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Isolation and Characterization of Lactate-Tolerant Mutants in *Bifidobacterium breve*

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Abstract The growth of *Bifidobacterium breve* strain HP2 was completely inhibited by the addition of lactate higher than 4.0% but not by the addition of acetate. Two kinds of lactate-tolerant mutants were isolated by the nitrosoguanidine treatment, enrichment on a liquid medium with 5% lactate, and selection on agar plates with 5% lactate. The mutants were not only able to grow in the presence of 5% lactate but also improved in viable cell stability in the acidic pH range. In a pH-controlled fermentor, mutant N-1-5 grew at a rate slower than that of the wild type but its growth yield was higher. Notably, mutants were more halotolerant and more osmotolerant than the wild type and they were able to grow in the presence of 3% NaCl or 25% lactose at which the wild type entirely stopped the growth. The enzyme activities involved in the lactose metabolism in *B. breve* were measured to elucidate the biochemical basis for lactate tolerance. In the mutants, activities of several enzymes including phosphoglucomutase decreased compared to the wild-type, which may explain their lower growth rate. However, the activity of lactate dehydrogenase or its nature of inhibition by lactate was not altered.

Key words: *Bifidobacterium breve*, lactate-tolerant mutant, fermentation.

Bifidobacteria which are gram-positive, non-motile, strictly anaerobic and rod-shaped bacteria [16] have attracted a great deal of attention due to their beneficial influence on human and animal health [7, 18]. The main health and nutritional benefits of bifidobacteria in the intestine include the following categories: (a) inhibition of the growth of many pathogenic and putrefactive bacteria by lowering the intestinal pH through the liberation of lactic acid and acetic acid [14]; (b) suppression of tumorigenesis by breaking down carcinogenic *N*-nitrosamines or by stimulation of immnological responses [6, 7]; (c) prevention or alleviation of antibiotic-associated diarrhea [11]; (d) improved lactose tolerance of milk products [7]; (e) synthesis of B-complex vitamins [3]; and, (f) reduction of the level of cholesterol in serum [4].

Bifidobacteria have been known generally to be the most predominant group of intestinal flora in infants and the third or fourth in adults as they reduce in number with aging [15]. Also, many endogeneous and exogeneous factors such as a variety of diseases, administration of antibiotics and emotional stress can generate an abnormal flora, which is generally characterized by the reduction or disappearance of bifidobacteria and by an increase in other pathogenic or putrefactive bacterial counts [16]. Therefore, a variety of probiotic preparations including foods fermented or supplemented with bifidobacteria and preparations of freeze-dried bifidobacterial cells have been made available commercially [5, 7, 17]. There are, however, some problems in the application of bifidobacteria for establishing balanced flora in the human intestine because bifidobacterial cells are relatively labile under the conditions encountered within the digestive tract and during the period from production of the products to consumption [20].

Recently, we reported the optimization of fermentation conditions for the production of cell mass and differential saccharide fermentation by *Bifidobacterium longum* and *B. breve* [10]. The aims of this research, therefore, are to isolate the mutant strains of *B. breve* which are tolerant to lactate and more stable under acidic conditions, and to characterize their properties to assess their potential for industrial application.

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MATERIALS AND METHODS

Bacterial Strains, Chemicals, and Media

B. breve HP2 was isolated from feces of an infant and identified according to the procedures described by Mitsuoka [15]. The strain was cultivated in MRL medium (pH 6.8) consisting of lactose 20 g, peptone 10 g, yeast extract 5 g, beef extract 10 g, sodium acetate 5 g, ammonium citrate 1 g, Tween 80 1 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, $MnSO_4 \cdot 7H_2O$ 0.05 g, and K_2HPO_4 2 g per liter of distilled water. Analytical coupling enzymes, coenzymes, and saccharides were obtained from Sigma Chemical Co. (St. Louis, U.S.A.). All other chemicals used were of reagent grade.

