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RESEARCH NOTE

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Stress Tolerance of *Bifidobacterium infantis* ATCC 27920 to Mild-heat Adaptation

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Abstract Two-dimensional gel electrophoresis (2-DE) was employed to assess the thermo-tolerance characteristics of *Bifidobacterium infantis* ATCC 27920 to mild heat adaptation. When exposed to various heat levels, pH, and hydrogen peroxide (H_2O_2) stress conditions, *B. infantis* ATCC 27920 exhibited high level of stress resistance. Under mild-heat treatment (46°C), no significant change in viability level was observed after 2 hr. Interestingly, improved viability was observed in mild-heat adapted (46°C for 1 hr) cultures exposed to 55°C, in comparison to control experiments. Viability was not affected by pH, bile, and H_2O_2 stress conditions. 2-DE analysis revealed those mild-heat adaptation up-regulated 4 proteins and down-regulated 3 proteins. Among these protein spots, isopropylmalate dehydratase (leuD), glycosyltransferase (glgA), and ribosomal protein L5 (rplE) were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS).

Keywords: *Bifidobacterium infants*, thermotolerance, mild heat stress, 2-dimensional gel electrophoresis

Introduction

The genus *Bifidobacterium* consists of Gram-positive, non-motile, strictly anaerobic microorganisms, which according to individual ecological niches, can be found in the human intestinal tract, oral cavity, animal gastrointestinal (GI) tract, insect intestine, and sewage (1).

In recent years, research on certain *Bifidobacterium* strains have confirmed that they contribute to maintenance of gastrointestinal health, including the prevention of diarrhea and balancing of intestinal microbiota (2,3). As a result, these strains are regarded as probiotic bacteria, and are often used as viable bacterial species in dairy products (e.g., yogurt) or as supplements in infant food production. The industrial application of these probiotic bacteria requires large scale processing. However, under stressful food processing conditions, including various acid, alkali, heat, and salt levels, the stability and viability of probiotic bacteria are dramatically reduced. Heat stress, in particular, may impose limitations on bacterial viability. As a result, the dairy industry uses capsule techniques, such as microencapsulation, in an effort to improve the survival and delivery of bacterial cultures (4).

Interestingly, it has been reported that certain Gram-positive bacteria, including *Bifidobacterium,* could develop adaptive responses when subjected to moderate stress conditions (5). However, specific bacterial responses under stress stimuli are not fully understood within genomic or proteomic levels. Recently, investigations have attempted to address the paucity of knowledge related to specific stress-associated proteins by focusing on DNA or protein levels, thereby highlighting the importance of chaperone proteins in stress tolerance of certain *Bifidobacterium* species (6,7).

Although heat shock proteins such as DnaK, DnaJ, GrpE, GroES, GroEL, and proteases (Clp, HtrA, FtsH) have been identified and found to be well conserved in lactic acid bateria (LAB), the stress-induced regulatory mechanisms are still unknown (8).

In particular, proteomic studies measuring functional protein expression levels have been extensively utilized for the characterization of bacterial responses to a variety of stimuli and stresses (5,9,10). However, proteome analysis using 2-dimensional gel electrophoresis (2-DE) have not been widely employed in relation to *Bifidobacterium* strains (7).

Employing 2-DE, we studied the thermo-tolerance characteristics of *Bifidobacterium infantis* ATCC 27920 to mild heat adaptation, and also conducted mild heat adaptation-associated protein profiling.

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Materials and Methods

Bacteria and culture medium *Bifidobacterium infantis* ATCC 27920 strains were routinely cultured at 37°C for 24 hr in blood-glucose-liver broth (BL) (11) without blood, overlaid with liquid paraffin oil, using an anaerobic chamber system (MACS 1000; Don Whitley Scientific Ltd., Shipley, UK). The bacteria were sub-cultured at least twice prior to use. All chemicals were obtained from the Sigma-Aldrich (St. Louis, MO, USA).

