Characterization and Purification of a Bacteriocin Produced by a Potential Probiotic Culture, *Lactobacillus acidophilus* 30SC

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ABSTRACT

Lactobacillus acidophilus 30SC was tested for its potential as a probiotic culture. The strain exhibited good acid tolerance in an artificial gastric solution as well as bile resistance in media containing 0.3% bile acids. The strain produced a heat-stable antimicrobial compound that was shown to be proteinaceous in nature and, therefore, referred to as a bacteriocin. The bacteriocin was active over a wide pH range and inhibited a number of Gram-positive bacteria including *Listeria ivanovii* and pathogenic strains. The bacteriocin was purified by 50% ammonium sulfate precipitation followed by hydrophobic interaction column chromatography. The SDS-PAGE of the active fractions resulted in a single band with estimated molecular mass of 3.5 kDa. These results demonstrate the potential of *L. acidophilus* 30SC as a probiotic culture that can be utilized in the manufacturing of dairy foods and dietary supplements.

(**Key words:** probiotics, bacteriocin, *Lactobacillus acidophilus*)

Abbreviation key: LAB = lactic acid bacteria

INTRODUCTION

Intestinal lactic acid bacteria (**LAB**) for humans are closely associated with the host's health because LAB is an important biodefense factor in preventing colonization and subsequent proliferation of pathogenic bacteria in the intestine (7, 18). A probiotic is defined as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (4). Some species of LAB have been claimed as probiotics, such as *Lactobacillus acidophilus, Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus casei*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, and *Lactobacillus reuteri*, and *Bifidobacterium* species. To be considered as probiotics, these bacteria should become a part of the normal microbial flora in the intestine, survive the gastrointestinal passage, and be able to adhere and colonize the intestinal tract (9).

The gastrointestinal tract of a healthy human is a harsh environment because it contains gastric juices, digestive enzymes, and bile acids. These conditions impose a significant threat to probiotic strains. In addition, low surface tension and immune response also affect the survival of probiotic strains (5). *Lactobacillus acidophilus* strains are widely used as probiotic cultures in dairy and pharmaceutical products because this species is one of the dominant lactobacilli in the human intestine (21).

Since one hundred trillion individual bacteria of 100 different varieties inhabit the intestine (16), it is challenging for probiotic strains to become established as gastrointestinal microflora. Thus, organisms that can produce a product that will inhibit the growth or kill existing organisms in the intestinal milieu have a distinct advantage (7).

LAB produce a number of antimicrobial substances, including organic acids, hydrogen peroxide, bacteriocins, and bacteriocin-like substances. Bacteriocins or bacteriocin-like substances are peptides or proteins, which exhibit inhibitory activity against sensitive strains of bacteria (11, 17). Antimicrobial peptides have been found to be widely distributed in microorganisms as well as some insects and rodents (17). Numerous LAB bacteriocins have been extensively studied. Bacteriocins have been grouped into three main classes based on their chemical and genetic properties (14). The first class, the lantibiotics, comprises small peptides with dehydrated or modified residues such as dehydroalanine and lanthionine (13). The second class includes small heat stable bacteriocins such as pediocin A, leucocin A, lactacin F, lactococcins, and carnobacteriocin A, BM1 and B2 (11). The third group comprises large heat labile bacteriocins such as helveticin J (14).

Some bacteriocins that are produced by strains of *L. acidophilus* have been purified and characterized

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including acidocin 8912 (24), lactacin B (2), acidophilin (23), acidolin (8), and lactocidine (25). However, few of these strains have been extensively studied for their potential probiotic use.

The purpose of this study was to evaluate the potential of *L. acidophilus* strains as a probiotic culture by characterizing the acid and bile tolerance as well as the bacteriocin produced by these strains in vitro.

MATERIALS AND METHODS

Bacterial Strains

Lactobacillus acidophilus 30SC was obtained from the Dairy Microbiology Laboratory in Oklahoma State University. *L. delbrueckii* subsp. *lactis* 4797 and pathogenic bacteria were obtained from American Type Culture Collections. LAB were grown for 18 h at 37°C in MRS broth (Difco, Detroit, MI), and the pathogens were incubated in trypticase soy broth (Difco) at either 30 or 37°C for 18 to 24 h. The strains were subcultured three times before use. Cultures were stored at –70°C in 10% skim milk with 30% glycerol as a cryoprotectant.