Cultivation Conditions

Organisms were maintained by anoxic and stringent culture techniques [8], and routinely grown at 37°C in anaerobic pressure tubes (Bellco Glass, Inc., Vineland, U.S.A.) containing 10 ml of MRL medium with a nitrogen gas headspace. The cultures were mixed with 30% glycerol at a ratio of 1:1, frozen, and stored in a deep freezer. Experimental cultures were grown without shaking in N_2-gassed 125-ml Wheaton serum bottles containing 50 ml of MRL medium. Fermentation time course studies were conducted in a 5-l jar-fermentor (Korea Fermentor Co., Inchon, Korea) that contained 2.5 l of MRL medium. Fermentors were agitated at 200 rpm, gassed initially and at the time of sampling with N_2 gas, and controlled at pH 6.0 with 10% sodium hydroxide solution.

Mutagenesis and Isolation of Mutants

The following procedures were performed using anaerobic techniques. The cultures grown on MRL medium to the mid-logarithmic growth phase ($O.D_{660} = 1.5$) were centrifuged, suspended in fresh MRL medium, and then treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG; 300 μg/ml) at 37°C for 4 h. NTG treatment resulted in the cell viability of less than 1%. The treated cells were washed twice with a diluent (pH 7.0) consisting of KH_2PO_4 4.0 g, Na_2HPO_4 6.0 g, L-cysteine·HCl 0.5 g, Tween 80 0.5 g, agar 1.0 g, and NaCl 8.5 g per l of distilled water, and then suspended in MRL medium. After incubation at 37°C for 4 h, the cells were transferred into MRL medium that contained different concentrations of lactate and the pH was adjusted to 7.0 with NaOH, and incubated until an increase of cell concentration was detected. The mutant cells enriched in lactate medium were plated on MRL agar medium with 4% lactate, placed in an anaerobic jar with an anaerobic system for generating H_2 and CO_2 (Difco Lab. Detroit, U.S.A.), filled with N_2 gas, incubated at 37°C for 2 days, and selected.

Quantification of Cell Growth, Lactate, Acetate, and Lactose

For determination of culture turbidities, culture broths were appropriately diluted with distilled water and the optical densities were measured at 660 nm using a Gilford spectrophotometer. To measure the stability of viable cells, the cultures were centrifuged, suspended in a solution that consisted of L-cysteine·HCl 0.1 g/l, NaCl 0.4 g/l, and HCl 1.13 g/l and the pH was adjusted to 2.0~5.0 with NaOH, and incubated at 37°C. During the incubation, the cells were removed, appropriately diluted with a diluent, plated on MRL agar medium, and incubated under the same conditions as above. Lactate was enzymatically determined using Sigma Diagnotics lactate reagents (Sigma Chemical Co.) and acetate was analyzed using a gas chromatograph (SRI Instrument, Inc., U.S.A.). Lactose was estimated either by the dinitrosalicylic acid method [1] or by the Somogi-Nelson method [19, 22].

Preparation of Enzymes

For preparation of the cell extracts, cells were grown in a 5 l fermentor (Korea Fermentor Co., Korea) containing 3 l of MRL medium, which was agitated at 200 rpm and gassed with N_2. The cells grown to the late exponential growth phase were harvested by centrifugation at 10,000×g for 10 min. Cell suspensions were prepared anaerobically by suspending about 3 g of wet cell paste in 15 ml of 2 mM $MgCl_2$ solution with 2 mM dithiothreitol. The cell suspension was disrupted by passage through a French pressure at 20,000 lb/in^2. The supernatant was collected by centrifugation at 30,000×g for 30 min at 4°C. The protein concentration in cell extracts was determined by the Lowry method [13].

Enzyme Assays

Specific activities of the enzymes involved in the conversion of lactose into fermentation products were determined in a range where linearity with protein concentration was established. One unit of enzyme activity was defined as the amount of enzyme catalyzing the conversion of 1 μmol of substrate into specific products. β-Galactosidase (EC 3.2.1.) activity was assayed by measurement of *o*-nitrophenol which was liberated during the reaction on *o*-nitrophenyl-β-D-galactopyranoside with the cell extract. The reaction mixture (1.5 ml) consisted of 80 mM potassium phosphate buffer (pH 7.0), 4 mM *o*-nitrophenyl-β-D-galactopyranoside and the appropriately diluted enzyme. After incubation at 37°C for 10 min, the reaction was stopped by adding 1.5 ml of 0.5 M Na_2CO_3. The released *o*-nitrophenol was determined by measuring absorbance at 540 nm using a spectrophotometer. Except for lactate dehydrogenase, all enzyme activities involved in the conversion of glucose and galactose into fermentation products were assayed aerobically at 37°C using cell

extracts by modification of the assay methods described previously [2].

