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Antimicrobial Substance from a Human *Lactobacillus* Strain

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***Lactobacillus* sp. strain GG, which was isolated from the feces of a normal person, produced a substance with potent inhibitory activity against a wide range of bacterial species. It inhibited anaerobic bacteria (*Clostridium* spp., *Bacteroides* spp., *Bifidobacterium* spp.), members of the family *Enterobacteriaceae*, *Pseudomonas* spp. *Staphylococcus* spp., and *Streptococcus* spp., as demonstrated by a microbiological assay; however, it did not inhibit other lactobacilli. The inhibitory activity occurred between pH 3 and 5 and was heat stable. Bactericidal activity against *Escherichia coli* was demonstrated at a dilution of 1:128. The inhibitory substance was distinct from lactic and acetic acids. It had a low molecular weight (<1,000) and was soluble in acetone-water (10:1). Because of these characteristics, the inhibitory material could not be considered a bacteriocin; it most closely resembled a microcin, which has been associated previously with members of the family *Enterobacteriaceae*.**

The genus *Lactobacillus* is widely distributed in nature. Several species, including *Lactobacillus acidophilus*, are components of the normal intestinal flora of healthy humans. Other species, such as *Lactobacillus bulgaricus* and *Lactobacillus casei*, are found in dairy products as well as in fruits and vegetables. The taxonomy is extremely complex, and complete agreement on the designation of specific strains cannot be found.

In some studies it has been shown that when lactobacilli are implanted in the intestinal tract, there seems to be a suppressive effect on other members of the microflora (13, 15). For example, it has been reported that antibiotic-associated diarrhea and colitis have been controlled (5, 11, 15). The metabolic activation of carcinogens by the intestinal flora of humans and animals is suppressed by feeding them lactobacilli (9). Similarly, chemically induced colon cancer is reduced in experimental animals given lactobacilli (10).

Previous investigators have described an antibacterial substance elaborated by certain *L. acidophilus* strains (4, 7, 11, 15, 16, 18). Although incompletely characterized, this substance seems to be active principally against other lactobacilli. We describe here a rather different antibacterial substance with broad-spectrum inhibitory properties produced by a human *Lactobacillus* strain designated GG and isolated and characterized in our laboratory.

MATERIALS AND METHODS

Lactobacillus sp. strain GG was isolated from a healthy person by screening stool specimens for strains that were stable to acid and bile, that adhered to the intestinal mucosa in an in vitro model system (8), and that produced an antibacterial substance. This organism grew better under anaerobic conditions, although it could grow in the presence of CO_2. It fermented xylose, trehalose, sorbitol, salicilin, ribose, rhamnose, melezitose, mannose, mannitol, glucose, fructose, and cellobiose. It did not ferment amygdalin, arabinose, erythritol, glycogen, inositol, lactose, maltose, melibiose, raffinose, or sucrose. The electrophoretic pattern of strain GG soluble proteins was most like that of *L. casei* subsp. *ramnosus*, but GG differed from that subspecies in that it could not ferment lactose, maltose, or sucrose. The gas-liquid chromatographic pattern, as determined by the methodology outlined by Holdeman et al. (12), revealed only a small peak of acetic acid among the volatile acids and a major peak of lactic acid among the nonvolatile acids. The fermentation pattern was determined by following the methodology described by the Virginia Polytechnic Institute, and it was confirmed by the Anaerobic Laboratory, Virginia Polytechnic Institute, Blacksburg, courtesy of L. V. Holdeman. They identified the organism as *Lactobacillus* species.

Isolation of inhibitory substance. Strain GG was grown in MRS (Difco Laboratories, Detroit, Mich.) for 48 h in an anaerobic chamber at 37°C. The bacterial cells were removed by centrifugation at 7,500 × *g*. The supernatant was filtered through a filter (pore size. 4.5 μm; Nalgene Labware Div., Nalgene/Sybron Corp., Rochester, N.Y.) and was concentrated 10-fold by rotary vacuum evaporation at 60°C. To control for inhibitory substances that could be present in the medium, a 10-fold sterile MRS concentrate was prepared by the same methodology. The concentrates were stored at 20°C, at which the GG concentrate retained activity for at least 1 year.

