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Purification and Partial Characterization of Novel Bacteriocin L23 Produced by *Lactobacillus fermentum L23*

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Abstract *Lactobacillus fermentum* strain L23 produced a small bacteriocin, designated bacteriocin L23, with an estimated molecular mass of < 7000 Da. Isolation, purification, and partial characterization of bacteriocin L23 are described. It displayed a wide inhibitory spectrum including both Gram-negative and Gram-positive pathogenic strains and two species of *Candida*. The antibacterial activity of cell-free culture supernatant fluid was not affected by catalase or urease but was abolished by the proteolytic enzymes trypsin and protease VI. Bacteriocin L23 was heat stable (60 min at 100°C) and showed inhibitory activity over a wide pH range (4.0 to 7.0). The proteinaceous compound was isolated from cell-free culture supernatant fluid and purified. Crude bacteriocin sample was prepared by a process of ammonium sulfate precipitation, gel filtration, thin-layer chromatography, bioautography, and reversed-phase HPLC.

Introduction

Facultative Gram-positive rods of the genus *Lactobacillus* (one of several genera of lactic acid bacteria) are the predominant organisms in the vaginal flora of healthy women. *Lactobacillus* species produce hydrogen peroxide, which helps prevent overgrowth of potentially pathogenic vaginal bacteria, and also produce lactic acid, lactocidin, acidolin, and lactacin B, which help maintain a healthy vaginal "ecosystem" [2, 24]. Other protective activities of lactobacilli are competition with pathogens for adherence to vaginal epithelium and stimulation of immune system cells, particularly lymphocytes and macrophages [26].

Bacteriocins are proteinaceous toxic compounds produced by bacteria that inhibit growth of closely related strains or unrelated species [13, 19]. Bacteriocins of lactobacilli are of interest because of their potential application for inhibition of pathogenic bacteria in humans [25].

Bacteriocins produced by lactic acid bacteria are divided into five classes based on primary structure, molecular mass, heat stability, and molecular organization [22, 31]: class I, lantibiotics; class II, nonlantibiotic peptides (subclass IIa, pediocin-like bacteriocins with strong antilisterial activity; subclass IIb, bacteriocins whose activity depends on complementary action of two peptides; subclass IIc, sec-dependent secreted bacteriocins); class III, large, heat-labile protein bacteriocins; class IV, bacteriocins consisting of an undefined mixture of protein(s), lipid(s), and carbohydrate(s); and class V, bacteriocins with circular, unmodified posttransductional structure (including AS-48, gasericine A, enterocin) [14, 16, 18].

Among the various lactic acid bacteria, bacteriocins have been isolated mainly from the genus *Lactobacillus*, because of the diversity of its species and habitats [13]. Recent attention has focused on bacteriocin production by strains of *L. fermentum* and *L. acidophilus*, which are the predominant lactobacilli in human intestine and display interesting probiotic properties [17, 29].

The majority of *L. fermentum* and *L. acidophilus* strains produce bacteriocins [28]. Lactacin B was the first

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antimicrobial peptide from *L. acidophilus* to be clearly identified as a bacteriocin [5]. Lactacin F was the first bacteriocin biochemically and genetically characterized from a strain of *L. acidophilus*, which was later reclassified as *L. johnsonii* [22]. Here, we describe the isolation, purification, and partial characterization of a bacteriocin from *L. fermentum*.

Materials and Methods

Strains and Culture Conditions

L. fermentum strain L23 was isolated from vaginal swabs of healthy, nonpregnant, premenopausal women admitted to the Department of Obstetrics and Gynecology, Río Cuarto Hospital. This strain was selected from among 100 strains of *Lactobacillus* on the basis of its bacteriocinogenic ability. It displayed properties relevant to colonization, i.e., self-aggregation, adherence to vaginal epithelial cells, and coaggregation with bacterial pathogens. The bacteria were grown in De Man/Rogosa/Sharpe (MRS) broth or agar at 37°C under a 5% CO_2 atmosphere for 24 h. Other bacterial strains isolated from genitourinary infections and used in this study are listed in Table 1. Purity of strains was confirmed by Gram-staining technique. Strains were identified by standard biochemical tests [7] and by the ID 32 E, rapid ID 2 STREP, ID 32 STAPH, ID 20 NE, API 50 CHL, and API 20 Candida systems (BioMèrieux, France). API 50 CH test kit and API CHL medium (BioMèrieux) were used to test the ability of the strains to cause fermentation of 49 different carbohydrates. Species identifications based on biochemical profiles were confirmed by identification software programs (BioMèrieux).