Fructose-6-phosphate phosphoketolase and D-xlulose-5-phosphate phosphoketolase were assayed by measurement of acetyl phosphate which was produced during the reaction and converted into acetohydroxamic acid by the reaction with hydroxylamine. For measurement of fructose-6-phosphate phosphoketolase, a reaction mixture (2 ml) consisting of 10 mM potassium phosphate buffer (pH 7.0), 10 mM sodium fluoride, 2.5 mM potassium iodoacetate, 2.5 mM fructose-6-phosphate, 0.05 mM thiamine pyrophosphate and the appropriately diluted enzyme was incubated at 37°C for 10 min. The reaction was stopped by adding 1 ml of 10% TCA (trichloroacetic acid), and then the reaction mixture was centrifuged at 10,000×g for 10 min to remove the insoluble precipitates. The color was developed by adding 1 ml of the supernatant to 2 ml of 5% $FeCl_3 \cdot 6H_2O$ solution in 1 N HCl and the absorbance was measured at 540 nm. The reaction mixture (0.5 ml) for measurement of D-xlulose-5-phosphate phosphoketolase consisted of 20 mM Tris·HCl (pH 7.5), 4 mM ribose-5-phosphate, 3.75 mM $MgSO_4$, 18 mM glutathione, 0.06 mM thiamine pyrophosphate, 3 units of phosphoriboisomerase, 0.4 unit of D-ribose-5-phosphate 3-epimerase, and the appropriately diluted enzyme.

The reaction conditions and acetyl phosphate analysis method for the assay of D-xlulose-5-phosphate phosphoketolase were the same as those for the assay of fructose-6-phosphate phosphoketolase. Galactose-1-phosphate uridylyltransferase (EC 2.7.7.12) was assayed by measurement of glucose-6-phosphate which was liberated during the reaction. The reaction mixture (0.5 ml) for the measurement of galactose-1-phosphate uridylyl transferase activity consisted of 2.0 mM galactose-1-phosphate, 0.02 mM glucose-1,6-diphosphate, 0.5 mM UDP-glucose, 20 mM $MgCl_2$, 2 units of phosphoglucomutase, and the appropriately diluted enzyme. The reaction was carried out at 37°C for 10 min, stopped by the addition of TCA, and the released glucose-6-phosphate was measured by the Somogi-Nelson method [19, 22].

Galactokinase (EC 2.7.1.6) was assayed by measurement of galactose-1-phosphate which was produced from galactose and ATP. A reaction mixture (2 ml) consisting of 15 mM maleate (pH 6.0), 1 mM galactose, 10 mM $MgSO_4$, 4 mM ATP, and the appropriately diluted enzyme was incubated at 37°C for 10 min, and the reaction was stopped by addition of 0.5 ml of 5% $ZnSO_4$ and 0.5 ml of 0.2 N $Ba(OH)_2$ solution. Then the mixture was centrifuged to remove the insoluble precipitates and the galactose-1-phosphate produced by the reaction was measured by the Somogi-Nelson method [19, 22]. For measurement of ribose-5-phosphate isomerase (EC 5.3.1.6), the reaction mixture (0.3 ml) consisted of 33 mM Tris·HCl buffer (pH 7.0), 20 mM ribose-5-phosphate, and the appropriately diluted enzyme. After incubation at 37°C for 10 min, the reaction was stopped by successively adding 3 ml of diluted H_2SO_4 solution (70%), 0.1 ml of 0.12% carbazole solution dissolved in absolute ethyl alcohol, and 0.1 ml of 0.15% cysteine·HCl solution. The reaction mixture was allowed to stand at 37°C for 30 min and its absorbance was measured at 540 nm.

