anaerobes that colonize the moist surface of the gastrointestinal tract, oral cavity, and vaginal epithelium of humans and nonhuman animals (2,3).

The *Lactobacillus* strains we used in this study had been isolated from Korean women's vaginas in a previous study (4). It was first identified in 1894 by a German physician A. Doderlein that *Lactobacillus* spp. was the predominant bacterium in the vaginal microbial flora found in women of reproductive age (2). *Lactobacillus* spp. help to maintain a low pH in the vagina (3,5), while producing various inhibitory compounds such as hydrogen peroxide which can prevent the growth of anaerobic pathogenic bacteria (6,7).

However, to be used as a probiotic, the microbe must endure severe acidic conditions in the digestive system (8). In addition, when they are produced for dietary supplements and dairy products, the microorganism suffers various stresses such as oxidative and temperature during the manufacturing process and storage. Therefore, selection and development of a specific strain with improved features is in need. The strain must possess a certain level of tolerance in the presence of environmental stresses so that it can successfully go through the industrial process, and colonize the target epithelium surfaces and provide benefit to the host.

The cell surface hydrophobicity (CSH) is believed to depend on several components of the cell wall and membrane such as (glycol-)proteinaceous materials, lipoteichoic acid, and lipopolysaccharide (9,10). CSH is believed to be an important factor if microorganisms are to fulfill their function and it has been shown that a hydrophobic cell surface is better than a hydrophilic one in terms of both cell adherence (9) and tolerance to stress (11).

Previous studies have suggested a possible correlation between CSH and stress tolerance in *Bifidobacterium* (11-13), a lactic acid bacterial flora found in the human intestine. Another research on *Salmonella typhimurium* has also indicated that acid adaptation increased in both tolerance towards various stresses and CSH of this bacterium (14). It was also reported that adaptation to benzalkonium chloride is accompanied by the alteration of CSH in *Pseudomonas aeruginosa* (15). Adaptive acquisition of novobiocin resistance in *Pasteurella multocida* resulted in an increase of CSH (16). It is also well known that, sessile cells in biofilm, which often have hydrophobic cell surfaces, tend to show stronger stress tolerance than their planktonic counterparts (17,18), but unfortunately, very little research has ever been done on the relationship between CSH and stress tolerance in planktonic cells. In this study, both strains for comparison belonged to *Lactobacillus fermentum*, but they showed substantial differences on CSH. We presumed that there might be some correlation between CSH and environmental stress tolerance. This study was aimed to find a possible correlation between CSH and tolerance to environmental stresses in vaginal *Lactobacillus* spp.

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Materials and Methods

Bacterial strains and medium The 2 lactobacilli strains used in this study had been isolated from Korean women's vaginas in a previous study (4), both of which were identified as *L. fermentum* by 16s rDNA sequencing. The sequences were deposited in GenBank as follows: *Lactobacillus* sp. KLB 231, *L. fermentum* (EF535258); *Lactobacillus* sp. KLB 261, *L. fermentum* (EF535257).

In this study, the medium used to culture KLB 231 and KLB 261 was MRS medium, which per L contained 20 g glucose (Sigma-Aldrich, St. Louis, MO, USA), 10 g peptone No.3 (Becton, Dickinson and Company, Sparks, MD, USA), 10 g beef extract (Becton, Dickinson and Company), 5 g yeast extract (Showa Chemical, Tokyo, Japan), 5 g sodium acetate (Showa Chemical), 2 g ammonium citrate (Sigma-Aldrich), 2 g potassium phosphate dibasic (Showa Chemical), 1 g Tween 80 (Showa Chemical), and 2 mL trace solution (Nano-Biotechnology Lab, Inha Univ., Incheon, Korea). Both strains yielded very similar growth curves under the culturing condition of this study.

Cell surface hydrophobicity (CSH) measurement CSH was measured according to the method of Rosenberg *et al.* (19) with some modifications. One % of the cells were inoculated, and after culturing for 20 hr, the cells were harvested by centrifugation at 18,000×g, washed, and resuspended twice in phosphate buffered saline (PBS) (pH 7.0) to give OD_{600} ca.1. The absorbance of the cell suspension was measured at 600 nm (A_0). One mL *n*-hexadecane was added to 3 mL cell suspension. The 2 phase system was mixed by vortexing for 2 min and the aqueous phase was removed after 20 min of incubation at room temperature. Its absorbance at 600 nm (A_t) was then measured. The percentage of bacterial adhesion to *n*-hexadecane was calculated as $(1-A_t/A_0)\times100$. The experiments were repeated 3 times and the data were graphed with SigmaPlot 2001.