Lactic acid bacteria were maintained in lyophilized form. Before experiments, cultures were propagated in duplicate in MRS broth [11]. *Escherichia coli* was used as the indicator strain for detection of antimicrobial activity and was maintained on nutrient agar slants and subcultured in tryptose soya broth (Oxoid).

For bacteriocin production, L23 strain was grown in MRS broth**.** *E. coli* was used as indicator strain for detection and quantitative determination of bacteriocin activity of *L. fermentum.* L23 MRS broth containing 1.2% agar (Oxoid) was used as solid medium. L23 were seeded in streaks on agar plates, incubated for 24 h at 37°C, and then exposed to chloroform for 20 min. Plates were seeded at a right angle to the original streak with *E. coli*, and incubated for 24 h at 37°C, by a modified version of Hall's method [3].

Table 1 Bacterial strains used in this study, and their sensitivity to the bacteriocin produced by *Lactobacillus fermentum* strain L23

Indicator strain	No. of strains	Diameter (mm) of inhibition zone[a]	% of strains inhibited
Escherichia coli	100	41	100
Pseudomonas aeruginosa	30	44	100
Proteus mirabilis	15	40	100
Proteus vulgaris	5	38	100
Klebsiella pneumoniae	10	34	100
Klebsiella oxytoca	4	30	100
Enterobacter aerogenes	8	49	100
Acinetobacter calcoaceticus	3	46	100
Serratia marsescens	4	54	100
Staphylococcus epidermidis	15	21	100
Staphylococcus saprophyticus	6	30	100
Staphylococcus aureus	15	36	100
Enterococcus faecalis	8	31	100
Gardnerella vaginalis	120	32	100
Candida albicans	30	15	95
Candida glabrata	8	13	62
Neisseria gonorrhoeae	26	30	100
Mycoplasma hominis	29	+[b]	100
Ureaplasma urealyticum	68	+[b]	100

[a] Average diameter (mm) of inhibition zones caused by 640 UA/ml of purified *Lactobacillus fermentum*

[b] Inhibition in liquid medium

Inhibitory Spectrum and Quantitative Determination of Bacteriocin Activity

To establish the inhibitory spectrum of bacteriocin, cell-free culture supernatant fluid adjusted to pH 5.5, derived from *L. fermentum* L23 culture incubated for 18 h at 37°C in MRS broth, was spotted onto indicator lawns of various strains of lactic acid bacteria and other Gram-positive and Gram-negative bacteria. Lawns were prepared by propagating fresh bacterial cultures to an optical density of 0.45 (at 600 nm), adding 200 µl of cell suspension to 3.5 ml of overlay agar, and incubating plates for 24 h at 37°C.

Bacteriocin activity was assayed quantitatively by agar spot test. Briefly, twofold serial dilutions in 50 mM KH_2PO_4 buffer of cell-free culture supernatant fluid containing bacteriocin were spotted (100 µl) onto fresh indicator lawns of *E. coli.* Bacteriocin activity was defined as the reciprocal of the highest dilution which produced complete inhibition of the indicator lawn, and is expressed as activity units (AU) per milliliter of culture medium [4, 12].

Preliminary Characterization of the Bacteriocin

The producer strain was cultivated in MRS broth for 18 h at 37°C, cells were removed by centrifugation (8500 *g*, 30 min, 4°C), and cell-free culture supernatant fluid was used as a crude bacteriocin sample for the following characterization steps. First, effects of trypsin, protease type VI, and urease on bacteriocin activity were tested. Each enzyme was prepared at a concentration of 1 mg/ml and added to the crude bacteriocin sample at a final concentration of 0.5–0.25 mg/ml. After incubation for 1 h at 37°C, inhibitory activity was tested. Next, the crude bacteriocin sample was treated with catalase (final concentration, 5 mg/ml) and dissolved in 50 mM^{-1} KH_2PO_4 buffer (pH 7.0) at 25°C to completely eliminate a possible inhibitory effect of hydrogen peroxide. Finally, heat sensitivity and pH stability were tested. The crude bacteriocin sample was heated for 60 min at 100°C and for 10 min at 121°C before testing inhibitory activity. To evaluate the influence of pH, the crude bacteriocin sample was adjusted to a pH of 2.0, 4.0, 5.0, 6.0, 7.0, or 9.0 with HCl or NaOH, mixed, left standing for 5 h, and inhibitory activity was tested [1, 6]. For testing temperature and enzymatic sensitivity and pH stability, the remaining activity was assayed by the agar well diffusion method and compared with the activity of a corresponding control. *E. coli* was used as indicator strain.

