

Isolation and Characterization of Acid- and Bile-Tolerant Isolates from Strains of *Lactobacillus acidophilus*¹

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ABSTRACT

Lactic acid bacteria have been reported to be useful as a health adjunct and are commonly added to food as the delivery mechanism. The literature contains many conflicting observations for their proposed benefits, and the mechanism of action is undefined. One source of variation is the large number of strains used without proper controls supplemented. Additionally, many of the organisms are not characterized for their acid shock response or the acid-tolerance response, which are known to vary among bacterial species. Our objective was to isolate acid-resistant and bile-resistant variants of *Lactobacillus acidophilus* and to determine the phenotypic changes. The acid- and bile-tolerant isolates were obtained using natural selection techniques after sequential exposure to hydrochloric acid (pH 3.5 to 7.0) and mixed bile salts. The acid- and bile-tolerant isolates retained their ability to grow at pH 3.5 with 0.3% bile after the selective pressure was removed and reapplied. Isolates varied from their parents for stability in freezing, lactose utilization, protease activity, aminopeptidase activity, plasmid profile, and cell-wall fatty acid profile. These data suggest that the isolated acid- and bile-tolerant isolates possess growth advantages over that of the parents under stress conditions and may be considered as candidates for probiotic strains after further characterization with animal models.

(**Key words:** acid-tolerant isolates, bile-tolerant isolates, probiotic, *Lactobacillus acidophilus*)

Abbreviation key: **A** = absorbance (subscript indicates wavelength in nanometers), **CFE** = cell-free extract, **OPA** = *o*-phthaldialdehyde, **p-NA** = *p*-nitroanilide.

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INTRODUCTION

Lactic acid bacteria are sometimes termed probiotic and are used as health adjuncts in food to provide a wide variety of health benefits (15). These bacteria, mainly lactobacilli and bifidobacteria, may have several therapeutic functions, including antimicrobial activity, anticholesterol activity, improved lactose utilization, and anticarcinogenic activity (4, 7, 8, 9, 10, 12, 17, 21, 22). There is little evidence that probiotic bacteria consistently improve health in animal models. Renner (19) suggested that lack of definitive evidence for the biological activity is due to improper experimental design and variation in strain characteristics used in clinical studies. Although this may not be true, it is clear that additional research is needed to firmly establish health benefits in animals using characterized strains (11).

Bacteria used as probiotic adjuncts are commonly delivered in a food system and, therefore, begin their journey to the lower intestinal tract via the mouth. As such, probiotic bacteria should be resistant to the enzymes in the oral cavity (e.g., lysozyme) (9) and should also have the ability to resist the digestion process in the stomach and the intestinal tract. Berada et al. (2) reported the time from entrance to release from the stomach to be 90 min. However, further digestive processes have longer residence times; hence, there is a need for the bacteria to be resistant to the stressful conditions of the stomach and upper intestine, which contain bile.

Cellular stress begins in the stomach, which has pH as low as 1.5 (14). After the bacteria pass through the stomach, they enter the upper intestinal tract where bile is secreted into the gut. The concentration of bile in the human gastrointestinal system is variable and is difficult to predict at any given moment (14). After traveling through this harsh environment, the organism colonizes the epithelium of the lower intestinal tract (5). Thus, strains selected for use as probiotic bacteria should be able to tolerate acid for at least 90 min, tolerate bile acids, attach to the epithelium, and grow in the lower intestinal tract before they can start providing any health benefits. In

TABLE 1. Strains of *Lactobacillus acidophilus* used in this study.

Strain	Original isolation source
ATCC 521	Unknown
ATCC 4796	Unknown
ATCC 4962	Unknown
ATCC 11975	Unknown
ATCC 4356	Human
ATCC 33200	Human
ATCC 43121	Pig rectum

this study, environmentally resistant isolates were isolated from *Lactobacillus acidophilus*. The phenotypes of the isolates were partially characterized for traits that are important in the physiology of lactic acid bacteria.

MATERIALS AND METHODS

Bacteria

Strains of *L. acidophilus* were purchased from the American Type Culture Collection (Rockville, MD) (Table 1). The strains were grown anaerobically (BBL® GasPak System) in MRS broth (Difco, Detroit, MI) for 16 to 24 h at 37°C. Stock cultures were prepared by growing the strains for 16 to 24 h and inoculating 0.3 ml into 1.2 ml of sterile reconstituted 12% NDM solution containing 2% glycerol. Each vial was frozen immediately and was stored at -70°C for further use. Each experiment used a stock freezer vial for medium inoculation.