Salt, H_2O_2, heat, cold, acid, and ethanol tolerance One % of the cells were inoculated, and cultured at 37°C for 20 hr. The cells were then harvested by centrifugation at 18,000×g, and then washed and resuspended twice in PBS (pH 7.0), and resuspended again in fresh modified MRS media. To measure the response to various stresses, cells were subjected to salt (2 M NaCl), acidity (pH 2), H_2O_2 (0.01%, v/v), ethanol (20%, v/v), heat (60°C), and cold (–20°C). Bacterial survival was determined by spot counting on MRS agar medium at different time intervals. Initial concentrations of all samples were 10^9-10^{10} CFU/mL. The experiments were repeated 3 times and the data were graphed with SigmaPlot 2001 (Systat Software Ind., San Jose, CA, USA).

Measurement of autoaggregation Autoaggregation assays were performed as previously described with certain modifications (10,20). Bacteria were grown for 20 hr at 37°C with MRS broth. The cells were harvested by centrifugation, washed twice, and resuspended in PBS. Cell suspensions (4 mL) were mixed by vortexing for 10 sec. Then 0.1 mL of the upper suspension was mixed with 0.9 mL PBS and the absorbance (A_0) at 600 nm was measured. In the same way, absorbance (A_t) was determined after 5 hr of incubation at room temperature. The autoaggregation percentage was expressed as $(1-A_t/A_0)\times100$. The experiments were repeated 3 times and the data were graphed with SigmaPlot 2001.

Morphological observation of KLB 231 and KLB 261 using SEM The samples were analyzed in a scanning electron microscope (SEM) (S-4200; Hitach, Tokyo, Japan) in order to examine the external appearance of KLB 231 and KLB 261. After 20 hr of culture at 37°C, the samples were washed twice and resuspended in PBS. Then 5 μL of resuspension was transferred onto a cover glass and air-dried at room temperature for 3 min. Samples were then fixed with 2.5%(w/v) glutaradehyde in PBS for 3 hr and then incubated in PBS for 12 hr. After that, samples were rinsed twice in the same buffer. Samples were dehydrated with ethanol (10, 25, 50, 75, 95, and finally 100%, v/v) serially and air-dried at room temperature. Cells coated with gold were examined and photographed with a FE (field emission)-SEM (S-4200; Hitach) operating at 10 kV×18 k.

Results and Discussion

CSH of KLB 231 and KLB 261 Previous studies have suggested a possible correlation between CSH and stress tolerance in *Bifidobacterium* (12-14). This study focused on trying to find whether such a relationship exists in *Lactobacillus* spp. The CSH of these 2 stains were tested by measuring their adhesion to *n*-hexadecane. After 20 min of incubation, nearly 80% of KLB 261 cells were absorbed into *n*-hexadecane, while no more than 3% of KLB 231 cells were absorbed, suggesting KLB 261 had a much more hydrophobic cell surface than KLB 231 (Fig. 1).

Survivability of KLB 231 and KLB 261 under salt stress Salt resistance of *Lactobacillus* is very important in food fermentation industry such as the manufacturing of *dongchimi*, a traditional Korean food, because food is often fermented in salty condition (21). In addition, to survive and to grow in the intestinal tract, microorganisms should

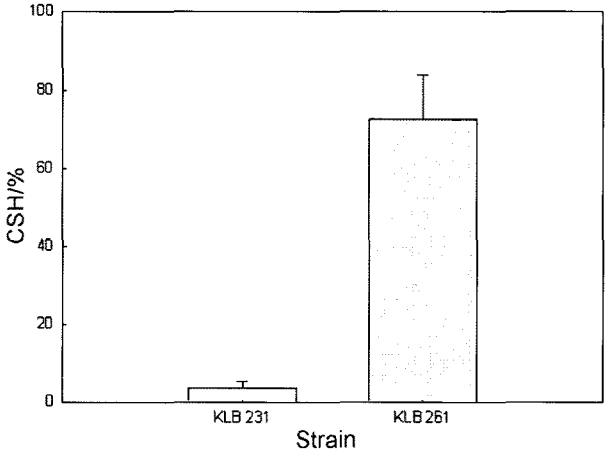

Fig. 1. Cell surface hydrophobicity (CSH) of KLB 231 and KLB 261.

