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Characteristics of Bacteriocin and Mucin Production Phenotypes in *Lactobacillus plantarum* 27

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Phenotypic changes after plasmid curing experiment suggested that the bacteriocin production phenotype (Bac$^+$) might be linked to a chromosomal DNA and the mucin production phenotype (Muc$^+$) might be linked to a 62.5 kilobase (kb) plasmid (pMUC62) in *Lactobacillus plantarum* 27 isolated from meat starter culture. The non-mucoid (Muc$^-$) variants were missing pMUC62 but they produced bacteriocin as the wild strain (Bac$^+$). There was no difference in antibiotic resistance and sugar fermentation patterns between the wild strain (Bac$^+$ Muc$^+$) and the non-mucoid (Bac$^+$ Muc$^-$) variants. Antimicrobial spectrum of bacteriocin produced by both wild strain and Muc$^-$ variant of *Lb. plantarum* 27 included strains of *Pediococcus acidilactici* (A, M, H), *Pediococcus* sp. isolated from meat, *Lactobacillus* sp. isolated from meat, *Lb. plantarum* NCDO 955 and *Staphylococcus aureus* 485. Neither of the tested Gram negative bacteria were inhibited by bacteriocin. Antimicrobial activity of crude bacteriocin was retained after autoclaving, DNase or catalase treatment and exposure from pHs 4 to 9 but was lost after treating with several proteolytic enzymes and exposure at pH 10.

The demands of using defined lactic starter culture bacteria in dairy and sausage fermentation industry is increasing. Along with lactic acid production, some species of lactic starter culture bacteria are known to produce bacteriocin or bacteriocin-like compounds (14, 16, 18). The bacteriocins are protein or protein-containing macromolecules which exerting bacteriocidal activity against species that are closely reated to the produced bacterium (29). Due to their antagonistic effect, especially against some health hazardous bacteria associated with food spoilage and food-borne diseases, the use of bacteriocins of lactic acid bacteria as food biopreservative has currently been an active area of study (8-11, 14, 16, 18).

Lactobacillus plantarum is one of the major starter culture bacteria used in the fermentation of meat (4, 28) and vegetable (15). Since the first description of bacteriocin-like compound from *Lb. plantarum* (20), few studies have been conducted on properties of bacteriocin produced by strains of *Lb. plantarum* from different origins (2, 3, 34). However, only one genetic study concerning bacteriocin production phenotype (Bac$^+$) in *Lb. plantarum* has been reported (M. A. Daeschel *et al*., 1986. Abstr. Ann. Meet. Am. Soc. Microbiol., Washington DC., USA, p. 14).

Extracellular ropy material (mucin) produced by several species of *Lactococcus* (21, 28) and *Lb. bulgaricus* (13) is important for the distinct texture of the products which are fermented by these strains. Several reports have indicated that the mucin production phenotype (Muc$^+$) in several *Lactococcus* are linked to a plasmid (24, 26, 32, 33) while there has been no reports regarding ropy characters in *Lb. plantarum*.

In the genetic studies of lactic starter culture for the strain development, it is important to understand the role of genetic determinants, either chromosomal DNA or plasmid, regarding the traits which give beneficial effects on fermented foods. The information derived from these studies will play a great role to develop genetically improved starter culture bacteria.

A strain of *Lb. plantarum* (designated as strain 27) that was isolated from meat starter culture and it was exerting both bacteriocin and mucin production phenotypes. The objectives of this study was: (a) to determine

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plasmid or chromosomal linkage of two industrially important traits (Bac$^+$ and Muc$^+$); (b) to characterize the properties of bacteriocin; and (c) to investigate the antimicrobial spectrum of bacteriocin produced by both wild and Muc$^-$ variant of *Lb. plantarum* 27.