Selection of Acid-Tolerant Isolates

Each parent strain of *L. acidophilus* was grown once in MRS broth from the stock freezer vial for 16 to 24 h at 37°C before use in an experiment. After this incubation, cells were harvested by centrifugation (4300 × *g* for 10 min at 4°C), washed three times in sterile saline (0.85% NaCl), inoculated (1%) into MRS broth acidified with concentrated hydrochloric acid to pH 3.5 or nonacidified MRS broth (pH 6.8), and incubated at 37°C in a temperature-controlled spectrophotometer (Beckman DU-8; Beckman Instruments, Fullerton, CA) for 90 min while the absorbance at 650 nm (A_{650}) was monitored at 15-min intervals. Before and after incubation, plate counts were done using MRS agar (pH 6.8) and the pour plate technique. Strains that showed little or no reduction in colony-forming units per milliliter of culture were considered to be candidates for selection of acid-tolerant strains and were used to isolate 10 single colonies per strain from acidified MRS agar.

Each acid-tolerant candidate was further investigated for its ability to grow in acidic conditions by streaking the organism onto acidified MRS agar (pH 3.5). The plates were incubated anaerobically at 37°C and were observed for growth after 24 to 96 h. Individual colonies were selected and were again grown in acidified MRS broth (pH 3.5) for 24 to 96 h at 37°C. Observed colonies were considered to be acid tolerant and were used for further study in acid- and bile-tolerant isolation experiments.

Selection of Bile-Tolerant Isolates

Each acid-tolerant isolate was screened for bile tolerance using the direct plate assay described by Christiaens et al. (3), except that MRS agar was used. The agar pH was adjusted to 4.0, 5.0, 6.0, or 7.0 and contained 0.3% of glycocholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, or oxgall, individually. Bile salt deconjugation was not tested below pH 4.0 because of bile salt precipitation. Each acid-tolerant isolate was streaked onto plates at each pH level of MRS agar containing individual bile salts and was incubated anaerobically for 24 to 96 h at 37°C. The plates were observed for growth and bile precipitation at 24-h intervals. If growth occurred, 10 individual colonies for each strain were selected and inoculated into MRS broth containing 0.3% of the specific bile salt at the specific pH from which the isolate was selected. If growth was observed in this broth, the isolate was considered to be both acid- and bile-tolerant.

Growth of Acid- and Bile-Tolerant Isolates

Acid- and bile-tolerant isolates were frozen at -70°C for 48 h, thawed at room temperature (25°C for about 5 min), and tested for growth. Thawed isolates were grown 16 to 24 h in their respective isolation broth to determine relative cell density changes during incubation at 37°C. The A_{650} before and after incubation was measured, and the change in cell density was determined by difference. The strains with the best growth were chosen for further study.

Additional tests for cell growth mimicked passage through the gut. Cells were grown in their respective media and were harvested by centrifugation (4300 × *g* for 10 min at 4°C), washed three times with PBS (pH 7.0), and resuspended in the same volume of saline (pH 7.0) as the original culture. The first portion of the suspension was inoculated (1%) into

acidified MRS broth (pH 4.5) and unacidified MRS broth (pH 6.8). Cultures were incubated at 37°C in a temperature-controlled spectrophotometer for 600 min; A₆₅₀ was monitored at 15-min intervals to determine the growth rate.

The second portion of the suspension was inoculated (1%) into acidified MRS broth (pH 4.5), and the A₆₅₀ was monitored at 15-min intervals. After 90 min of incubation at 37°C, sterile 1*N* NaOH was added to the broth to achieve a final pH of 6.8, and each bile salt was added to a final total concentration of 0.2% with individual bile salts of equal concentration. The A₆₅₀ was monitored for an additional 510 min at 15-min intervals at 37°C after adjustment of pH and bile salt.

Phenotypic Characterization

Preparation of cell-free extracts. Cell-free extracts (CFE) from cultures grown in their respective isolation media were prepared as described by Dias and Weimer (6). Briefly, cultures were incubated for 14 h in their respective isolation media at 37°C and were harvested from 10 ml of culture by centrifugation (7000 × *g* for 10 min at 4°C). The cell pellet was collected, washed twice with 0.05 *M* sodium phosphate buffer (pH 7.2), and resuspended in 1 ml of 3 *mM* sodium phosphate buffer (pH 7.2) containing 200 U/ml mutanolysin and 40,000 U/ml of lysozyme. Cells were incubated in this lysis buffer for 1 h at 37°C. Glass beads (106 μm and finer; Sigma Chemical Co., St. Louis, MO) were added, and the sample was vortexed at high speed for 2 min at room temperature (25°C) to complete cell lysis. After centrifugation (7000 × *g* for 15 min at 4°C), the supernatant was collected. This supernatant was considered the CFE and was used for intracellular enzyme assays.