Molecular Size Estimation

Dialysis membranes of MWCO 10,000 and 7000 Da were placed in agar MRS plates to separate the producer streak from the sensitive strain when an inhibitory halo was observed [3].

Bacteriocin Isolation and Purification

Separation of Bacteriocin from Bacterial Culture

Bacterial strains were cultured in MRS broth for 18 h at 37°C. Cells were removed by centrifugation, and the pH of the cell-free culture supernatant fluid was adjusted to 5.5. The supernatant fluid was precipitated with ammonium sulfate (60% saturation) overnight at 4°C, with gentle stirring, and then centrifuged for 45 min at 33,000 *g* [15, 31].

Sephadex G-25 Chromatography

The solution from step a was chromatographed on a Sephadex G-25 column (35 × 2 cm) and eluted with the same phosphate buffer. Column fractions were assayed for bactericidal activity in **p**etri plates. The precipitate was redissolved in a minimal volume of phosphate buffer (pH 6.5). Final purification was performed by thin-layer chromatography **(TLC)** [8, 10, 15**]**.

HPLC

Bacteriocin produced by *L. fermentum* strain L23 was purified by the three-step procedure developed for class II bacteriocins [16], i.e., ammonium sulfate precipitation, Sephadex G-25 chromatography, and C-18 reversed-phase HPLC. Selected fractions were further purified to homogeneity on HPLC with an isocratic gradient. HPLC-purified bacteriocin preparations were assayed for inhibitory activity by agar diffusion test [21].

Thin-layer Chromatography

Fractions obtained by chromatography as above that showed antibacterial activity were analyzed by TLC on silica gel plates (20 × 10 cm) using a solvent system of diethyl ether/toluene/ethanol/acetic acid (40:50:2:0.2). TLC plates were run in duplicate, and one set was used as the reference chromatogram. Spots and bands were visualized by UV irradiation at 254 nm [9**]**.

Bioautographic Assays

Developed chromatograms were placed in sterile **p**etri glass plates with covers. An inoculum of *E. coli* containing 10^6 CFU/ml in liquid MRS broth containing 1.2% agar was poured over the plates. After the medium solidified, the TLC plate was incubated overnight at 37°C [30**]**.

Results

Inhibitory Spectrum

L. fermentum strain L23 was incubated in MRS broth for 18 h at 37°C, producer cells were removed, **the** pH of the cell-free culture supernatant fluid was adjusted to pH 5.5, and inhibitory effects on various bacterial strains were tested. Results are **reported** in Table 1. The inhibitory spectrum of strain L23 was quite broad, including Gram-negative and Gram-positive pathogenic strains and *Candida* species. Accordingly, strain L23 was subjected to quantitative determination of bacteriocin activity.

Table 2 Characterization of the substance with antibacterial activity produced by *Lactobacillus fermentum*

Effect of treatment on antibacterial activity	Size of inhibitory zone (average, mm)
Temperature	
60 min at 100°C	27
10 min at 121°C	27
Control (37°C)	27
pH, 5 h	
2	0
4	13[a]
5	21[b]
7	9[a]
9	0
Control (5.5)	23
pH, 15 h	
2	0
4	9[a]
5	19[b]
7	0
9	0
Control (5.5)	23
Enzymes	
Trypsin	0
Protease VI	0
Control	24

[a] Significant difference compared to control ($p < 0.05$)

[b] No significant difference compared to control ($p > 0.05$)

Preliminary Characterization of Crude Bacteriocin

To evaluate the proteinaceous nature of the antibacterial substance, the effect of proteolytic enzymes (trypsin, protease VI) was tested. Incubation of samples for 1 h at 37°C with these enzymes completely abolished the antibacterial activity (Table 2). Catalase and urease had no effect on the activity.

The inhibitory activity of the crude bacteriocin was not significantly altered by heat treatment. After 60 min at 100°C, the activity was about 640 AU/ml, the same as the activity observed after 10 min at 121°C. These results suggest that the antibacterial substance produced by strain L23 is resistant to heat.

The effect of pH variation (2.0, 4.0, 5.0, 6.0, 7.0, 9.0) on bacteriocin activity was tested after 5 h of incubation in MRS agar. All samples showed inhibitory activity in a wide pH range, from 4.0 to 7.0, with optimal activity at pH 5. Bacteriocin activity was most stable at acid or neutral pH. At alkaline pH, the bacteriocin became progressively inactivated (Table 2).

Molecular size estimation using dialysis membranes indicated that the antibacterial substance was a dialyzable molecule smaller than 7000 Da.