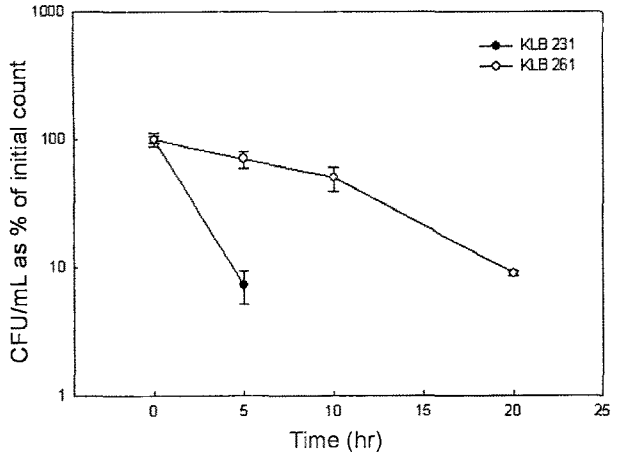

Fig. 2. Survivabilities of KLB 231 and KLB 261 under salt stress.

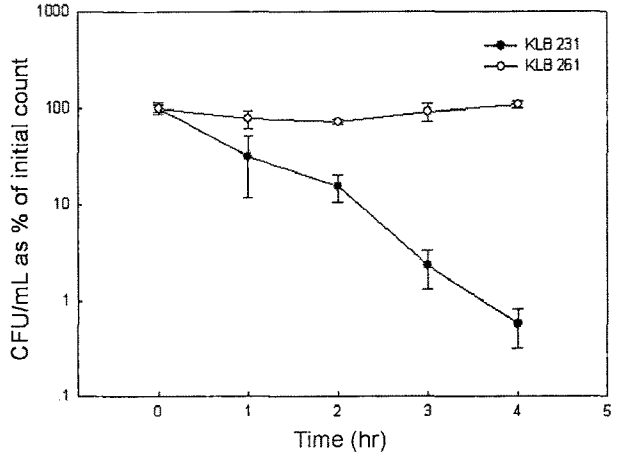

Fig. 3. Survivabilities of KLB 231 and KLB 261 under oxidative stress.

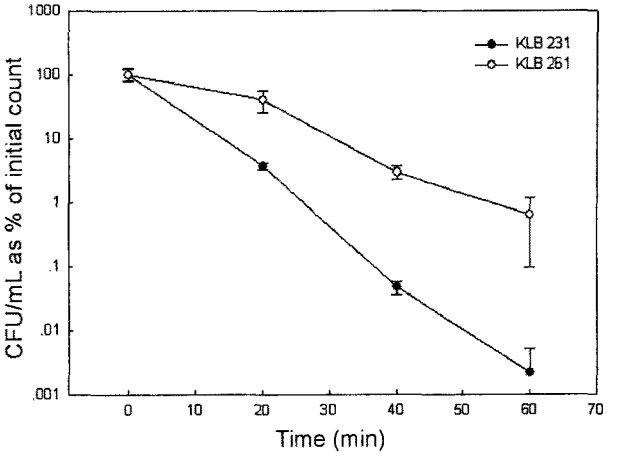

Fig. 4. Survivabilities of KLB 231 and KLB 261 under heat stress.

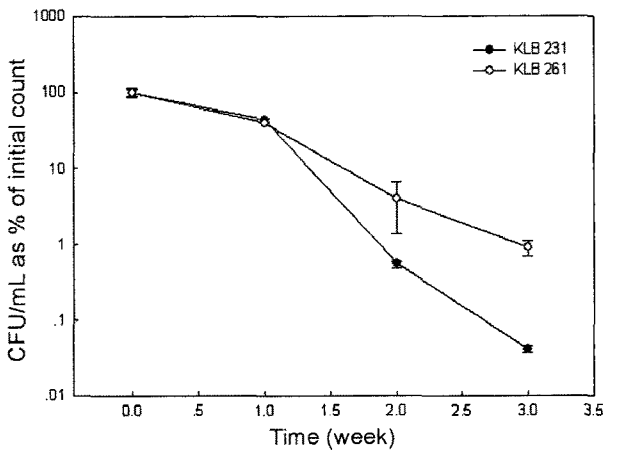

Fig. 5. Survivabilities of KLB 231 and KLB 261 under cold stress.

be salt resistant. Salt tolerance is considered to be an important characteristic of lactobacilli that enables it to survive, to grow and to exert its action in the human body (22). In this study, KLB 231 and KLB 261 showed an obvious difference in salt tolerance after 10 hr incubation in 2 M NaCl. KLB 261 and KLB 231 showed 50 and 0% survivability, respectively, after 10 hr incubation (Fig. 2).