D-ribulose-5-phosphate 3-epimerase (EC 5.1.3.1) was assayed using a reaction mixture (2 ml) that consisted of 120 mM glycylglycine buffer (pH 7.5), 6 mM ribose-5-phosphate, 3.4 units of ribose-5-phosphate isomerase, and the appropriately diluted enzyme. After incubation at 37°C for 10 min, the reaction was stopped by adding 50 µl of 10% TCA. After centrifugation at 12,000 rpm for 10 min, 200 µl of the supernatant was taken and transferred to 200 µl of 0.15 M sodium bicarbonate solution. Xylulose-5-phosphate concentration in the mixture was enzymatically determined as follows. The reaction mixture (0.8 ml total volume) consisting of 50 µl of the above xylulose-5-phosphate supernatant, 50 mM Tris·HCl (pH 7.5), 6.25 mM $MgCl_2$, 10 mM disodium arsenate, 0.125 mM thiamine pyrophosphate, 1 mM NAD, 0.03 unit of transketolase, 1.25 units of glyceraldehyde-3-phosphate dehydrogenase, and 0.625 mM ribose-5-phosphate was incubated until absorbance did not increase more.

The compositions of the reaction mixtures (total volume, 0.8 ml) for the enzymes were as follows: phosphohexoisomerase (glucose-6-phosphate isomerase, EC 5.3.1.9), 50 mM Tris·HCl buffer (pH 7.0), 25 mM $MgCl_2$, 4.25 units of glucose-6-phosphate dehydrogenase, 0.5 mM NADP, and 2 mM fructose-6-phosphate; transaldolase (EC 2.2.1.2), 50 mM Tris·HCl buffer (pH 7.5), 6.25 units of phosphohexoisomerase, 4.25 units of glucose-6-phosphate dehydrogenase, 1.5 mM NADP, 0.4 mM sedoheptulose-7-phosphate, and 1 mM glyceraldehyde-3-phosphate; transketolase (EC 2.2.1.1), 50 mM Tris·HCl buffer (pH 7.5), 3 units of glyceraldehyde-3-phosphate dehydrogenase, 10 mM disodium arsenate, 1 mM NAD, 1.25 mM ribose-5-phosphate, and 0.5 mM xylulose-5-phosphate; 3-phosphoglycerate kinase (EC 2.7.2.3), 50 mM potassium phosphate buffer (pH 7.0), 1 mM glyceraldehyde-3-phosphate, 2 units of glyceraldehyde-3-phosphate dehydrogenase, 5 mM $MgSO_4$, 0.75 mM ADP, cysteine·HCl (0.2 g/l),and 0.75 mM NAD; and, UDP-galactose 4-epimerase (EC 5.1.3.2), 50 mM glycine buffer (pH 8.7), 0.5 units of UDP-glucose dehydrogenase, 1 mM NAD, and 0.1 mM UDP-galactose. Pyridine dinucleotide oxidation or reduction reactions were measured at 340 nm ($E_{340} = 6.10\ mM^{-1}Cm^{-1}$).

Hexokinase (EC 2.7.1.1), phosphoglucomutase (EC 5.4.2.2), acetate kinase (EC 2.7.2.1), glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), enolase (EC 4.2.1.11), and lactate dehydrogenase (EC 1.1.2.3) activities were determined by the methods described previously [9].

Table 1. Effect of end-product concentrations on the growth of *B. breve*.[a]

End-products	Concentration (%)	Growth ($O.D_{660}$) at 25 h	Growth ($O.D_{660}$) at 50 h	Final pH
Acetate	0.0	3.4	3.8	4.0
	1.0	3.2	3.4	4.1
	2.0	3.0	3.5	4.3
	3.0	4.1	3.5	4.3
	4.0	4.1	4.0	4.7
	5.0	4.5	3.7	4.9
Lactate	0.0	3.5	4.1	3.8
	1.0	2.4	2.1	4.1
	2.0	2.0	1.7	4.3
	3.0	0.7	1.0	4.8
	4.0	0.1	0.0	5.3
	5.0	0.0	0.0	5.2

[a]Cells were grown at 37°C in 30-ml Wheaton serum bottles containing 20 ml of MRL medium (pH 6.0) supplemented with lactate or acetate to the concentrations as indicated.

