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Screening of *Lactobacilli* Derived from Fermented Foods and Partial Characterization of *Lactobacillus casei* OSY-LB6A for Its Antibacterial Activity against Foodborne Pathogens

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Abstract

Various fermented foods were screened in search of food-grade bacteria that produce bacteriocins active against Gram-negative pathogens. An isolate from a mold-ripened cheese presented antibacterial activity against Gram-positive and Gram-negative bacteria. The most active isolate was identified as *Lactobacillus casei* by a biochemical method, ribotyping, and membrane lipid analysis, and was designated as OSY-LB6A. The cell extracts of the isolate showed inhibition against *Escherichia coli* p220, *E. coli* O157, *Salmonella enerica* serovar Enteritidis, *Salmonella* Typhimurium, and *Listeria monocytogenes*. The antibacterial nature of the cell extract from the isolate was confirmed by eliminating the inhibitory effects of acid, hydrogen peroxide, and lytic bacteriophages. The culture supernatant and cell extract retained antibacterial activity after heating at 60~100°C for 10~20 min. The activity of the cell extract from *Lb. casei* was eliminated by pronase and lipase. Finally, the cell extract showed a bactericidal mode of action against *E. coli* in phosphate buffer solution, but it was bacteriostatic in broth medium and food extracts.

Key words: *Lactobacillus casei*, antibacterial activity, foodborne pathogens, bactericidal, bacteriostatic

INTRODUCTION

In recent decades, food safety and food control have been important issues in many countries. Foodborne diseases cause approximately 76 million illnesses, resulting in 325,000 hospitalizations, and 5,000 deaths each year in the US alone (1). Foodborne pathogens such as *Salmonella* spp. and pathogenic *E. coli* have raised concerns about the safety and quality of food, especially minimally processed foods, as they may multiply in these products during extended refrigerated storage (2).

To inactivate foodborne pathogens, novel technologies including nonthermal technologies such as high pressure processing or pulsed electric fields, or combined treatments with a biopreservation system, have been studied using the hurdle concept (3). With increasing consumer demands for natural products and increasing concerns about foodborne disease, recent approaches in food preservation are increasingly being directed towards biocontrol using protective microflora, usually lactic acid bacteria (LAB), to inhibit the growth of foodborne pathogens. LAB are important groups of microorganisms in food fermentation with GRAS (generally recognized as safe) status and have been associated with the production of fermented foods for many centuries. In addition to their health and nutritional benefits (4), LAB contribute desirable effects on food flavor and texture and inhibit undesirable microflora by producing compounds like organic acids, diacetyl, hydrogen peroxide, and antibacterial proteins during fermentation (5,6), thus extending product shelf-life. For this reason, the use of bacteriocins, as well as the organisms that produce them, is attractive to the food industry to control the growth of pathogenic and spoilage organisms in a variety of foods (7,8).

So far, bacteriocins from LAB have been studied against Gram-positive spoilage and pathogenic bacteria and only a few studies have examined their inhibition of Gram-negative bacteria. This is because Gram-negative bacteria are resistant to bactericins *per se*, due to their outer membrane acting as a permeability barrier (9-11). Thus, experiments have been conducted to sensitize Gram-negative bacteria to bacteriocins by exposing them to physical or chemical antibacterials at sublethal levels (12,13).

The objectives of this study were to screen for food-grade microorganisms that produce antibacterials against foodborne pathogens, particularly Gram-negative bacteria, and to determine the efficacy of a strain against

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Gram-positive and -negative bacteria.

MATERIALS AND METHODS

Screening for antibacterial-producing bacteria

The products and environmental samples screened for bacteriocin-producing LAB were derived from food (Parmesan, Cheddar, mold ripened cheeses, and specialty cheeses) and different environments (meat and dairy plants, food processing facilities, and home kitchens). The samples were collected and their microbiota were screened for the ability to produce antibacterials against a selected indicator strain. The screening and isolation of bacteria with antibacterial activity was performed using hydrophobic grid membrane filters, as described by Ryser and Richard (8) with modifications. Briefly, the samples were homogenized and diluted and the dilutions were filtered through a hydrophobic-grid filter (QA Life Sciences Inc., San Diego, CA) mounted on an autoclaved filtering apparatus. The filters were overlaid on tryptose agar (TA; Difco laboratories, Detroit, MI, USA) and the agar plates were incubated at 37°C for 18 hr. Next, the filters were removed and the incubated agar plates, which potentially contained antibacterial metabolites, were overlaid with soft TA (0.75% agar) seeded with *E. coli* p220 (~10^6 CFU/mL), the indicator bacterium. The inoculated plates were incubated at 37°C for 18 hr and were assessed for zones of inhibition after incubation.