β-galactosidase activity. The ability to utilize lactose was determined in the CFE of each selected isolate and in its parent by use of automated reflectance colorimetry (Omnispec[®] 4000 Bioactivity Monitor; Wescor, Inc., Logan, UT) with *p*-nitrophenyl-β-D galactopyranoside as the substrate (Sigma Chemical Co.). A 1.5 *mM* stock solution of the substrate was prepared in sterile 0.05 *M* sodium phosphate buffer (pH 7.2) and was frozen at -20°C. Prior to the enzyme assay, each stock substrate solution was thawed and equilibrated to the assay temperature. The CFE was added to begin the test. Each assay mixture contained 100 μl of 1.5 *mM* chromogenic substrate and 100 μl of CFE. Assays were carried out at 37°C in 96-well microtiter plates (Baxter Diagnostics, Inc., McGaw Park, IL). Hydrolysis was measured at

10-min intervals for 10 h by monitoring the increase in yellowness (b*) by methods similar to that of Dias and Weimer (6). Briefly, β-galactosidase activity was induced by the addition of 1 *mM* isopropyl-β-D-thiogalactopyranoside as described by Miller (16). Three controls containing 100 μl of 1-*mM* chromogenic substrate in sodium phosphate buffer (0.05 *M*; pH 7.2), 100 μl of buffer, and 0.5 *mM* of *p*-nitroanilide (**p-NA**) in 0.05 *M* sodium phosphate buffer (pH 7.2) were also included. Means of duplicate assays are reported.

Protease activity. The extracellular protease activity was determined using *o*-phthaldialdehyde (**OPA**) as described by Oberg et al. (18). The strains were grown anaerobically overnight at 37°C in their respective isolation broth, harvested by centrifugation (7000 × *g* for 10 min at 4°C), washed three times with sterile saline (0.85% NaCl), and resuspended in sterile saline to an A₅₉₀ of 0.4. The washed cell solution (200 μl) was inoculated into 10 ml of 10% reconstituted NDM and was incubated for 4.5 h at 37°C. Plate counts were conducted after the incubation, and TCA was added to a final concentration of 6% to stop the enzyme reaction and to precipitate nonhydrolyzed protein. The assay mixture was filtered (number 1 Whatman filter paper; Whatman Corp., Clifton, NJ) and the collected supernatant was used to react with OPA reagent. The supernatant and OPA reagent mixture (1:1, vol/vol) was incubated at room temperature (25°C) for 5 min, and the absorbance at 340 nm was determined. Sterile reconstituted NDM served as the control, and blank OPA reagent was used to calibrate the spectrophotometer. Means of duplicate assays are reported.

Amino peptidase activity. Amino peptidase activity was determined with chromogenic substrates by use of automated reflectance colorimetry as described by Dias and Weimer (6). Stock solutions (10 *mM*) of *p*-NA-L amino acid derivatives (Sigma Chemical Co.) of arginine, leucine, lysine, alanine, valine, proline, methionine, glycine, and γ-glutamyl were prepared in sterile 0.05 *M* sodium phosphate buffer (pH 7.2). The *p*-NA L-amino acid derivatives (Sigma Chemical Co.) of tyrosine, phenylalanine, and S-benzyl-cysteine were dissolved in the minimum amount of 0.5 ml of *N,N*-dimethyl formamide before addition of sterile 0.05 *M* sodium phosphate buffer (pH 7.2) to a final volume of 10 ml. Aliquots (1 ml) of 10 *mM* stock solutions were stored at -20°C. The aliquots were thawed and diluted immediately prior to use.

Each assay mixture contained 100 μl of 1 *mM* chromogenic substrate in 0.05 *M* sodium phosphate

TABLE 2. Plate counts¹ of *Lactobacillus acidophilus* cultures before and after 90 min of incubation at pH 6.8 or 3.5 at 37°C.