Table 2. Comparison of growth of *B. breve* wild type and mutant strains in the presence of lactate.[a]

Strain	Lactate conc. (%)	Growth ($O.D_{660}$) at 24 h	Growth ($O.D_{660}$) at 48 h	Final pH
Wild type	0.0	3.20	3.20	3.9
	4.7	0.00	0.00	6.0
	5.6	0.00	0.00	6.0
Mutant N-1-3	0.0	2.40	2.25	4.2
	4.7	1.45	1.46	4.6
	5.6	0.00	0.15	5.5
Mutant N-1-5	0.0	2.95	2.65	4.1
	4.7	1.50	1.45	4.5
	5.6	0.00	0.80	4.8

[a]Cells were grown at 37°C in 30-ml Wheaton serum bottles containing 20 ml of MRL medium (pH 6.0) supplemented with lactate to the concentrations as indicated.

RESULTS AND DISCUSSION

Effect of End Product Concentrations on the Cell Growth

In the previous studies [10], it was shown that the maximum cell concentration of *B. breve* HP2 grown in a pH-controlled fermentor was 10.0 ($O.D_{660}$) implying that the cessation of growth might be due to the accumulation of some end-products. The effects of end-products on the growth of *B. breve* were examined. As shown in Table 1, the growth of the organism was inhibited in proportion to the increasing concentration of lactate and the organism did not grow on MRL medium with higher than 4.0% of lactate. In contrast to lactate, the addition of acetate did not affect the growth of *B. breve*. Based on these fundamental results, it was assumed that selection of lactate-tolerant mutants through mutagenesis might yield an enhanced growth yield and an improved viable cell stability.

Isolation of Lactate-Tolerant Mutants

Lactate-tolerant mutants were isolated through mutagenesis with NTG, enrichment in MRL medium with 4% lactate, and selection of colonies on MRL agar plate containing 4% lactate. About 40 colonies were tested for alteration in lactate tolerance and two major mutants were chosen and further characterized. Effects of lactate concentration on the growth of *B. breve* wild type and mutant strains were examined and are compared in Table 2. These mutants were confirmed to be stable by testing whether they maintain the lactate tolerance (based on growth rate and lag time) after at least 10 culture transfers on MRL medium containing 4% lactate. The data showed that both mutants, N-1-3 and N-1-5 are lactate-tolerant because they grew on MRL medium with 4.7% lactate at which the wild type did not grow. Notably, mutant N-1-5 was able to grow even in the presence of 5.6% lactate but with a long lag time and a very low growth yield.

Comparison of Lactose Fermentation Time Courses in Wild Type and Mutant Strains

This study was performed to examine whether the acquisition of lactate tolerance in the mutant strains resulted in higher growth yields compared to the wild type. Figures 1A, 1B, and 1C show the fermentation time course of *B. breve* wild-type, mutant N-1-5, and mutant N-1-3, respectively. In a pH-contolled fermentor, the wild type strain (Fig. 1A) rapidly grew until the cell concentration ($O.D_{660}$) reached about 12.0 and then growth ceased. In mutant N-1-5 (Fig. 1B) the final cell concentration ($O.D_{660}$) was enhanced to 13.5 but the specific growth rate was slightly lower than the wild type. It is notable that the mutant produced less acetate than the wild type. These data indicate that the enhanced growth yield in mutant N-1-5 might be due to the less production of lactate compared to the wild type. Mutant strain N-1-3 (Fig. 1C), however, showed much lower growth rate and final cell concentration comparing to the wild type.

Comparison of Survival of Wild Type and Mutant Strain

The culture viability is an important factor for effective use of bifidobacteria in commercial dairy products as well as probiotic preparations. Therefore, the viable cell stabilities of *B. breve* wild type and mutant strains were studied using the cells suspended in artificial stomach solutions with different pHs. As shown in Fig. 2, lactate-tolerant mutant N-1-5 was significantly improved in viable cell stability compared to the wild type and maintained about 500-fold higher viable cell counts than