Microorganisms

Lactobacillus casei OSY-LB6A was isolated from food and identified during the course of this study. *Lb. casei* ATCC 334, *Lb. curvatus* OSY-HJC6, *Lb. curvatus* ATCC 25601, *Lb. leichmannii*, and *Lactococcus lactis* ATCC 11454 were obtained from the culture collection of the Food Safety Laboratory, Ohio State University (OSU, Columbus, OH, USA). All strains were grown in MRS broth (Difco). The working cultures were maintained on slants of MRS agar, and stored at 4°C. *E. coli* (395T4958, K12, p220, O157:H7, O157:H12, O157-M1, and O157:M2), *Listeria monocytogenes* Scott A, *Bacillus cereus* 14579, *Pseudomonas fluorescens, Salmonella enterica* serovar Enteritidis, and *Salmonella* Typhimurium were obtained from the culture collection of the Food Safety Laboratory at Ohio State University (Columbus, OH, USA). The pathogens were grown in Trypticase Soy Broth (BBL, Sparks, MD, USA) and supplemented with 0.6% Yeast Extract (Difco) (TSBYE). The working cultures were maintained on slants of Trypticase Soy Agar supplemented with 0.6% Yeast Extract (TSAYE) and stored at 4°C. Each stock culture was maintained in the respective growth media, containing 20% glycerol (v/v), and stored at -80°C. Before use in subsequent experiments, the strains were transferred into fresh media and incubated at 37°C for 18 hr; this was followed by two consecutive transfers in the medium and incubation under the conditions just indicated. The final counts of cultures were approximately 10^9 CFU/mL.

Characterization and identification of an antibacterial-producing isolate

An isolate showing the strongest antibacterial activity against the indicator microorganisms was identified using colony and cell morphology, biochemical analysis (API 50 CH strip, bioMérieux, Inc., Hazelwood, MO), membrane lipid profile analysis (Microbial ID, Inc., Newark, DE), and ribotyping (RiboPrinter® system, DuPont Qulicon, Wilmington, DE). The antibacterial-producing isolate was identified as *Lactobacillus casei* OSY-LB6A. *Lb. casei* OSY-LB6A was compared to *Lb. casei* ATCC 334 and other lactobacilli using a pulsed-field gel electrophorertic typing technique as described by Tynkkynen et al. (14) with slight modification. Briefly, bacterial DNA was prepared from *Lactobacillus* spp. and embedded in agarose gel. Glycine and phenylmethylsulphonyl fluoride were omitted from the growth medium and the wash buffer, respectively. Agarose-imbedded DNA for PFGE analysis was digested with 2 restriction enzymes, ApaI and SmaI (Invitrogen Life Technologies, Carlsbad, CA). The resulting restriction fragments were separated in agarose gel by PFGE (Electrophoresis Cell, Bio-Rad, Hercules, CA). The electrophoresis conditions were 1.6% agarose gel, 0.5X TBE buffer (45 mM Tris-borate, 1 mM EDTA), 5 V/cm, and 1~15 sec switching time for 42 hr (15).

Antibacterial cell extract of *Lb. casei* OSY-LB6A

Cell extract of *Lb. casei* was prepared according to the procedure described by Chung and Yousef (11). *Lb. casei* was inoculated into 2 L of MRS medium, supplemented with 1% NaCl (16), and the mixture was incubated at 30°C for 18 hr. The cells were harvested by centrifugation at 13,200×*g* for 20 min at 4°C and washed twice in 20 mM sodium phosphate buffer (SPB), pH 7.0. The cell pellet was treated with 5 mg/mL lysozyme (Sigma, St. Louis, MO), 0.6 M sucrose, and 5 mM $MgCl_2$, and reacted at 30°C for 3 hr. After the reaction, the pellet was washed twice in 20 mM sodium phosphate buffer (pH 7.0), suspended in 50 mL of the same buffer, cooled in a water/ice mixture bath, and sonicated (Torbeo ultrasonic processor, Cole Parmer, Vermon Hill, IL) at setting 5 for 5 min while maintaining cooling. The supernatant was collected by centrifugation at 34,200×*g* for 20 min, freeze-dried, and stored at -18°C

until use. The freeze-dried powder (0.2 g) was re-suspended in 1 mL of the same buffer; this solution was designated as the cell extract (11).