Survivability of KLB 231 and KLB 261 under oxidative stress For lactobacilli, accumulation of hydrogen peroxide was found to be the principal reason for anaerobiosis (23). Moreover, *Lactobacillus* produces H_2O_2 as an antimicrobial (6,7). Therefore, it should be oxidative resistant itself. This study showed that KLB 261 was more tolerant to H_2O_2 than KLB 231 was, with the viable cell count of KLB 261 being increased to 108.2% while that of KLB 231 dropped to 0.6% after 4 hr incubation (Fig. 3).

Survivability of KLB 231 and KLB 261 under heat stress Tolerance to heat is important because *Lactobacillus* is often processed industrially in a heated state. In the manufacturing and processing of cheese milk, *Lactobacillus*, which is preferably heat resistant, can be used as adjunct cultures to improve flavor development (24). In this study, heat shock was administered in 60°C water. After 20 min incubation, the viable cell count of KLB 261 dropped to 40.2%, while that of KLB 231 dropped more dramatically to 3.7%. This result suggested that KLB 261 was more heat tolerant than KLB 231 (Fig. 4).

Survivability of KLB 231 and KLB 261 under cold stress Tolerance to cold is also important if *Lactobacillus* is processed industrially in a cold state. Take yogurt fermentation for example, *Lactobacillus* has to go through a cold storage process (25). In this study, an obvious difference on survivability between KLB 231 and KLB 261 could also be seen in cold storage. After 2 weeks storage at −20°C storage, KLB 261 survived at the rate of 4%, while KLB 231 at 0.55% (Fig. 5).

Survivability of KLB 231 and KLB 261 under acid stress Acid stress tolerance is particularly important to *Lactobacillus* because after entering human body it encounters an acidic condition in the digestive system, such as the gastric acid in the stomach. Besides that, lactobacilli are also found in very acidic vaginal tract (3,5). *Kimchi* is another traditional Korean food, which is produced by fermentation with *Lactobacillus* (26). For application on

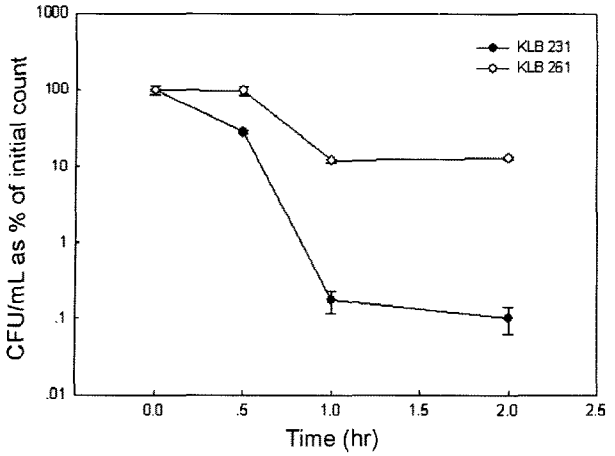

Fig. 6. Survivabilities of KLB 231 and KLB 261 under acid stress.

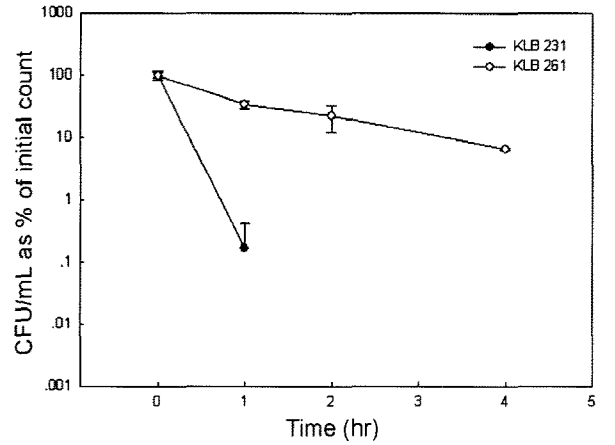

Fig. 7. Survivabilities of KLB 231 and KLB 261 under ethanol stress.