Acid Tolerance

The artificial gastric juice was prepared by supplementing MRS broth (Difco) with pepsin (Sigma Chemical Co., St. Louis. MO). The MRS broth was prepared by adjusting to pH 2.5 with 1*N* HCl and was then sterilized by autoclaving for 15 min at 121°C. The pepsin solution was filter sterilized using a 0.22-μm membrane filter and was then added to MRS broth (pH 2.5) to a final concentration of 1000 units/ml.

The strains were incubated at 37°C for 18 h and then centrifuged (4,000 × *g* for 10 min, 4°C). The collected cells were then resuspended in sterile saline (0.85% NaCl). The cells were inoculated at 10^6 cfu/ml into artificial gastric juice and incubated at 37°C for 1, 2, and 3 h, respectively. The bacterial counts were determined with MRS plates containing 2% β-glycerophosphate (Sigma Chemical Co.).

Bile Tolerance

Bile tolerance was determined in MRS broth containing 0.3% bile acids (oxgall; Difco). Before testing for bile tolerance, all strains were incubated at 37°C for 18 h in MRS broth without bile. After centrifugation (4,000 × *g* for 10 min, 4°C), the collected cells were resuspended in sterile saline (0.85% NaCl) and then inoculated into MRS broth containing 0.3% bile acids. Cultures were incubated at 37°C. The bacteria were plated and enumerated after 24 and 48 h of incubation.

Bacteriocin Production and Activity

The five strains of *L. acidophilus* were screened for bacteriocin production against a range of indicator bacteria (Table 1) using the spot-on-lawn assay (1). Before testing, cells were removed from the growth medium by centrifugation (6,000 × *g* for 20 min, 4°C). Bacteriocin activity was quantitated by spotting 20-μl aliquots of twofold serial dilutions of the culture supernatant that was adjusted to pH 6.5 using 10*N* NaOH and spotted onto the surface of MRS agar. The spotted agar was then overlayed with 0.8% MRS agar inoculated with 1% of the indicator strain, *Leuconostoc* sp. K2 isolated from Kimchee, a traditional Korean fermented cabbage product. The plates were incubated at 37°C for 24 h. The bacteriocin activity was determined by the highest twofold dilution showing a clear inhibition zone on the MRS agar.

Sensitivity to heat, pH, and proteases

Samples of crude bacteriocin were used for these tests. Aliquots of the semi-purified bacteriocin were exposed to heat treatments of 65°C for 40 min, 95°C for 20 min, and 121°C for 20 min, and then were tested for remaining antimicrobial activity. Semipurified preparations of the bacteriocin were adjusted to various pH values in the range of 3 to 10. The pH-adjusted bacteriocin samples were incubated at 37°C for 20 min and then neutralized to pH 6 and tested for bacteriocin activity. Susceptibility of bacteriocin to various proteases was performed by incubating the bacteriocin preparation in the presence of pronase E (1 mg/ml) and proteinase K (1 mg/ml) at 37°C for 1 h. After incubation, the enzymes were inactivated by heat treatment at 65°C for 30 min and tested for bacteriocin activity.

Purification of Bacteriocin

Culture supernatant was obtained by centrifugation (8,000 × *g* for 30 min, 4°C) of *L. acidophilus* 30SC inoculated MRS broth incubated at 37°C for 24 h. Ammonium sulfate was added to reach 50% (wt/vol) and allowed to stir overnight at 4°C. The ammonium sulfate precipitate was collected by centrifugation at 10,000 × *g* for 20 min and resuspended with 2-(4-morpholino)-ethane sulfonic acid (FisherBiotech, Fair Lawn, NJ) buffer (50 m*M*, pH 6.5). The crude bacteriocin solution was subjected to hydrophobic interaction column chromatography using Octyl-Sepharose CL-4B (Pharmacia Biotec AB, Uppsala, Sweden). The column was equilibrated with 1.7 *M* $(NH_4)_2SO_4$ and then eluted with a linear increasing gradient using H_2O and ethanol at a flow rate of 1 ml/min. The absorbance was a monitored at

Table 1. Antimicrobial spectrum of crude bacteriocin from *Lactobacillus acidophilus* 30SC against Gram-positive and Gram-negative bacteria.