Purification of the Bacteriocin Produced by Strain L23

The cell-free culture supernatant fluid (640 AU/ml) was precipitated with 40% ammonium sulfate saturation. The bacteriocin activity of the supernatant was also 640 AU/ml.

The partially purified bacteriocin was further purified by chromatography gel filtration (Sephadex G25). The sample was concentrated by evaporation and diluted in a small volume of phosphate buffer at pH 6.5.

Fractions of 2.0 ml were collected, and their activity toward the indicator strain was tested. Fractions F15, F16, F17, and F18 displayed inhibitory activity against *E. coli*. They were pooled and concentrated.

The fraction collected after C18 reversed-phase HPLC exhibited activity against the indicator strain *E. coli.* The corresponding elution profile from reversed-phase HPLC recorded at 220 nm revealed one peak collected in the fraction eluted at 30 min. When an aliquot of the 30-min fraction was subjected to agar well diffusion assay, a zone of inhibition was produced in the agar.

The fractions with antibacterial activity (F15, F16, F17, F18) obtained by chromatography assays were analyzed by TLC on silica gel plates. A single spot was visualized by UV irradiation (254 nm). This spot was subjected to bioautography and showed an inhibition zone against *E. coli*, indicating the presence of active compound. The compound having antibacterial activity was identified as bacteriocin L23, produced by *L. fermentum* strain L23.

Discussion

The bacteriocin produced by *L. fermentum* strain L23 was sensitive to several proteases, indicating that the inhibitory material was proteinaceous. Its antibacterial activity was not abolished by treatment with catalase and urease. Like most of the known bacteriocins produced by *L. fermentum* strains, it is a heat-stable and low molecular mass (<7000-Da) peptide. The bacteriocin activity was stable at a wide range of nonalkaline pH levels (4.0 to 7.0). According to the classification in Ref. 18, these biochemical characteristics indicate that the bacteriocin produced by strain L23 belongs to the class II lactic acid bacterium bacteriocins.

The number of chromatographic steps varies from three or more, e.g., for acidocin LF221 A and B [8], to only one, e.g., for bacteriocin L23 in the present study, in which antibacterial activity was recovered after simple

precipitation with ammonium sulfate from the cell-free culture supernatant fluid. The final reversed-phase HPLC step of the purification procedure led to isolation of a single active fraction having antibacterial activity. Bioautography on thin-layer chromatograms, a method previously used for detecting antibacterial and antifungal substances for control of pathogens in the urogenital tract, was useful to reveal the active fraction.

The bacteriocin L23 produced by *L. fermentum* strain 23 showed a wide inhibitory spectrum, including some lactobacilli. A noteworthy observation was the inhibition of the Gram-negative, pathogenic bacteria *E. coli*, *Proteus vulgaris*, *P. mirabilis, Klebsiella pneumoniae*, and *Neisseria gonorrhoeae*. In general, bacteriocins from lactic acid bacteria are active only toward Gram-positive bacteria. Our results are not consistent with those of Klaenhammer [19]. A wide inhibitory spectrum, as observed here for *L. fermentum*, seems to be common among bacteriocin-producing isolates from the genus *Lactobacillus* (group III). For example, lactacin F from *L. johnsonii V*Pl 11088 displays bactericidal activity toward other lactobacilli and *Enterobacter faecalis* [22]. Acidocins J1129 and J 1132 [28], and acidocins LF221 A and LF213 B [8], are active against closely related lactic acid bacteria. Many low molecular mass, proteinaceous, antibacterial compounds from *L. acidophilus* showing broad inhibitory spectra, including inhibition of Gram-positive and Gram-negative bacteria, have been classified as small, heat-stable, bacteriocin-like peptides [12]. These findings are consistent with those of the present study. Bacteriocin L23 did not show inhibitory activity against species of normal vaginal flora, including lactobacilli. The amino acid composition of the antibacterial component secreted by *L. fermentum* strain L23 remains to be determined. Strain L23 secretes an antibacterial substance(s) other than lactic acid that is heat stable and only moderately sensitive to enzyme treatment. Several characteristics of the component(s) responsible for the antibacterial activity suggest that it contains an unusual acidic amino acid present in a novel peptidic agent. Further research is necessary to confirm whether bacteriocins from probiotic lactic acid bacteria are among the compounds responsible for inactivation of pathogens in urogenital tracts.

In conclusion, bacteriocin L23 appears to be a novel inhibitor of bacterial infection, and its potential antibacterial activity should be further explored in animal models.

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