Determination of inhibitory activity by microbiological assay. Inhibitory activity was determined against various bacterial strains: anaerobic bacteria (*Clostridium* spp., *Bacteroides* spp., *Bifidobacterium* spp.), members of the family *Enterobacteriaceae*, *Staphylococcus* spp., *Pseudomonas* spp., and *Streptococcus* spp. Facultative bacteria were grown in Mueller-Hinton broth for 4 to 5 h, streptococci were grown in brain heart infusion broth, and anaerobes were grown in brain heart infusion broth supplemented with yeast-vitamin K-hemin. Cultures were adjusted to 10^8 CFU/ml, and 0.5 ml of the adjusted culture was added to molten agar (brucella agar for streptococci and anaerobic bacteria, MRS agar for lactobacilli, and Mueller-Hinton agar for the *Enterobacteriaceae*). Plates were allowed to solidify, and wells (diameter, 4 mm) were cut into the agar and filled with the 10-fold concentrate of the GG strain or the MRS 10-fold concentrate. The plates were incubated aerobically and anaerobically at 37°C for 24 h. Zones of inhibition were

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TABLE 1. Inhibitory effect of *Lactobacillus* sp. strain GG 10-fold concentrate on other bacteria

Bacterium (no. of strains tested)	No. of strains at the following inhibitory zone diam (mm):						
	0	7	10	12	14	16	>16
Escherichia coli (11)			4	7			
Streptococcus spp. (12)				8	4		
Pseudomonas spp. (2)			2				
Salmonella spp. (2)		2					
Bacillus fragilis group (8)				3	2	1	2
Clostridium spp. (18)			4	5	7	2	
Bifidobacterium spp. (1)				1			
Lactobacillus spp. (9)	9						

read with a caliper. Determinations of the inhibitory activity of different concentrations of lactic and acetic acids against *Escherichia coli* B-44 were performed by the same methodology.

Thermal stability. The GG 10-fold concentrate was heated at 90°C for 1 h and autoclaved at 121°C for 15 min. Inhibitory activity was then determined by using *E. coli* B-44 as an indicator.

pH stability. The pH of the GG 10-fold concentrate was raised from 4.5 to 5.3 and 6.7, with 1 N NaCl. Inhibitory activity was tested at different pHs by using *E. coli* B-44 as an indicator.

Stability to proteases. The pH of an acetone extract of the MRS broth supernatant of *Lactobacillus* sp. strain GG was adjusted to pH 7.4 with NaOH. Trypsin, proteinase K, α-chymotrypsin, carboxypeptidase, and insoluble *Streptomyces griseus* protease were added to a final concentration of 1 mg/ml. Bromelin was mixed with the extract without altering the original pH (3.5 to 4.5). All enzymes were obtained from Sigma Chemical Co., St. Louis, Mo. After a 2-h incubation at 37°C, 1 μl of a saturated solution of phenol red was added and HCl was added until the indicator turned yellow. The inhibitory activity of the enzyme-treated preparations was measured against *E. coli* B-44 by using the diffusion assay in Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.). Controls included the original extract, with and without pH adjustments. Because the strain GG extract might have inhibited the proteolytic enzymes, the enzymatic activity was measured against casein (ICN Nutritional Biochemicals, Cleveland, Ohio) by using an agar diffusion assay (6, 14) at pH 7.4 and 4.1. All of the soluble proteases demonstrated caseinolytic activity at the appropriate pH, indicating that the GG extract did not inactivate the enzymes.

Determination of bactericidal activity against *E. coli* B-44. Serial twofold dilutions were made of the strain GG 10-fold concentrate in Trypticase soy broth at pH 5. *E. coli* B-44 and *Lactobacillus* sp. strain GG were grown in Trypticase soy broth and incubated anaerobically at 37°C for 24 h. The cultures were adjusted by using McFarland standards to a density equivalent to 10^7 CFU/ml, and 0.5 ml of *E. coli* B44 or *Lactobacillus* sp. strain GG was added to the diluted GG 10-fold concentrate. The tubes were incubated anaerobically at 37°C; *E. coli* was incubated for 24 h, and *Lactobacillus* sp. strain GG was incubated for 48 h. A total of 25 μl from each tube was plated; *E. coli* was plated in Mueller-Hinton agar and incubated at 37°C aerobically, and GG was plated in MRS agar and incubated anaerobically for 48 h. Positive controls, Trypticase soy broth plus the bacteria, either *E. coli* or strain GG, and negative controls, GG 10-fold concentrate and Trypticase soy broth, were also plated. The MBC was defined as the highest dilution of GG concentrate that reduced the original bacterial inoculum by 99.9%.

TABLE 2. Lactic and acetic acids in the 10-fold concentrates of three *Lactobacillus* strains

Lactobacillus strain	Concn (meq/100 ml) of:	
	Lactic acid	Acetic acid
Lactobacillus sp. strain GG	27.54	5.92
Lactobacillus acidophilus ADH	26.33	1.55
Lactobacillus bulgaricus	17.71	16.53
MRS broth (control)	1.41	4.23

RESULTS

The zones of inhibition (diameter in millimeters) against the bacteria tested are shown in Table 1. The strain GG substance was inhibitory against strains of *E. coli*, *Streptococcus*, *Pseudomonas*, *Salmonella*, *Bacteroides fragilis*, *Clostridium*, and *Bifidobacterium*. The inhibitory activity of the GG 10-fold concentrate when measured against *E. coli* B-44 was always greater under anaerobic conditions, producing larger zones of inhibition than under aerobic conditions. No inhibition was demonstrated against any of the nine strains of *Lactobacillus* tested. Wells containing MRS 10-fold concentrate showed no inhibitory effect.