Bacteria	Inhibition[1]	Reference
Gram-positive		
Bacillus cereus ATCC1 1778	+	ATCC
Bacillus subtilis 1A650	+	(20)
B. subtilis 1A651	+	(20)
Lactobacillus acidophilus ATCC 43121	–	ATCC
Lactobacillus casei Y2	+	Lab. isolate
Lactobacillus delbruekii subsp. *lactis* ATCC 4797	+	ATCC
Lactobacillus fermentum ATCC 11931	+	ATCC
Lactobacillus helveticus 1213	–	Lab. isolate
Lactobacillus plantarum	+	Lab. isolate
Lactococcus lactis ATCC 1145	–	ATCC
Lactococcus sp. CU216	–	Lab. isolate
Leuconostoc sp. K2	+	Lab. isolate
Listeria innocua	–	Lab. isolate
Listeria ivanovii	+	Lab. isolate
Listeria monocytogenes	–	Lab. isolate
Staphylococcus aureus	+	Lab. isolate
Streptococcus faecalis	–	Lab. isolate
Gram-negative		
Acinetobacter baumani	–	Lab. isolate
Escherichia coli O157 ATCC 43889	–	ATCC
E. coli O157 ATCC 43893	–	ATCC
E. coli O157 ATCC 43894	–	ATCC
E. coli O157 ATCC 43895	–	ATCC
Klebsiella pneumoniae	–	Lab. isolate
Salmonella typhimurium	–	Lab. isolate
Yersinia enterocolitica	–	Lab. isolate

[1]+, Inhibited by crude bacteriocin; – not inhibited. ATCC, American Type Culture Collection.

280 nm and bacteriocin activity of each fraction was determined by the spot-on-lawn assay.

SDS-PAGE

Active fractions from the Octyl-sepharose CL-4B column were pooled and concentrated by rotary evaporation. The concentrated sample was electrophoresed on 16.5% tricine-SDS polyacrylamide gel (22). A duplicate gel was washed three times in 250 ml of H_2O for 30 min, and the gel was placed onto MRS agar medium and overlayed with soft MRS agar inoculated with 1% *Leuconostoc* sp. K2.

RESULTS

Acid and Bile Tolerances

All experiments were replicated four times, and each viable cell count was performed in duplicate. As shown in Figure 1, a relatively high population of *L. acidophilus* survived under acidic conditions. *Lactobacillus acidophilus* NCFM was found to be the least acid tolerant, with a final population of 6.0×10^4 cfu/ml, whereas the population of *L. acidophilus* 30SC and ATCC 43121 remained relatively constant.

As shown in Figure 2, all five strains of *L. acidophilus* exhibited excellent bile tolerance. *Lactobacillus acido-*

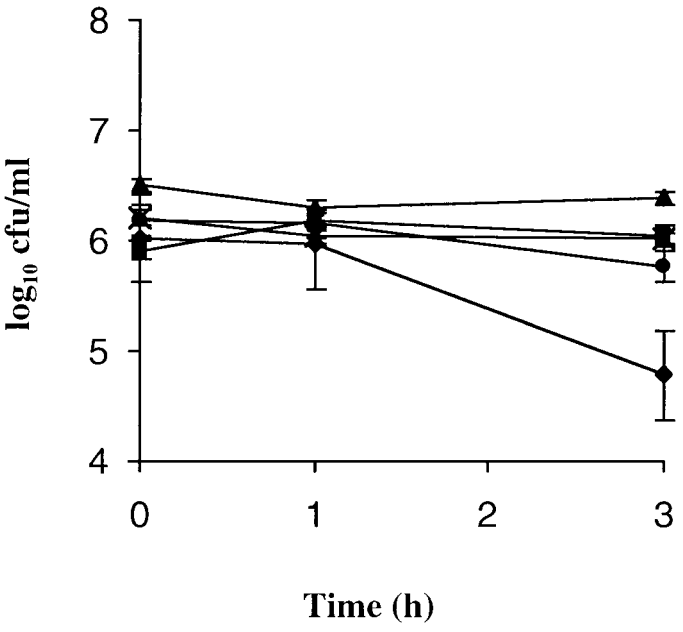

Figure 1. Acid tolerance of *Lactobacillus acidophilus* strains. *L. acidophilus* 30SC (■), *L. acidophilus* ATCC 4356 (●), *L. acidophilus* ATCC 43121 (*), *L. acidophilus* GP1B (▲), *L. acidophilus* NCFM (◆).