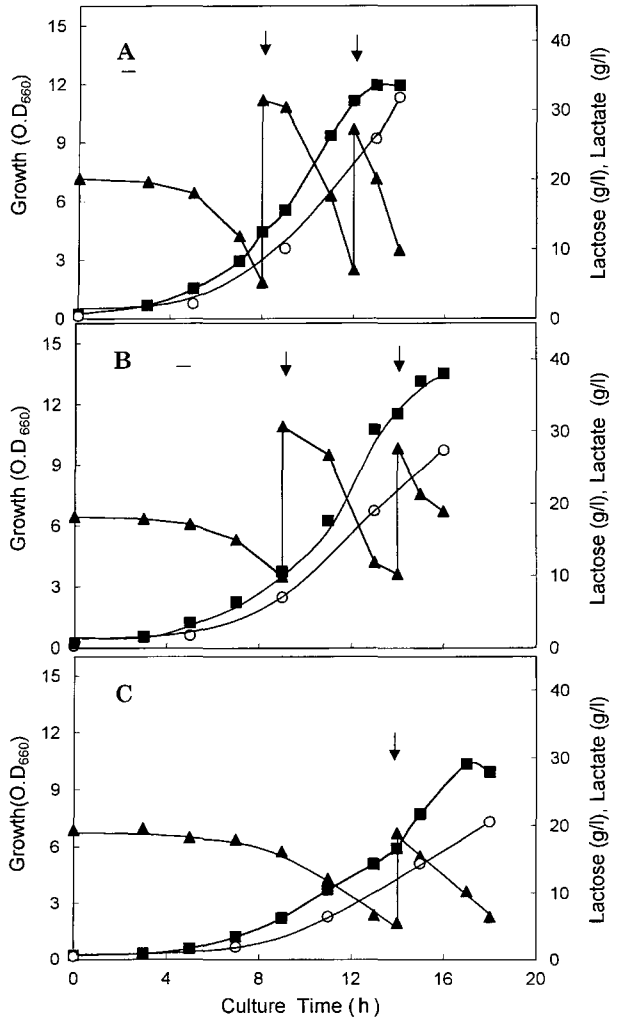

Fig. 1. Comparison of lactose fermentation time courses in *B. breve* wild type (A), mutant N-1-5 (B), and mutant N-1-3 (C).

Experiments were performed in a 5 l jar fermentor containing 2.5 l of MRL medium, which was pH-controlled, agitated at 200 rpm, and not gassed. The arrows indicate the addition of lactose. Symbols: ■, growth ($O.D_{660\ nm}$); ▲, lactose concentration; ○, lactate concentration.

the wild type after incubation at pH 4.0 and 4°C for a day (Fig. 2C) and 10^4-fold higher viable cell counts after incubation at pH 5.0 and 4°C for 30 h (Fig. 2D). The viable cell stability of the mutant strains in a culture broth was also studied and found to be greatly enhanced compared to the wild type (data not shown), implying that the rapid death of *B. breve* cells after cessation of growth may be related to the accumulation of lactate. It is of great interest that the lactate tolerant mutant acquired the nature of improved stability because improvement of culture stability by the acquisition of lactate tolerance has never been reported to our knowledge although the improvement of culture stability by the isolation of hydrogen peroxide-resistant mutants in *Bifidobacterium longum* was reported [12].