The antimicrobial material was active between pH 3 and 5. When the pH was raised to 5.3, the inhibitory activity was lost. Back-titration of the substance to a lower pH allowed the inhibitory effect to be retrieved.

The 10-fold concentrates of GG retained good activity after they were heated at 90°C for 1 h and at 121°C for 15 min. They were also stable to protease inhibitors such as trypsin, proteinase K, α-chymotrypsin, bromelin, carboxypeptidase A, and *S. griseus* protease.

The amounts of lactic and acetic acids present in the 10-fold concentrates of three *Lactobacillus* strains grown in MRS broth, as determined by gas-liquid chromatography determinations, are listed in Table 2. The concentration of lactic acid in the GG concentrate was 27.54 meq/100 ml, which was the highest concentration among the three *Lactobacillus* strains tested. The acetic acid concentration of GG concentrate was 5.92 meq/100 ml, which was greater than the concentration produced by strain ADH (kindly supplied by North Carolina State University, Raleigh) but less than that produced by *L. bulgaricus*. The zones of inhibition against *E. coli* B-44 obtained at lactic acid concentrations of 50 and 100 meq/100 ml were 4.5 and 7.5 mm, respectively (Table 3). Similarly, the zones of inhibition with acetic acid were 10 and 8 mm, respectively. The GG 10-fold concentrate produced a zone of inhibition of 12 mm against this organism. When lactic and acetic acids were tested at the concen-

TABLE 3. Lactic acid and acetic acid inhibition against *E. coli* B-44

Sample (meq/100 ml)	Inhibitory zone (mm)[a]:	
	Lactic acid	Acetic acid
100	7.5	10
50	4.5	8
20	0	6
5	0	0

[a] A 10-fold GG concentrate, containing 27.54 meq/100 ml of lactic acid and 5.92 meq/100 ml of acetic acid, produced an inhibitory zone of 12 mm.

trations produced by GG (27.54 and 5.92 meq/100 ml; Table 2), no inhibitory activity was observed.

Bactericidal activity against *E. coli* B-44 was demonstrated at a dilution of 1:128.

DISCUSSION

It has been reported by several investigators that lactobacilli are able to produce antimicrobial substances when grown in specific media (4, 7, 11, 15, 17, 18). Vincent et al. (18) described in 1959 what was called lactocidin, a substance obtained from solid agar medium seeded with *L. acidophilus*. This substance was more active against gram-negative than gram-positive bacteria.

Barefoot and Klaenhammer (4) reported a substance produced by *L. acidophilus* N2 which was active against *L. leichmannii*, *L. helveticus*, *L. lactis*, and *L. bulgaricus*. It was pH dependent with maximum activity detected in broth cultures at pH 6. This substance was classified as a bacteriocin, because it had a molecular weight of 6,000 to 6,500; however, its activity was restricted to members of the family *Lactobacillaceae*, and it had a peptide structure.

In 1974, Hamdan and Mikolajcik (11) isolated acidolin from skim milk cultured for 48 h with *L. acidophilus*. This substance was acidic with a molecular weight of 200 and was inhibitory to enteropathogenic and spore-forming organisms. It also showed limited activity against lactic acid bacteria.

Other antibacterial substances have been isolated from various types of bacteria. Microcins, for example, are produced mostly by members of the family *Enterobacteriaceae*. First described by Asensio et al. (1), microcins are peptide antibiotics with low molecular weights. Their production is mediated by plasmids, and they are not inducible by DNA-damaging agents (3). They are also insensitive to proteases (2).

In contrast, the bacteriocins, which are inhibitory substances that are generally produced by gram-positive bacteria, have high molecular weights and are susceptible to proteases; and their spectrum of antimicrobial activity is limited to related species (17).

The inhibitory substance produced by *Lactobacillus* sp. strain GG, even though it is produced by a gram-positive organism, has a low molecular weight and is active against a broad spectrum of gram-negative and gram-positive organisms, including lactic acid bacteria, but not against other lactobacilli. These characteristics make the substance different from the bacteriocins. It is resistant to various proteases such as proteinase K, α-chymotrypsin, bromelin, trypsin, and carboxypeptidase A. It is also heat resistant. The low molecular weight and its narrow range of pH preference, in the acidic range, suggest that the antibacterial substance may be a short-chain fatty acid. Certainly, it is not lactic or acetic acid, the principal acids produced by GG according to gas-liquid chromatographic determinations, because the concentrations of these acids required for antibacterial activity were considerably higher than the amounts produced by strain GG. Our laboratory is attempting to characterize and purify this antibacterial substance.

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