<i>L. acidophilus</i>	Control (pH 6.8)		Treatment (pH 3.5)	
	0 min	90 min	0 min	90 min
ATCC 521	8.5	8.3	8.4	8.1
ATCC 4796	8.5	9.0	9.0	8.9
ATCC 4962	8.2	9.0	8.9	8.6
ATCC 11975	8.1	8.5	8.1	8.3
ATCC 4356	8.0	8.8	8.8	8.7
ATCC 33200	9.1	9.2	9.3	9.2
ATCC 43121	9.4	9.4	9.1	9.1

¹Counts are converted to log₁₀ cfu/ml; MRS agar was used for growth medium.

buffer (pH 7.2) and 100 μ l of CFE. Assays were carried out at 37°C in 96 well microtiter plates with sterile tape coverings. The plates were preincubated at 37°C for 15 min before addition of the CFE. Hydrolysis of the chromogenic substrates was measured at 15-min intervals for 12 h by the increase in yellowness using the Omnispec[®] 4000 Bioactivity Monitor (Wescor, Logan, UT). The controls contained 100 μ l of 1 mM chromogenic substrate and 100 μ l of sodium phosphate buffer (0.05 M, pH 7.2). Also, a 0.5-mM solution of *p*-NA in 0.05 M sodium phosphate buffer (pH 7.2) served as a blank control. Means of duplicate assays are reported.

Protein determination. The protein concentration in CFE was determined according to the instructions of the manufacturer using the bicinchoninic acid method (Pierce Chemical, Rockford, IL) with BSA as the standard. Means of duplicate assays are reported.

Plasmid analysis. Small-scale plasmid isolation was done as described by Anderson and McKay (1). After the isolation of plasmid DNA, the pellet was dried in a vacuum desiccator for at least 30 min, resuspended in 14 μ l of TE buffer (10 mM Tris, pH 8.0, and 1 mM EDTA), electrophoresed (0.6 to 0.7% agarose) at 80 V for 2 h, and stained in a 1- μ g/ml solution of ethidium bromide to visualize the plasmids.

Fatty acid analysis. Selected strains were sent to Analytical Services, Inc. (Essex Junction, VT) to determine the content and identification of the fatty acids in the cell wall.

RESULTS AND DISCUSSION

Selection of Acid-Tolerant Isolates

The food transit time through the human stomach is about 90 min (19). Therefore, each ATCC parent

strain was tested for tolerance to hydrochloric acid at pH 3.5 for 90 min. All strains tested were tolerant to pH 3.5 for 90 min at 37°C (Table 2). *Lactobacillus acidophilus* ATCC 11975 grew slightly at pH 3.5 during the 90-min incubation but produced no colonies on acidified MRS agar after 96 h, suggesting that this strain had the ability to survive short-term acid stress but not long-term exposure to acidic environments. This observation was noted for many of the strains tested. Total plate counts (Table 2) yielded the same information as the growth curves measured at A₆₅₀ (data not shown). These data indicate that acid-tolerant variants exist in the parent population, and, presumably, these variants can be isolated.

Acid-tolerant variants were isolated from agar plates and were screened again by growth in broth. If growth occurred on acidified MRS agar (pH 3.5), 10 single colonies were transferred to acidified MRS broth and were incubated for 24 h at 37°C. If the isolate did not grow within 24 h of incubation in broth, it was discarded. Only *L. acidophilus* ATCC 43121, 33200, and 4962 produced colonies on acidified MRS agar and grew within 24 h in acidified MRS broth (Table 3). Therefore, these colonies were used in further isolation.

Selection of Acid- and Bile-Tolerant Isolates

Each parent strain (*L. acidophilus* ATCC 43121, 33200, and 4962) and the acid-tolerant isolates from these strains were inoculated onto MRS agar containing individual bile salts at varying pH levels (pH 4.0 to 7.0) in an effort to isolate bile-tolerant variants. Two distinctly different results were observed: growth indicated by a colony and growth with bile salt deconjugation indicated by a white precipitate surrounding the colony. However, no predictive pattern was observed for any selection conditions. After 24 h of

TABLE 3. Examples of acid- and bile-tolerant derivatives isolated from parent strains of *Lactobacillus acidophilus*.