Characteristics of cell extract

The inhibitory effect of the *Lb. casei* cell extract was evaluated in terms of inhibitory responses that may be attributable to H_2O_2 from the producing cells. The possible interference of a lytic bacteriophage was tested; portions of the cell extract were subjected to heat (60～100°C for 10 min), or were treated with enzymes including pepsin, trypsin, protease, chymotrypsin, bromelin, lipase, α-amylase, pronase, ficin, and papain at 37°C for 2 hr. The enzymes were obtained from Sigma, except for pronase, ficin, and papain, which were from Calbiochem (Sandiago, CA). The enzymes were applied at a final concentration of 1 mg/mL, with the exception of lipase, which was added at 0.1 mg/mL. Fifty μL of the supernatant and enzyme mixture was assayed for residual activity using the well diffusion assay (17).

Antibacterial activity assay using a microtiter plate

The antibacterial activity of the cell extract was tested in a microtiter plate assay system, as described by Jimenez-diaz et al. (18) with modifications. Each well of the microtiter plate (Becton Dickinson, Franklin lakes, NJ) contained 25 μL of 2x TSBYE medium, 25 μL of cell extract or its two-fold dilutions, and 10 μL of the indictor strain, *E. coli* p220 (10^6 CFU/mL). The positive and negative control wells contained 25 μL of TSBYE medium or 25 μL of SPB, with or without added inoculums (10 μL), respectively. The microtiter plate cultures were incubated at 37°C for 15 hr, and growth inhibition of the indicator strain was assessed by measuring the absorbance at 600 nm in a microtiter plate reader (Vmax Kinetics Microplate Reader, Molecular Devices, Sunnyvale, CA). The absorbance value of each dilution without the indicator was subtracted from the corresponding value of the same dilution containing the indicator microorganism. The antibacterial activity of colicin (Sigma) against *E. coli* p220 was used as a reference for measuring the activity in the cell extract. Commercial colicin produced by *E. coli* contains 20,000 units/mg of pure proteins. One unit per mL is the minimal concentration required to cause a zone of inhibition on a lawn of *E. coli* ATCC 9637 cells. In this study, the highest dilution of the cell extract causing inhibition of the indicator, compared to the positive control, was converted to colicin-equivalent activity units (CEAU) (11).

Antibacterial activity of *Lb. casei* cell extract against *E. coli* p220

The freeze-dried cell extract of *Lb. casei* (0.2 g) was prepared in 1 mL of 20 mM SPB (pH 7.0) and two-fold dilutions were prepared in the same buffer. An overnight culture of *E. coli* p220 was diluted into fresh TSBYE medium or 20 mM SPB (pH 7) to a final concentration of ～10^6 CFU/mL. Aliquots (1 mL) of 3 different concentrations of the *Lb. casei* cell extract (20, 80, and 320 CEAU/mL) were added to 9 mL of the *E. coli* p220 cell suspension. The control received 1 mL of buffer instead of the cell extract. The mixtures were incubated at 37°C, and aliquots were taken at 3 hr intervals, during 18 hr of incubation. The number of viable bacterial cells was determined by spread-plating on TSAYE agar. The plates were incubated at 37°C and the colony forming units were counted.

RESULTS AND DISCUSSION

Isolation and identification of an antibacterial agent-producing bacterium

Analysis of more than 100 food and environmental samples produced a limited number of isolates exhibiting antibacterial activity against a Gram-negative indicator bacterium, *E. coli* p220. An isolate from a mold-ripened cheese showed broad antibacterial activity against *E. coli* p220, *E. coli* O157 strains (O157:H7, O157:H12, O157-M1, and O157-M2), selected *Enterobactericeae*, and Gram-positive bacteria including *L. monocytogenes* Scott A and *B. cereus* (Table 1). The Gram-positive bacteria tested in this study were more sensitive to the antibacterial agent produced by the isolate than Gram-negative bacteria. For the Gram-negative bacteria, *Salmonella* spp. and *Pseudomonas fluorescens* were less sensitive than *Escherichia* spp. The isolate was characterized by

Table 1. Inhibitory spectrum of cell extract from *Lb. casei* OSY-LB6A by a microtiter plate method at 37°C

Microorganisms	Media[1)]	Relative sensitivity[2)]
Gram-positive bacteria		
Listeria monocytogenes Scott A	TSBYE	+++
*Bacillus cereus*14579	TSBYE	+++
Gram-negative bacteria		
Escherichia coli p220	TSBYE	++
E. coli K12	TSBYE	++
E. coli O157:H7	TSBYE	++
E. coli O157:H12	TSBYE	++
E. coli O157-M1	TSBYE	++
E. coli O157-M2	TSBYE	++
Salmonella Enteritidis	TSBYE	+
Salmonella Typhimurium	TSBYE	+
Pseudomonas fluorescens	TSBYE	+

[1)]TSBYE=Trypticase soy broth + 0.6% yeast extract, MRS=de man, Rogosa, and Sharpe.
[2)](+) Inhibitory properties; +++, strong; ++, medium; +, mild.