Heat adaptation In order to assess the tolerance of *B. infantis* ATCC 27920 against various temperatures, bacteria were inoculated in BL broth and incubated at 37, 46, and 55°C for 2 hr in a water bath, respectively. Subsequently, the serial dilutions (anaerobic) of these suspensions were

plated onto BL agar, and the surviving bacteria were counted after 48 hr at 37°C under anaerobic conditions, as described above. For mild-heat adaptation, diluted *B. infantis* ATCC 27920 (ca. 10^7 CFU/mL) in BL broth was transferred to 46°C for 1 hr. Control cultures or mild-heat adapted cultures were then harvested by centrifugation, resuspended in fresh medium, and exposed to 55°C. The survival rates were normalized as 100% viability by control survival.

Preparation of protein samples Overnight cultured *B. infantis* ATCC 27920 cells were inoculated in 100 mL of BL medium and incubated at 37°C (control) and 46°C (mild-heat shock) for 1 hr, respectively. Total proteins were prepared by previously described methods (12).

2-DE In order to perform the first dimension, each of the samples was mixed with rehydration solution [8 M urea, 2% CHAPS, 50 mM dithiothreitol (DTT), 10% isopropanol, 5% glycerol, 0.5% ampholytes at pH of 3-10, and a trace of bromophenol blue], resulting in a final protein content of 700 μg in a 300 μL total volume. Isoelectric focusing (IEF) was conducted using a PROTEAN™ IEF cell (Bio-Rad, Hercules, CA, USA), in accordance with the manufacturer's instructions, under the following conditions: 250 V (30 min), 500 V (30 min), 1,000 V (30 min), 8,000 V (2 hr), 8,000 V (35,000 Vhr), and 500 V (15 min). After IEF separation, the strips were incubated for 10 min in equilibration buffer I [6 M urea, 2% sodium dodecyl sulfate (SDS), 20% glycerol, 130 mM DTT, 0.375 M Tris-HCl, pH 8.8], and then for an additional 15 min in equilibration buffer II (6 M urea, 2% SDS, 20% glycerol, 135 mM idoacetamide, 0.375 M Tris-HCl, pH 8.8). After equilibration, the strips were transferred to 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) gels (20×22 cm) for running of the second dimension. Separation was conducted using a PROTEAN™ II xi system (Bio-Rad) with 10 mA/gel for 1 hr, and thereafter with 20 mA/gel at 4°C. The protein spots were visualized by blue-silver staining (19). The stained gels were then scanned with a densitometric scanner (800×1,600 dpi, UTA 2100XL; UMAX, Dallas, TX, USA), and the spots analyzed using PDQuest software (Bio-Rad) in accordance with the manufacturer's instructions. Four gels resulting from 2 independent experiments were acquired, while 2 gels of good quality were utilized for analysis. Only significant spot intensity changes (at least 2.5-fold) were considered and selected for MALDI-TOF/MS analysis.

Protein identification by MALDI-TOF/MS The sample preparation for MALDI-TOF/MS in-gel digestion was conducted by previously described methodology (12). An Ettan MALDI-TOF (Amersham Pharmacia Biotech, Uppsala, Sweden) system with an UV nitrogen laser (337 nm), delayed extraction, low mass rejection, and a harmonic reflection was employed in positive ion reflector mode for peptide mass mapping. The ion acceleration voltage was 20 kV. The spectra were calibrated with internal calibration using trypsin autodigestion fragments. Three-hundred single shots were accumulated for each spectrum. The obtained spectra were subjected to a ProFound (http://129.85.19.192/profound_bin/WebProFound.exe) search for protein identification.

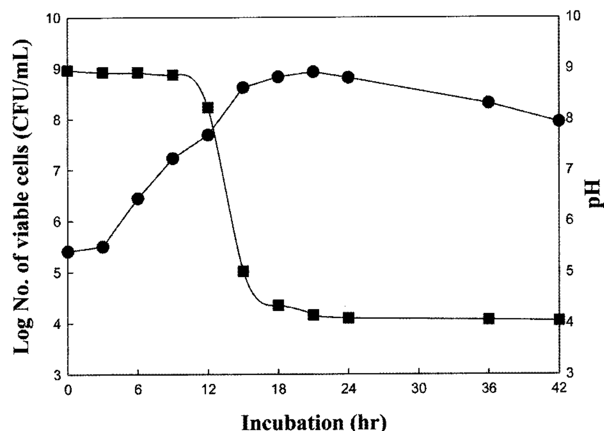

Fig. 1. Changes of viability (●) and pH (■) in *Bifidobacterium infantis* ATCC 27920 during incubation at 37°C in BL medium.