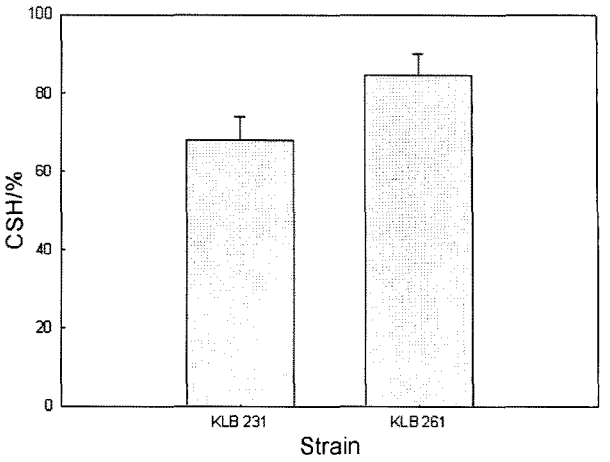

Fig. 8. Autoaggregation abilities of KLB 231 and KLB 261.

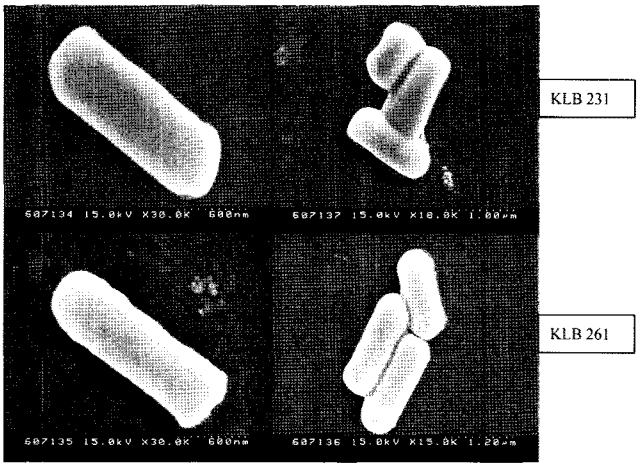

Fig. 9. Morphology of KLB 231 and KLB 261.

kimchi fermentation, probiotics must possess a strong viability in an extremely acidic gastric juice and bile juice (27). In this study, after 2 hr of acid exposure in MRS medium of pH 2, KLB 261 survived at a level of 12.9%, while KLB 231 exhibited 0.1% survivability (Fig. 6).

Survivability of KLB 231 and KLB 261 under ethanol stress Since ethanol is one of the main products in most brewing and fermentation process, when used in brewing and fermentation, high tolerance to ethanol stress of lactobacilli is necessary. Alcohol was identified as one of the flavor compounds of *dongchimi* (21). Therefore, only lactobacilli with strong resistance to ethanol can be possibly used in this fermentation process. The 2 strains exhibited great difference regarding survivability under ethanol stress. KLB 261 had strong tolerance to ethanol, exhibiting a 33.8% survival rate after 1 hr of incubation in 20%(v/v) ethanol, while KLB 231 survived at only 0.2% (Fig. 7).

Autoaggregation of KLB 231 and KLB 261 The mechanisms by which CSH affects stress response are not completely understood, and the effects can be direct or indirect. Cell surface hydrophobicity (CSH) can affect autoaggregation ability of microorganisms (10,20). We were interested in whether autoaggregation ability explains the differences of stress response between strains with high and low CSH. In this study, autoaggregation of KLB 261 turned out to be 25% higher than that of KLB 231 (Fig. 8). Cells with high CSH retain a higher ability to aggregate, and it is also reasonable to assume that compared with individual cells, aggregates of cells can create a more suitable microenvironment for survival.

Morphological observation of KLB 231 and KLB 261 using SEM In terms of the mechanism by which CSH affects stress tolerance, no direct explanation has been made in previous studies. We hypothesized that the differences on CSH and stress tolerance between KLB 231 and KLB 261 were due to differences of their cell surface structure. SEM pictures of both strains were taken in an effort to observe the surface structural differences. The pictures revealed that KLB 231 had a smooth cell surface, while KLB 261 had a rough one. Additionally, the cell surface of KLB 231 was translucent, while the cell surface of KLB 261 appeared coated by a thick layer of opaque material (Fig. 9). We believed that some components of the cell surface could make the sell surface hydrophobic, while serving as 'protectors' at the same time. More convincing explanations, however, need and deserve more research in the future.

Acknowledgement

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