Parent strain	Isolate	Isolation pH	Bile salt used
ATCC 43121	LSC2	3.5	None
	LSC2-1 GD4	4.0	Glycodeoxycholic acid
	LSC2-2 GD4	4.0	Glycodeoxycholic acid
	LSC7	3.5	None
	LSC7-1 GD4	4.0	Glycodeoxycholic acid
	LSC7-2 GD4	4.0	Glycodeoxycholic acid
	LSC7-3 GD4	4.0	Glycodeoxycholic acid
ATCC 33200	LSC13	3.5	None
	LSC13-1 GD4	4.0	Glycodeoxycholic acid
	LSC13-2 GD4	4.0	Glycodeoxycholic acid
	LSC15	3.5	None
	LSC15 GD4	4.0	Glycodeoxycholic acid
ATCC 4962	LSC20	3.5	None
	LSC20 GD4	4.0	glycodeoxycholic acid
	LSC20A	3.5	None
	LSC20A GD4	4.0	Glycodeoxycholic acid

incubation, most strains grew at pH 5.0 to 7.0 in the presence of individual bile salts. However, growth at pH 4.0 in the presence of bile salts was the target of this experiment. In media at pH 4.0, all isolates tested were inhibited by glycocholic acid and oxgall but most strains grew with glycodeoxycholic acid added. Therefore, colonies of each acid-tolerant isolate were picked and were designated GD4 after each isolate number (Table 3). For example, colonies from acid-tolerant isolate LSC2 (derivatives from parent ATCC 43121) that grew in MRS agar containing glycodeoxycholic acid at pH 4 were designated as LSC2-1 GD4 and LSC2-2 GD4, respectively (Table 3). Based on these observations, we hypothesized that isolation of acid- and bile-tolerant colonies was possible. Further, these data suggested that the phenotypic traits of probiotic bacteria might change during the digestion process, leaving a distinctly different population in the digestive tract than originally consumed. Therefore, extensive strain characterization would be required to define the phenotype of acid- and bile-tolerant isolates.

Results demonstrated that with longer incubation times, bile-tolerant isolates grew, which is contrary to results of Lankaputhra and Shah (14). An important difference in this study compared with previous reports is that we combined two factors, acid and bile, during screening. A common observation among these studies, despite major design differences, is that acid and bile have separate and combined effects on the growth of bacteria. These data suggest that selection of acid- and bile-tolerant probiotic bacteria as a dairy adjunct be part of a regimen to aid in reducing undefined phenotypic shifts after ingestion.

Growth and Characterization of Acid- and Bile-Tolerant Isolates

Isolation conditions in this study defined acid- and bile-tolerant variants by their ability to grow at low pH in the presence of bile salts within 24 h. Prior to determining the phenotypic differences between the parent and the variants, each isolate was subjected to an experiment in which the strain was frozen and then thawed to determine strain stability after freezing. Subsequently, the most stable strains were used to determine growth at pH 6.8 followed by growth at pH 4.5 and the intracellular enzymology known to be critical for the growth of lactic acid bacteria.

Influence of freezing. Survival and rapid growth after freezing are essential characteristics for the selection of probiotic bacteria because the mode of delivery is often in frozen dairy desserts. These characteristics are also critical for the storage of frozen stock cultures used in industrial production. To investigate this ability, 18 isolates that were determined to be acid- and bile-tolerant after extended incubation on acidified MRS agar (pH 3.5) were frozen at -70°C for 2 d, thawed within 5 min, and regrown on MRS broth at pH 3.5 and 6.7. Many isolates readily grew in MRS agar at pH 6.7 after freezing; however, some of the isolates lost the ability to grow at pH 3.5 after freezing (Figure 1). Only isolates LSC2-1 GD4 and LSC13-1 GD4 grew equivalently or better at pH 3.5 than at pH 6.7. Strain LSC20A GD4 grew equally at both pH conditions but was not studied further because the other two strains had significantly larger increases (Figure 1). Therefore, isolates LSC2-1 GD4 and LSC13-1 GD4 were used in further characterization studies. These data

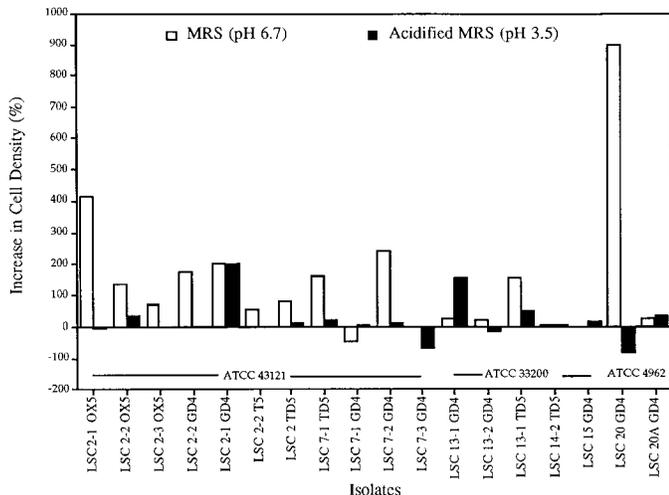


Figure 1. The effect of freezing on the ability of acid- and bile-tolerant mutants to grow in MRS broth at pH 6.7 or 3.5. The percentage increase in cell density was calculated by [(cell density after freezing) - (cell density before freezing)]/(cell density before freezing effect) \times 100.