MATERIALS AND METHODS

Bacterial Strains

Wild strain *Lb. plantarum* 27 was obtained from Dr. Lücke (Federal Meat Research Institute, Germany) and all test bacteria used in this study were isolated and maintained in our stock culture (Food Micro. Lab., University of Wyoming, Laramie). The test bacteria used were: *P. acidilactici* (strain M, A and H), *Pediococcus* sp. and *Lactobacillus* sp. both isolated from meat which were vacuum packaged and stored in refrigerator, *Lb. plantarum* NCDO 955, *Staphylococcus aureus* 485, *Escherichia coli* (enterotoxigenic), and *Salmonella typhimurium*. Both wild strain (Bac$^+$ Muc$^+$) and non-mucoid but bacteriocin producing (Bac$^+$ Muc$^-$) variants of *Lb. plantarum* 27, and other strains of lactic acid bacteria were maintained by weekly transfers in lactobacilli MRS broth (Difco Lab. Detroit, MI). Non-lactic acid bacterial strains were maintained in Tryptic Soy (TS) broth (Difco Lab).

Plasmid Curing

The wild strain *Lb. plantarum* 27 (Bac$^+$ Muc$^+$) was grown for 16 to 18 h at 30℃ in MRS broth containing one of the following chemicals: acriflavine (40 μg/m*l*), acridine orange (40 μg/m*l*), ethidium bromide (40 μg/m*l*), novobiocin (40 μg/m*l*). Also wild strain was grown at temperature (40℃) higher than their optimal temperature (30℃) in MRS broth for plasmid curing. Cells were maintained in the same environment by transferring three times in broth containing either curing chemicals or incubating at 40℃ before test. From each treatment, cells were serially diluted and surface plated (30 plates) on MRS agar to give 30 to 40 colonies per plate. After incubating at 30℃ for 48 h, percentage of non-mucoid (Muc$^-$) colonies were calculated from total colonies counted. Non-mucoid cells were purified and antibiotic sensitivity test, sugar fermentation test and plasmid profile were investigated. Also cells from selected dilutions, where population of wild strain and plasmid cured variants may coexist, were pour plated on MRS agar (approximately 100 cells per plate, 10 plates each treatment), incubated for 24 h at 30℃ and then 5 m*l* MRS soft agar containing 10 μ*l* of over night grown indicator *P. acidilactici* A was overlaid on it. Plates were then incubated for further 24 h at 30℃ and examined for the presence or absence of clear zone of inhibition around each colony. Similarly, purified Muc$^-$ variants, grown in MRS broth over night, were also pour plated in the same way and examined for the zone of inhibition around each colony.

Determination of Bacteriocin Activity against Test Bacteria

The wild strain (Muc$^+$) and non-mucoid (Muc$^-$) variants of *Lb. plantarum* 27 were grown in MRS broth for 16 to 18 h at 30℃ and the supernatant fluid was obtained by centrifugation of the culture broth at 12,000 rpm for 20 min. The pH of the supernatant fluid was adjusted to pH 5.5 with 3 N NaOH and finally filter sterilized (0.22 μm, Millipore). To determine bacteriocin activity by disc assay method (9), 30 μ*l* of the pH adjusted cell-free culture broth was transferred onto a sterile paper disc (5 mm in diameter) placed on soft agar (MRS broth containing 0.75% agar) lawn of test bacteria. To prepare a soft agar lawn of test bacteria, 10 μ*l* of a 16 to 18 h grown culture (approximately 5×10^6 cells in 10 μ*l*) in appropriate media (MRS for lactic acid bacteria, TS broth for non-lactic acid bacteria) was mixed well in 5 m*l* tempered (50℃) soft agar and this was evenly poured on empty petri dish. The plates were incubated at 37℃ for 16 to 18 h and observed for the presence or absence of zones of inhibition around the discs.

Preparation of Crude Bacteriocin

Cell-free culture broth, prepared by centrifugation and membrane filtration of over night grown cultures of wild strain and Muc$^-$ variant of *Lb. plantarum* 27, were precipitated by adding 70% ammonium sulfate (w/v) and the precipitates were collected by centrifugation (20,000 rpm). Residual ammonium sulfate in the precipitate was removed by dialysis (14,000 molecular weight cut-off) against deionized water. Ammonium sulfate free sample in the sac were then lyophilized and finally dissolved in 5 mM potassium phosphate buffer (pH 7.0) to give a 10X concentration of the original culture volume. These concentrates, designated as crude bacteriocin, were used in next study.