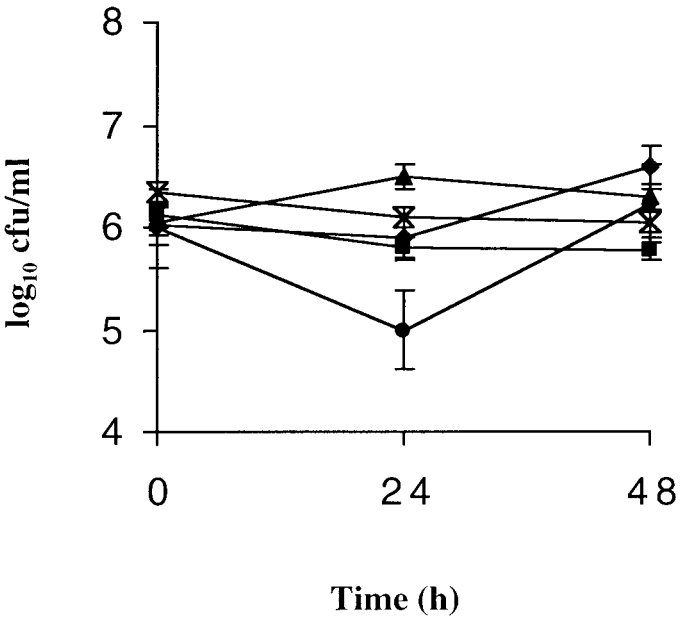

Figure 2. Bile tolerance of *Lactobacillus acidophilus* strains. *L. acidophilus* 30SC (■), *L. acidophilus* ATCC 4356 (●), *L. acidophilus* ATCC 43121 (*), *L. acidophilus* GP1B (▲), *L. acidophilus* NCFM (◆).

philus ATCC 43121 showed enhanced growth in media containing 0.3% bile acids after 24 h of incubation, while *L. acidophilus* 30SC survived at a level of 7.0×10^5 cfu/ml under the same conditions.

Inhibitory Spectrum

Only *L. acidophilus* 30SC was found to produce a bacteriocin that displayed antimicrobial activity against some of the tested indicator strains (Table 1). The bacteriocin produced by *L. acidophilus* 30SC was capable of inhibiting Gram-positive pathogenic bacteria including *Bacillus cereus* ATCC 11778, *Listeria ivanovii*, and *Staphylococcus aureus*. Other Gram-positive strains inhibited were primarily LAB. Like other LAB bacteriocins, the *L. acidophilus* 30SC bacteriocin did not inhibit Gram-negative bacteria.

Effects of Enzymes, pH, and Heat on the Bacteriocin

Partially purified bacteriocin was found to be sensitive to pronase E and proteinase K, but a bacteriocin solution treated with catalase did not affect the activity (Table 2). The fact that catalase had no effect on the bacteriocin inhibitory activity suggests that the inhibition is not due to hydrogen peroxide. These data indicate that the inhibitory substance is proteinaceous in nature. Inhibitory activity was unaffected by heating

Table 2. Characteristics of bacteriocin.

Treatment	% Activity[1]
Heat Treatment	
65°C/40 min	100
95°C/20 min	100
121°C/20 min	50
pH	
3	50
4	50
5	50
6	100
7	100
8	50
9	50
10	50
Enzymes[2]	
Pronase E	0
Proteinase K	0

[1]Bacteriocin activity is expressed as the % of residual activity.
[2]The enzyme concentration was 1 mg/ml.

up to 95°C for 20 min, and 50% of activity still remained after a heat treatment of 121°C for 20 min. The bacteriocin produced by *L. acidophilus* 30SC was completely stable at pH 6 and 7, and 50% of activity remained after subjection to the various pH values between 3 and 10.