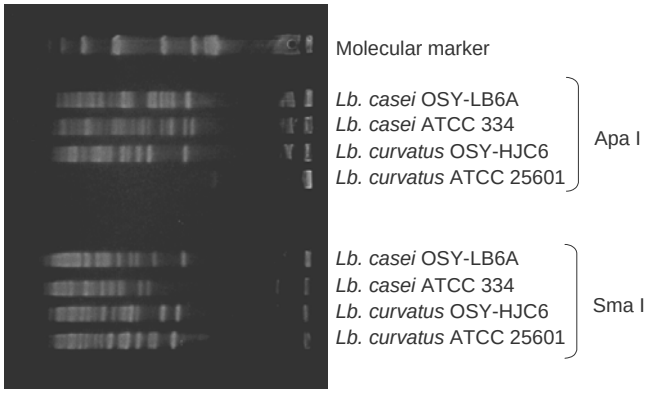

Fig. 1. Comparison of *Lb. casei* OSY-LB6A and other lactobacilli by genetic typing using pulsed-field gel electrophoresis.

biochemical testing, using an API system (API 50 CH strip), ribotyping, and membrane lipid profile analysis, and it was identified as *Lb. casei*. The isolate was compared with antibacterial agent-producing and non-producing *lactobacilli*, using PFGE, a genetic typing technique. The restriction enzymes generated different DNA banding patterns for the food isolate as compared with the other strains (Fig. 1). The isolate was designated as *Lb. casei* OSY-LB6A.

Inhibitory characteristics of the cell extract

The inhibitory activity of the cell extract from *Lb. casei* OSY-LB6A was tested under conditions that eliminated possible inhibitory effects of organic acids by testing both a neutralized cell extract and acidified broth. The natural pH of the *Lb. casei* OSY-LB6A cell extract was 4.2. When neutralized to pH 7.2, the cell extract retained most of its inhibitory activity when tested using an agar diffusion assay, confirming that the source of the antibacterial activity could not be attributed to the low pH of the *L. casei* cell extract. Similar results were reported for several bacteriocins produced by lactic acid bacteria (5,19). To eliminate a possible inhibitory effect by hydrogen peroxide, the cell extract was treated with catalase and the activity was tested by an agar diffusion assay. The catalase-treated *Lb. casei* cell extract did not lose any activity compared to an untreated cell extract. In addition, plaques were not detected on lawns of indicator strain prepared by mixing the indicator cells with a portion of agar that had previously been removed from the clear zones of inhibition by the cell extract. This result rules out the possibility of a lytic phage causing the inhibitory activity.

Next, the nature of the antibacterial agent was evaluated by treating with various enzymes. The activity of the *Lb. casei* cell extract was eliminated by pronase, but showed resistance to most proteolytic enzymes. Lipase also had an influence on the activity of the cell extract, indicating that a lipid is critical to its biological activity (Table 2).

Table 2. Effect of heat and enzyme treatments on the activity of cell extract from *Lb. casei* OSY-LB6A

Treatment		Relative activity[1)]
None		++
Temperature	60°C for 10 min	++
	70°C for 10 min	++
	80°C for 10 min	++
	90°C for 10 min	++
	100°C for 10 min	++
Enzymes[2)]	Pepsin	++
	Trypsin	++
	Protease	++
	Chymotrypsin	++
	Papain	++
	Bromelin	++
	Lipase	−
	Pronase	−
	Ficin	++
	Amylase	++

[1)](+) most of the activity retained; (−) activity lost.
[2)]Enzymes were applied at a final concentration of 1 mg/mL, except lipase was added at 0.1 mg/mL.

In terms of heat sensitivity, when the *Lb. casei* cell extract (pH 4.2) was heated at 60, 80, 90, and 100°C for 10 min, it retained inhibitory activity against *E. coli* p220 as compared to an unheated control (Table 2). All together, the data show that the inhibitory activity of the cell extract from *Lb. casei* OSY-LB6A is from a bacteriocin-like substance (5).