suggest that sensitivity to freezing is also an important characteristic in a probiotic selection program.

Growth in neutral and acidified conditions.

To differentiate the characteristics of the acid-tolerant isolate from those of its parent, growth in both neutral and acidified conditions were investigated for changes in growth rate. Acid-tolerant isolate LSC13 was derived from ATCC 33200 by incubation on MRS agar at pH 3.5. In this verification step, only the influence of pH on the growth of LSC13 and its parent, ATCC 33200, was investigated.

Lactobacillus acidophilus ATCC 33200 and its acid-tolerant derivative LSC13 were inoculated into MRS broth at pH 6.8 and 4.5 and were monitored for growth by measuring the A_{650} (Figure 2A). The parent grew well at pH 6.8 but grew only slightly at pH 4.5, and the reduction in A_{650} after 7.5 h of incubation was observed for both pH conditions. Conversely, the acid-tolerant isolate LSC13 grew as rapidly as its parent strain in nonselective conditions (pH 6.8), and the population did not decrease as with the parent. When LSC13 grew in acidified MRS broth (pH 4.5), it had a slightly longer lag time, but later, the strain grew equally well at pH 4.5 or pH 6.8. Also, inoculation of LSC13 into MRS broth (pH 6.8), followed by growth in acidified MRS (pH 3.5), demonstrated that the isolate grew equally well (data not shown). These observations suggest that LSC13 was acid-tolerant because it grew equally well in selective and nonselective conditions and did not lyse upon entering the stationary phase.

Growth in selective conditions was investigated further. The transit time, pH, and bile concentration in the human digestive tract were mimicked to compare the growth of parent ATCC 33200, acid-tolerant isolate LSC13, and acid- and bile-tolerant isolate LSC13-1 GD4 (derived from LSC13). The cultures were inoculated into acidified MRS broth (pH 3.5) for 90 min, followed by a pH increase to 6.8 with the addition of NaOH and 0.2% mixed bile salts (Figure 2B). After the lag phase, *L. acidophilus* ATCC 33200 grew to a maximum cell density, and the density dropped quickly (Figure 2B). However, the acid-tolerant isolate LSC13 grew as fast as the parent and remained in a stationary phase over the incubation period, indicating that this isolate may contain certain functional proteins that protect the cells and allow better survival than its parent strain. The acid- and bile-tolerant isolate LSC13-1 GD4 had a longer lag time and grew more slowly than did LSC13 and ATCC 33200 but grew to a high cell density during