Purification of Bacteriocin

The majority of the bacteriocin activity was eluted at approximately 60% ethanol from Octyl-Sepharose CL-4B, indicating that the antimicrobial compound is hydrophobic in nature (Figure 3). SDS-PAGE of the pooled active fractions resulted in a single band with the estimated molecular mass of 3.5 kDa. Incubation of the electrophoresed gel on the agar seeded with indi-

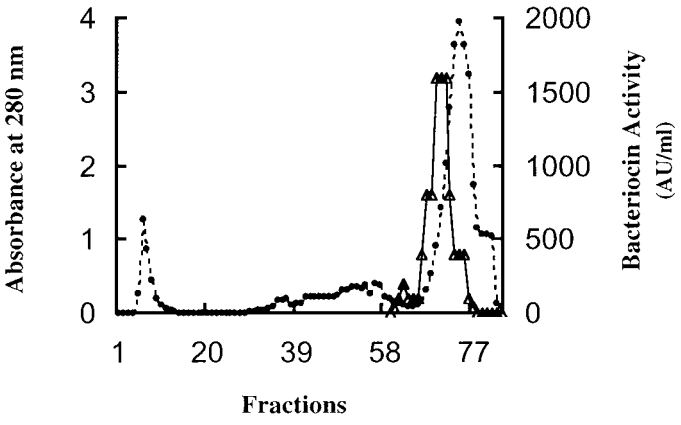

Figure 3. Elution pattern of *Lactobacillus acidophilus* 30SC bacteriocin on Octyl-Sepharose CL4B. Absorbance at 280 nm (●); Bacteriocin activity (△).

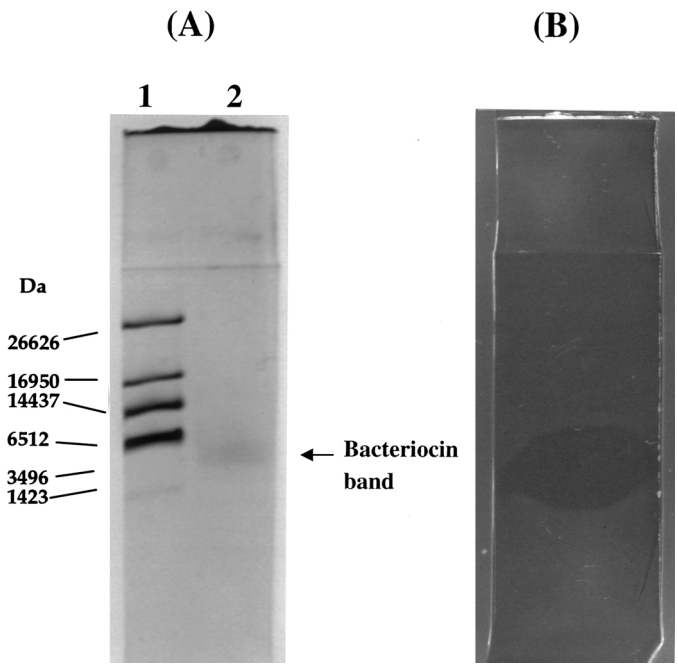

Figure 4. Tricine-SDS-PAGE and detection of antimicrobial activity of the purified *Lactobacillus acidophilus* 30SC bacteriocin. (A) gel stained with Coomassie blue stain; lane 1, low molecular mass protein standards (Bio-Rad, Hercules, CA); lane 2, purified *L. acidophilus* 30SC bacteriocin. (B) gel overlayed with cells of *Leuconostoc* sp K2 inoculated in MRS soft agar.

cator strain confirmed the inhibitory action of the corresponding protein band (Figure 4).