Antibacterial mode of the *Lb. casei* cell extract against *E. coli*

The cell extract's mode of action was investigated using *E. coli* p220, a nonpathogenic strain. Here, a suspension of *E. coli* p220 (10^6 CFU/mL) in SPB or TSBYE was treated with 32, 8, and 2 CEAU/mL at 37°C for 18 hr. The cell extract (32 CEAU/mL) was bactericidal against the *E. coli* cells in buffer, showing a reduction of approximately 5 log cycles in *E. coli* cells within 3 hr of incubation. Treatment of the cultures with low concentrations of the cell extract (8 and 2 CEAU/mL) seemed to stimulate the growth of *E. coli* cells in SPB, as these low concentrations of the cell extract may have served as a source of nutrients for the growing cells (Fig. 2A). When *E. coli* was suspended in a growth-permitting medium (TSBYE), the cell extract of *Lb. casei* (32 CEAU/mL) exhibited a bacteriostatic mode of action against the indicator during 18 hr of incubation. This may have been due to a possible interaction of the cell extract with the ingredients of the nutritionally complex media, modifying its antibacterial activity (20). A low

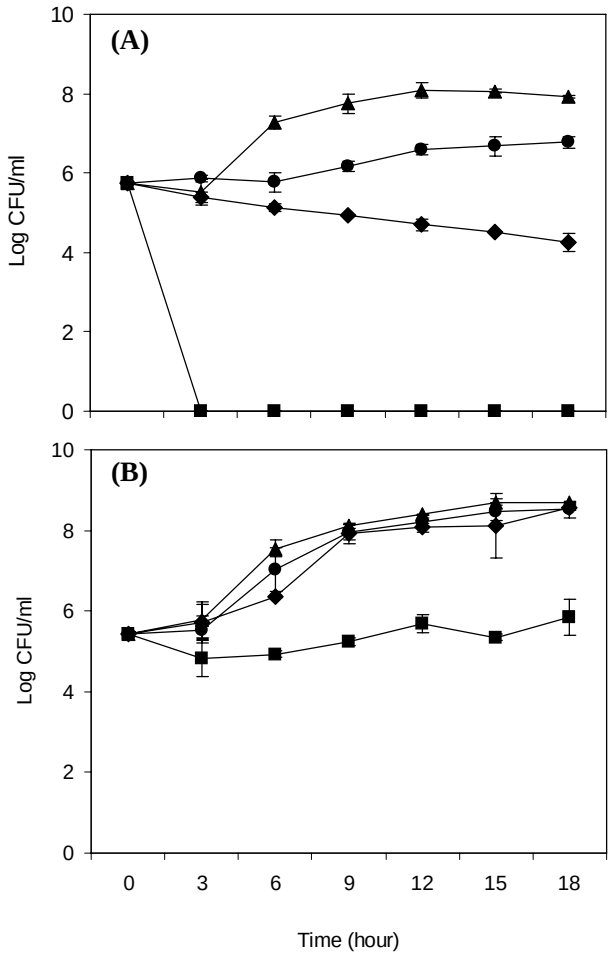

Fig. 2. Bactericidal and bacteriostatic effects of cell extract from *Lb. casei* OSY-LB6A on *E. coli* p220. A. sodium phosphate buffer (20 mM, pH 7). B. TSBYE. Culture of *E. coli* p220 was incubated with various concentrations of cell extract, and survivors were counted. (●) Control cells (without cell extract), (■) 32 CEAU/mL, (◆) 8 CEAU/mL, and (▲) 2 CEAU/mL.

concentration of the cell extract (8 CEAU/mL) presented an inhibitory effect but the 2 CEAU/mL concentration did not (Fig. 2B). Thus, it is likely that this cell extract behaves bacteriostatically under growth permitting conditions such as in foods.

Thus far, bacteriocins and bacteriocin-like substances (5) produced by strains of *Lb. casei* have been reported. They include caseicin 80 (21), lactocin 705 (22), and antibacterial substances produced by *Lb. casei* ssp. *casei* LC-10 and *Lb. casei* ssp. *pseudoplantarum* LB1931 (23). These antibacterials are active against strains closely related to the producer, but they were not active against Gram-negative bacteria (21-23). In that sense, these antibacterial activities against Gram-negative bacteria are quite an intriguing discovery.

In conclusion, a new *Lactobacillus* strain, isolated from a mold-ripened cheese, produced a bacteriocin-like substance with inhibitory activity against Gram-positive and Gram-negative bacteria, including *L. monocytogenes* and *E. coli* O157 strains, respectively. Overall, the cell extract from *Lb. casei* is potentially useful as a biopreservative in food.

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