Statistical analysis All experiments were triplicated. Mean and standard deviation of each treatment was acquired using the SAS software package (Version 9.1; SAS Inc., Cary, NC, USA). The level of significance was established at $p<0.05$.

Results and Discussion

Growth of *B. infantis* ATCC 27920 Viable cells and pH levels of *B. infantis* ATCC 27920 were recorded during fermentation, as shown in Fig. 1. Rapid increase of *B. infantis* ATCC 27920 was observed from 3 to 15 hr. Viable cells were initially estimated to be 5.4 log CFU/mL at 0 hr, eventually reaching a maximum of 8.9 log CFU/mL at 21 hr. It took approximately 6 hr to reach mid-log phase, and approximately 21 hr to establish stationary phase. The process of acidification caused a drop in pH values over the 42 hr fermentation period, with a more rapid decline between 3 and 18 hr.

Effect of heat adaptation on the survival of *B. infantis* ATCC 27920 Figure 2 shows the survival of heat-adapted and non-adapted *B. infantis* ATCC 27920 in MRS broth during exposure at 55°C. The viable population of *B. infantis* ATCC 27920 declined as exposure period extended, regardless of heat adaptation. However, viable population of heat-adapted cells declined less rapidly. After 2 hr of exposure, heat-adapted *B. infantis* ATCC 27920 showed a viable population of ca. 3.5 log CFU/mL, which was significantly ($p<0.05$) higher than that of 1.3 log CFU/mL found with non-adapted *B. infantis* ATCC 27920. Viable population of *B. infantis* ATCC 27920 was not affected by the exposure of 1% H_2O_2 and acidic pHs (data not shown). Similarly, heat shock at 45°C for 30 min did not influence the growth rate of *L. rhamnosus* HN001. Also osmotic shock of *L. rhamnosus* HN001 with 0.3M NaCl did not affect growth (13).

Comparative proteomes of *B. infantis* ATCC 27920 Protein expression profiles between conventional culture *B. infantis* ATCC 27920 and heat adapted *B. infantis* ATCC 27920 were analyzed using 2-DE and MALDI-TOF/MS. Figure 3 shows 2-D gels analyzed together, while

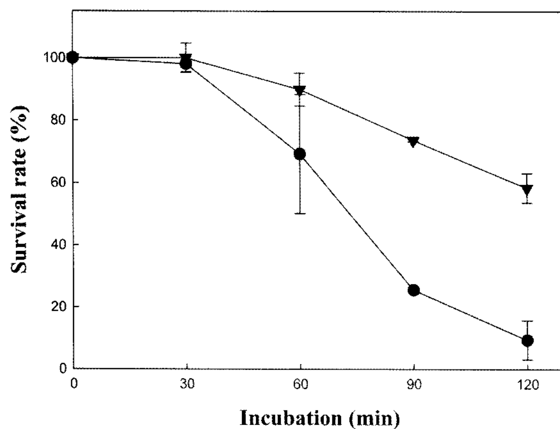

Fig. 2. Changes of viability in *Bifidobacterium infantis* ATCC 27920 exposed to heat treatment (55°C). (●) Control (unadapted) cells; (▼) heat adapted cells (46°C, 60 min). Overall means and standard errors were calculated from triplicate trials.

approximately 300 protein spots were visualized by CBB G-250 staining. Comparative proteomes obtained under heat adaptation showed that the intensity of four protein spots (A, B, C, and D) increased (≥2), while 3 spots (E, F, and G) decreased. Spot D appears to be the ribosomal protein L5, which is an important component of the ribosome and is most probably required for the translation of most, if not all, natural mRNAs (14).

Ribosomal protein L5 is induced in *Bacillus subtilis* in response to various stresses such as salt, heat, and nutrient limitations (15). The high expression level of ribosomal protein after heat stress could indicate that protein synthesis is sustained post shock.

However, Ventura *et al.* (16) reported that in case of *Bifidobacterium breve*, the DnaK operon was not induced at mild-heat shock temperatures of 43°C (16). *Bifidobacterium adolescentis* have demonstrated that the expression of the DnaK can be induced by both bile salts and heat-shock treatments (17). The different regulatory patterns displayed by these 2 major chaperone systems probably reflect their distinct and complementary roles in stress responses as already described in other bacterial species (18).