DISCUSSION

In general, strains of *L. acidophilus* exhibit more acid and bile resistance than other lactic acid bacteria (5). The strains of *L. acidophilus* tested in this study have been isolated from feces from human or various animals and showed acid resistance. The acid tolerance of LAB is dependent upon the pH profile of H^+-ATPase and the composition of the cytoplasmic membrane, which is largely influenced by the type of bacteria, type of growth media, and the incubation conditions (9, 10). Strains of *L. acidophilus* as well as the strains of *L. casei* and *L. plantarum* have been shown to survive at pH 3.0 or less. Some were able to pass through the stomach without losing viability (10, 19). Because there was significant correlation between in vivo and in vitro experiments, the acid tolerance test with an artificial gastric juice is frequently used to select for probiotic strains.

Bile tolerance is one of the most essential criteria for a strain to be used as a probiotic culture (5). Bile salts are surface-active chemicals produced in the liver from the catabolism of cholesterol. The bile acids consist of chenodeoxycholic acid, cholic acid, deoxycholic acid and other minor components secreted from spleen into duodenum of the small intestine (3). Bile acids have been shown to inhibit microorganisms and their inhibitory activity is greater than organic acids (19). LAB isolated from the intestine, do not grow well in the media containing 0.15% bile acids (6). However, LAB isolated from intestinal sources such as *L. acidophilus* and *L. casei*, are capable of surviving in the presence of bile due to their ability to deconjugate bile acids. Bile resistance and the ability of LAB to inhabit the intestinal tract appear to be correlated (6, 10).

L. acidophilus 30SC exhibited excellent acid resistance and bile tolerance compared with commercial probiotic strains that are presently used such as *L. acidophilus* NCFM. For prolonged survival of probiotic strains in the host body, LAB should have the ability to colonize the intestine (7). Adherence and colonization are considered important properties for probiotic strains (9). Colonization of a probiotic strain in an already existing microbial ecosystem requires more than adherence alone. Probiotics with the ability to compete against other microorganisms, for example by the production of antimicrobial substances, increases their chance to colonize this ecosystem (9). The ability of probiotics to establish themselves in the gastrointestinal tract should enhance their ability to eliminate competitors using bacteriocin(s).

The bacteriocin produced by *L. acidophilus* 30SC displays antimicrobial effects on bacteria such as *Listeria* and *Bacillus* species; spore-forming bacteria, *B. cereus* and *B. subtilis* were sensitive to the bacteriocin produced by *L. acidophilus* 30SC. The bacteriocin may have potential use as a food additive in the food industry, particularly processed food, in which some *Bacillus* species are potential spoilage microorganisms.

The *L. acidophilus* 30SC bacteriocin was active over a wide range of pH, and was stable to various heat treatments. The loss of antimicrobial activity following treatment with proteinase K and pronase E indicated that the active component secreted extracellullarly by *L. acidophilus* 30SC was proteinaceous in nature. Similar properties have been reported for other bacteriocins including lactacin, lactacin 27, acidolin, pediocin A, and pediocin PA-1 (11, 14). These bacteriocins were also stable over a wide range of pH. This heat and pH stability may be useful if the bacteriocin is to be used as an antimicrobial agent in fermented foods or thermally processed foods.

The molecular mass of the *L. acidophilus* 30SC bacteriocin was estimated at 3.5 kDa, whereas acidocin 8912 (24) and lactacin B (2) were reported to be 5.4 and 6.5 kDa, respectively. However, acidocin B migrated with a molecular mass of 2.4 kDa on SDS-PAGE, but the

molecular mass was subsequently calculated to be 5.8 kDa from its DNA sequence (15). Similarly, the molecular mass of gassericin A was estimated by SDS-PAGE to be 3.8 kDa, but the mass spectroscopy showed that the molecular mass of gassericin A is 5652 Da (12). To characterize the structure and function of the bacteriocin produced by *L. acidophilus* 30SC, further investigation is required and is presently being performed.

In conclusion, *L. acidophilus* 30SC meets several of the criteria for use as a probiotic culture, which includes acid and bile tolerances, as well as the production of antagonistic substances. These characteristics may be advantageous for a probiotic culture to be successful in colonizing and to compete with pathogens in the gastrointestinal environment. The ability to survive acidic conditions, bile resistance, and the production of a bacteriocin that is active against food-related pathogens and spoilage microorganisms, contributes to the ability of *L. acidophilus* 30SC as a probiotic culture that may have potential applications in the production of cultured foods and dietary adjuncts.

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