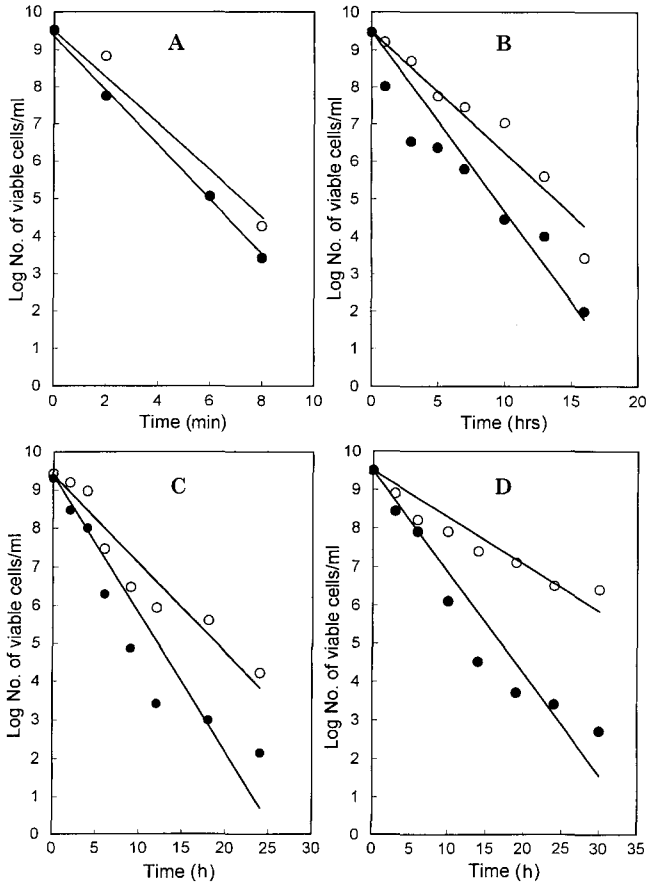

Fig. 2. Comparison of survival of *B. breve* wild type (●) and mutant N-1-5 (○), at pH 2.0 (A), 3.0 (B), 4.0 (C), and 5.0 (D).

General Characterization of Mutants

The mutants were similar to the wild type in morphology, utilization of carbon sources, and lactate and acetate production. In view of end product formation pattern, the two mutants were very similar to the wild type in the yield and ratio of lactate and acetate produced from lactose (Table 3). These results suggest that lactate-tolerant mutants were altered in some properties other than metabolic pattern.

Other experiments were performed in order to investigate whether mutants were altered specifically in lactate tolerance alone or also in halotolerance or osmotolerance. Table 4 demonstrates that mutants, N-1-3 and N-1-5 aremuch more halotolerant than the wild type but mutant N-1-5 is less halotolerant than N-1-3 because mutant N-1-3 grew immediately and mutant N-1-5 grew after a long lag time (~40 h) when inoculated on MRL medium with 3% NaCl at which the wild type displayed no growth at all. Table 5 also compares the growth of *B. breve* wild type and mutant strains on high lactose-containing medium. Notably, the data indicate that mutants are slightly more osmotolerant than the wild type because both mutants initiated growth at about 36 h after inoculation on MRL

medium with 25% of lactose and showed 2.40 of $O.D_{660}$ value but the wild-type strain did not grow within 60 h.

Here, it is worth emphasizing that these lactate-tolerant mutants acquired halotolerance and osmotolerance. In general, many of Korean traditional fermented foods, such as kimchi and fermented soybean paste, contain a high concentration of salt (about 2.5%), and hence the development of a halotolerant strain is essential for the practical application of bifidobacteria to those fermented foods. Therefore, further enhancement of halotolerance in these mutant strains will advance the practical potential of using bifidobacterial fermentations for the production of fermented foods.

Table 3. Comparision of growth and fermentation product formation by *B. breve* wild type and mutant strains when grown on MRL medium containing lactose as the sole carbon source.[a]

Strain	Growth ($O.D_{660}$)	Lactose consumption (mmol/l)	End products (mmol/l)	
			Lactate	Acetate
Wild type	1.76	10	18	30
Mutant N-1-3	1.85	10	16	30
Mutant N-1-5	1.60	10	19	9

[a]Cells were cultivated at 37°C for 24 h in anaerobic pressure tubes containing 10 ml of MRL medium (pH 6.0).

Table 4. Comparison of growth of *B. breve* wild type and mutant strains on MRL media containing NaCl.[a]

Strains	NaCl conc. (%)	Growth ($O.D_{660}$) at		
		22 h	44 h	68 h
Wild type	0	4.85	3.60	3.35
	3	0.10	0.25	0.25
	4	0.00	0.10	0.10
Mutant N-1-3	0	3.45	2.55	2.60
	3	2.00	2.05	2.05
	4	0.15	0.45	0.05
Mutant N-1-5	0	3.20	3.00	3.30
	3	0.00	0.55	2.00
	4	0.05	0.10	0.05

[a]Cells were grown at 37°C in 125-ml Wheaton serum bottles containing 50 ml of MRL medium (pH 6.0) supplemented with various concentrations of sodium chloride as indicated.