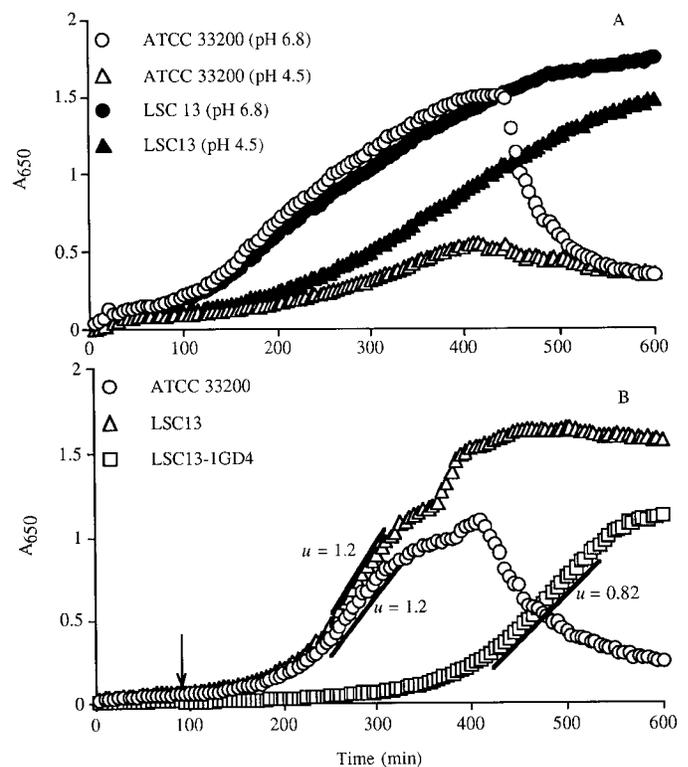


Figure 2. Growth of *Lactobacillus acidophilus* ATCC 33200 and acid-tolerant mutant LSC13 in MRS broth (pH 6.8) or acidified MRS broth (pH 4.5) (A). Growth of ATCC 33200, acid-tolerant mutant LSC13, and acid- and bile-tolerant mutant LSC13-1 GD4 in simulated gastric conditions (B). Arrow indicates time of addition of NaOH and mixed bile salts to result in a 0.2% bile salt concentration at pH 6.8. Straight lines indicate the portion of the curve used to calculate growth rate (u). A_{650} = Absorbance at 650 nm.

the assay time. Based on the growth curve, lag time, and generation time (Figure 2B), the acid-tolerant isolate LSC13 and the acid- and bile-tolerant isolate LSC13-1 GD4 may be candidates for use as probiotic adjuncts in the future.

Characterization of Enzyme Activity

The strains used in these experiments were limited to *L. acidophilus* ATCC 43121, ATCC 33200, and their isolates LSC2, LSC2-1 GD4, LSC13, and LSC13-1 GD4, respectively, based on growth after freezing (Figure 1).

β -galactosidase activity. The activity of β -galactosidase was investigated because it is an essential enzyme for lactobacilli to utilize lactose and has been shown to reduce the symptoms of lactose intolerance in animals (19). The acid- and bile-tolerant isolate LSC13-1 GD4 had lower β -galactosidase activity than its parent ATCC 33200 or acid-tolerant isolate LSC13, but the activity remained inducible (Table 4). In the ATCC 43121 strain series, all three strains showed lower β -galactosidase activity than that of the ATCC 33200 series, and the activity was not inducible.

Protease activity. Protease activity is required by lactic acid bacteria for fast growth and acid production in milk fermentations (13, 20). Probiotic bacteria are often delivered in dairy products and must maintain their population during the shelf-life;

presumably, protease activity is important in this respect. Additionally, the role of protease in colonization and survival in the gut is unknown. Therefore, we examined the influence of acid and bile tolerance on protease activity. The activities of ATCC 33200 and its isolates were similar. Strain ATCC 43121 and its isolates had either very low or undetectable activity (Table 4). These data suggested that the ATCC 43121 series would grow more slowly than the ATCC 33200 series in a complex protein substrate such as milk because the low protease activity leads to slow growth and acid production.

Growth in MRS and milk. The generation time of each series was tested in both milk and MRS broth (pH 6.8). *Lactobacillus acidophilus* ATCC 33200 grew ($P = 0.05$) faster in MRS broth than in milk (Table 4). However, LSC13-1 GD4 grew significantly faster in milk than in MRS broth. This difference in growth rate between milk and MRS broth suggests LSC13 1 GD4 has a trait that is useful for dairy fermentation and may play a role in its ability to survive in dairy products for delivery as a probiotic agent. Based on the limited characteristics tested, it is plausible that high protease and an inducible β -galactosidase system may partially explain this difference, despite low total aminopeptidase activity. The ATCC 43121 series appeared to be deficient in β -galactosidase and protease activity; this deficiency could account for their slow growth in milk and sug-

TABLE 4. Enzyme activity and specific growth rate of selected parent strains, acid-tolerant isolates, and acid- and bile-tolerant isolates.

Enzyme activity ¹	<i>Lactobacillus acidophilus</i> strain											
	ATCC 33200		LSC13		LSC13-1 GD4		ATCC 43121		LSC2		LSC2-1 GD4	
β -D-Galactosidase (uninduced) ²	22		20		1		1		1		7	
β -D-Galactosidase (IPTG ³ induced)	77		69		29		1		1		9	
Protease activity ⁴	24		21		38		1		ND ⁵		NA ⁶	
Total AP ³ activity ²	113		86		14		192		171		87	
	\bar{X}	SEM	\bar{X}	SEM	\bar{X}	SEM	\bar{X}	SEM	\bar{X}	SEM	\bar{X}	SEM
Generation time/min												
MRS broth	22 ^b	2	28 ^b	1	45 ^a	3	29 ^b	1	34 ^b	2	61 ^a	3
12% NDM	38 ^a	4	37 ^a	3	25 ^b	2	42 ^a	3	42 ^a	4	44 ^b	5

^{a,b}Means with the same superscript within a column are not different ($\alpha = 0.05$).