In relation to down regulated proteins, 2 regulatory factors (spot F and G), isopropylmalate dehydratase and glycosyltransferase were identified. Isopropylmalate dehydratase and glycosyltransferase catalyze the transfer of a glycosyl moiety, either through retention or inversion of configuration, such as peptidoglycan biosynthesis.

The preparation of bifidobacteria-containing products may

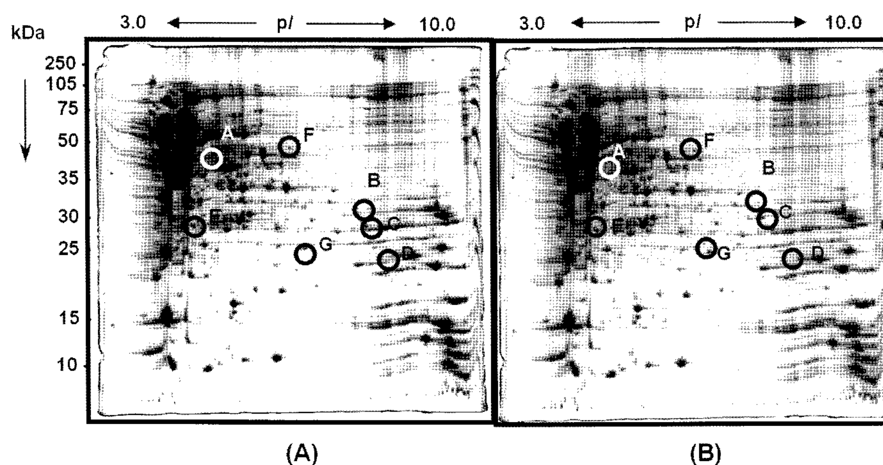

Fig. 3. Two-dimensional gel electrophoresis images of *B. infantis* ATCC 27920 in control (A; 37°C) and mild-heat shock (B; 46°C) conditions. The crude protein extracts were separated on pH 3.0 to 10.0 non-linear IPG strips, followed by 12.5% SDS-PAGE. Gels were stained with colloidal Coomassie blue (G250). Induced and repressed proteins are encircled with a line.

Table 1. Identification of up-regulated *B. infantis* ATCC 27920 proteins under exposure to mild-heat shock (46°C)

Spot No.[1]	Gene	Protein identification	Regulation	Mr (Da)[2]	Sequence coverage[3] (%)	Accession No.[4]
A	-	Unidentification	↑ (-2.5)			
B	-	Unidentification	↑ (-2.5)			
C		Unidentification	↑ (-2.7)			
D	*rplE*	Ribosomal protein L5	↑ (-2.7)	21.39	48	gil23336513
E	-	Unidentification	↓ (+2.5)			
F	*leuD*	Isopropylmalate dehydratase	↓ (+2.8)	50.49	33	gil23335994
G	*glgA*	Glycosyltransferase	↓ (+2.5)	72.14	16	gil23464826

[1]The spot numbers indicate CBB G-250 stained spots labeled in Fig. 3.
[2]Theoretical molecular weight.
[3]The sequence coverage provides an indication of confidence of identification.
[4]Accession No. refers to the SWISS-Prot/TrEMBL databases.

require microbes to survive industrial food manufacturing processes involving heat shock treatments and dehydration. Therefore, understanding the genetic basis of heat stress response may be crucial for selection of new bifidobacterial strains displaying probiotic properties. Stress adaptation mechanisms in *Bifidobacterium* strains are still poorly characterized and understood. Studies characterizing stress responses at molecular or physiological levels have focused on heat, acid, oxygen, and bile-adaptation processes (5,6,9, 20).

In relation to heat shock genes, transcription analysis of the major chaperones and protease encoding genes, using traditional techniques such as Northern blot hybridization and primer extension assay, have been performed by Ventura *et al.* (21-23). These analyses targeted predicted *hsp* genes such as *groEL, groES, dnaK, grpE, dnaJ1, dnaJ2, clpB, clpC*, and *clpP1P2*. In this study, rRNA binding protein RplE may have been identified as being responsible for the stress response protein. This protein specifically binds to the conserved site of rRNA and is only produced by certain bacteria under stress conditions.

Acknowledgments

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