Table 5. Effect of lactose concentration on the growth of *B. breve* wild type and mutant strains.[a]

Strains	Lactose conc. (%)	Growth ($O.D_{660}$) at		
		15 h	40 h	65 h
Wild type	2	2.35	3.05	3.20
	20	0.10	0.30	2.65
	25	0.10	0.10	0.45
	30	0.10	0.10	0.10
Mutant N-1-3	2	1.15	2.15	3.10
	20	0.10	2.25	2.70
	25	0.10	0.15	2.40
	30	0.10	0.10	0.10
Mutant N-1-5	2	2.15	3.05	3.30
	20	0.75	3.05	3.50
	25	0.10	0.45	2.40
	30	0.10	0.10	0.10

[a]Cells were grown at 37°C in 30-ml Wheaton serum bottles containing 20 ml of MRL medium (pH 6.0).

Comparison of Lactose Fermentation Enzyme Activities between Wild Type and Mutant Strains

In order to elucidate the biochemical basis for the lactate tolerance of mutants, key enzyme activities involved in lactose metabolism by *B. breve* were measured. The data in Table 6 indicate that β-galactosidase activity in mutant N-1-3 is higher than that of the wild type but the activities of phosphoglucomutase, transaldolase, transketolase, 3-phosphoglycerate kinase and lactate dehydrogenase (NADH-oxidizing) were lower. In mutant N-1-5, the activities of phosphoglucomutase, transaldolase, transketolase, and acetate kinase were lower compared to those of the

Table 6. Comparison of lactose-fermenting enzyme activities in wild type and mutant strains of *B. breve*.[a]

Enzyme	Specific activity (μmol/min/mg-protein)		
	Wild type	N-1-3	N-1-5
β-Galactosidase	5.229	16.007	6.647
Hexokinase	0.031	0.023	0.015
Glucose-6-phosphate isomerase	7.942	5.711	6.287
Fructose-6-phosphate phosphoketolase	0.214	0.204	0.112
Galactokinase	0.875	0.995	0.993
Galactose-1-phosphate uridyltransferase	0.428	0.344	0.238
Phosphoglucomutase	0.408	0.058	0.074
UDP-galactose-4-epimerase	0.342	0.420	0.286
Transaldolase	0.014	0.002	0.002
Transketolase	0.013	0.003	0.004
Ribose-5-phosphate isomerase	0.050	0.030	0.026
Ribulose-5-phosphate-3-epimerase	0.019	0.010	0.013
Xylulose-5-phosphate phosphoketolase	0.052	0.027	0.012
Glyceraldehyde-3-phosphate dehydrogenase	0.060	0.109	0.033
Acetate kinase	14.143	12.874	2.600
3-Phosphoglycerate kinase	0.085	0.011	0.078
Enolase	0.909	0.607	0.383
Lactate dehydrogenase (NADH-oxidizing)	0.737	0.166	0.447
Lactate dehydrogenase (NAD^+-reducing)	0.060	0.109	0.033

[a]Enzyme activities were determined at 25°C as described in Materials and Methods.

wild type. The lower activities of these metabolic enzymes observed in mutant strains may explain the lower growth rate of mutants compared to the wild type. In fact, we have investigated whether lactate dehydrogenase (both NADH-oxidizing and NAD^+-reducing) of the mutant strains was altered in the extent of inhibition by lactate or not, but no change was observed (data not shown). Therefore, we concluded that growth inhibition by lactate in *B. breve* may be due to other factors than inhibition of lactate dehydrogenase by lactate.

Conclusively, the lactate-tolerant mutants obtained here displayed improved viable cell stability, enhanced halotolerance as well as enhanced osmotolerance as compared to the wild type. This is an encouraging result because desired bifidobacteria for effective use in fermented food products must be of human origin, possess enhanced stability under the acidic conditions encountered within the digestive tract, remain viable during the storage or fermentation period of the fermented products, and possess salt resistance. Therefore, the mutants derived from infant-originated *B. breve* HP2 may be a potential candidate strain for wide use in the fermented food industry as a consequence of enhanced culture stability, lactate tolerance and halotolerance.

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