¹Enzyme activities are means of duplicate assays.

²Unit is change in yellowness, expressed per colony-forming unit per milliliter.

³IPTG = Isopropyl β -D-thiogalactopyranoside; AP = aminopeptidase.

⁴Unit is change in absorbance at 340-nm wavelength expressed per colony-forming unit per milliliter.

⁵Not detected.

⁶Not analyzed.

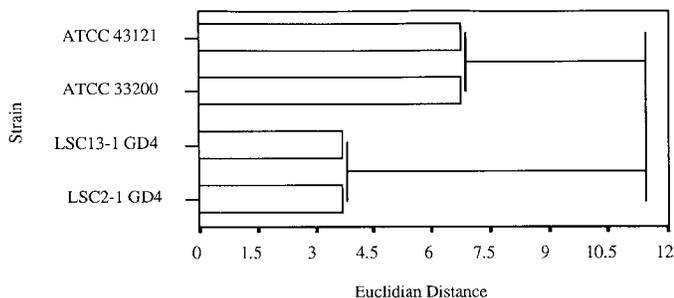


Figure 3. Dendrogram comparison chart of acid- and bile-tolerant isolates and parents by fatty acid analysis. Less than or equal to 2.0 is the same strain, less than or equal to 6.0 is the same subspecies or biotype, less than or equal to 10.0 is the same species, and less than or equal to 25.0 is the same genus.

gests the series may lack the plasmid or plasmids containing these genes.

Amino-peptidase activity. The ability to hydrolyze peptides once inside the cell was investigated by monitoring the intracellular aminopeptidase activity (Table 4). The hydrolysis of each substrate varied, and no predictive pattern was found (data not shown). Therefore, the individual activities were summed and were used to compare the isolates (Table 4). The isolates consistently contained less intracellular aminopeptidase activity than the parents.

Plasmid profile. Plasmid analysis of ATCC 43121 found this strain to contain a 7.0-kb plasmid and a 3.9-kb plasmid. Each of the acid- and bile-tolerant isolates lost these plasmids (data not shown), which may account for the change in protease activity (Table 4). Analysis of ATCC 33200 found plasmids of 6.0 and 3.5 kb. The acid- and bile-tolerant isolates lost these two plasmids but retained inducible β -galactosidase and protease activities. These data suggest the plasmids were integrated into the chromosome or were lost and were not linked to the activities.

Cell-wall fatty acid analyses. Cell-wall fatty acid analyses further characterized the isolates to distinguish them from their parents. This technique is commonly used to identify bacteria because organisms have unique patterns of fatty acids in their cell walls, which allows identification when grown in the same conditions. We hypothesized that, if the cell-wall fatty acids were the same, then the isolates were environmentally adapted variants with widely varying phenotypic traits.

Isolate LSC2-1 GD4 did not contain any of the fatty acid $C_{14:1}$, but its parent ATCC 43121 contained 0.9%. The dendrogram comparison chart (Figure 3) from fatty acid analysis showed that isolate LSC2-1

GD4 and its parent ATCC 43121 were not the same strain. The same results were observed for isolate LSC13-1 GD4 and its parent ATCC 33200 (Figure 3). These data suggest that isolates LSC2-1 GD4 and LSC13-1 GD4 are not acid- or bile-adapted variants of the parents but are distinctly different strains.

CONCLUSIONS

Acid-tolerant isolates and acid- and bile-tolerant isolates were obtained from *L. acidophilus*. These isolates were capable of rapid growth in MRS at pH 3.5 containing 0.2% mixed bile salts. Initial screening for acid resistance for 90 min did not accurately predict acid tolerance, but extended incubation in selective conditions successfully isolated acid-tolerant and acid- and bile-tolerant colonies. Isolates were verified as being different from their parental strains rather than being environmentally adapted variants based on growth in selective and nonselective conditions, phenotypic characterization, plasmid profiles, and fatty acid profiles of the cell wall. These data indicate that the use of natural selection with consideration to the environment in the gastrointestinal tract is useful in isolating acid- and bile-tolerant strains for use as probiotic bacteria. Further work is needed to explore the usefulness of these characterized strains as probiotic strains in animal models.

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