Effect of Enzymes and Heat, and pH on Crude Bacteriocin Activity

In order to investigate the chemical and physical nature of crude bacteriocin, several enzyme solutions, prepared in 10X concentration (in 5 mM potassium phosphate buffer, pH 7.0), were added to 2 m*l* crude bacteriocin to make the final enzyme concentration of 400 μg/m*l* and the mixture was digested for 30 min at 37℃. To remove the enzyme activity, samples were heated in a water bath (95℃ for 5 min). After cooling, 30 μ*l* of enzyme treated bacteriocin was transferred to a sterile disc placed on the MRS soft agar lawn of indicator. Different enzymes used in this study was: Catalase, DNase, Lysozyme, Trypsin, Papain, Ficin, Protease (type XIV)

and α-Chymotrypsin (all Sigma Chemical Co.).

To examine the pH effect on crude bacteriocin activity, 2 m*l* portion of the crude bacteriocin was adjusted at pHs from 4.0 to 10.0 by adding 3 N HCl or 3 N NaOH, kept at room temperature for 2 h, and finally readjusted at pH 7.0 with 5 mM phosphate buffer. The samples were then sterilized by membrane filtration (0.22 μm, Millipore) and 30 μ*l* of each was seeded on indicator lawn by disc assay method.

To investigate effect of heat on crude bacteriocin, a portion was autoclaved for 15 min at 121℃, rapidly cooled and examined by disc assay against the lawn of indicator. The bacteriocin sensitive indicator used in this studies was *P. acidilactici* A.

Plasmid Isolation, Purification and Agarose Gel Electrophoresis

Basically, plasmid isolation protocol developed by Anderson and McKay (1) for *Lactococcus* was used with the following modifications: cells, grown in lysis broth (MRS broth containing 1% glucose and 20 mM D,L-threonine, pH 6.5) until $OD_{600\,nm}$ of 0.6 were collected, washed, resuspended and incubated with self-digested pronase (100 μg/m*l*, final concentration) for 1 h at 37℃ to hydrolyze any surface layer protein exist (7). Residual pronase was removed by washing three times with sucrose buffer (6.7% sucrose, 50 mM Tris, 1 mM EDTA, pH 8.0) and lysozyme (4 mg/m*l*, final concentration) treatment lasted for 1 h at 37℃. Following 20% SDS treatment and adjusting the lysate at pH 12.5, sample was vortexed at full speed for 30 sec, heated at 65℃ for 30 min, cooled at room temperature and then neutralized at pH 8.75 by adding 2 M-Tris (pH 7.0). To remove mechanically sheared chromosomal DNA, 5 M NaCl solution was added to the sample and supernatant fluid was obtained by centrifugation (12,000 rpm, 20 min). The phenol and chloroform treatments were repeated twice and crude plasmid was collected by precipitating at −70℃ in 2X. vol. of ethanol. Plasmid was dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and agarose gel electrophoresis (0.7% agarose, 5 v/cm, TBE buffer, pH 8.3) was performed. To determine molecular weight of each plasmid in wild and Muc^- variant of *Lb. plantarum* 27, molecular weight standard plasmids of *Escherichia coli* (22) were isolated by the method developed by Birnboim and Doly (12). A modified CsCl-ethidium bromide centrifugation method (27) was used for the purification of covalently closed circular plasmid DNA.

RESULTS

The wild strain of *Lb. plantarum* 27 formed large mucoid colonies on MRS agar plates (Fig. 1A). Similarly,

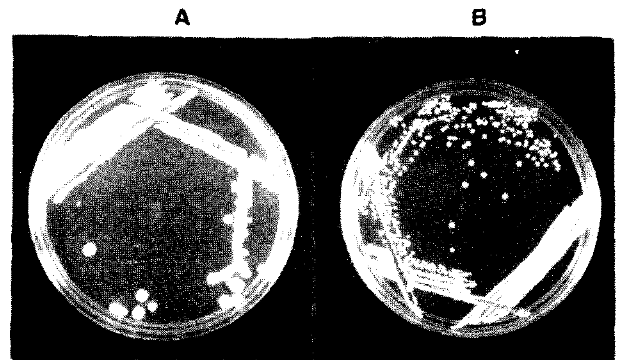

Fig. 1. Colony characteristics of the wild strain (A; Bac^+ Muc^+) and non-mucoid (B; Bac^+ Muc^-) variant of *Lb. plantarum* 27 grown on MRS agar.

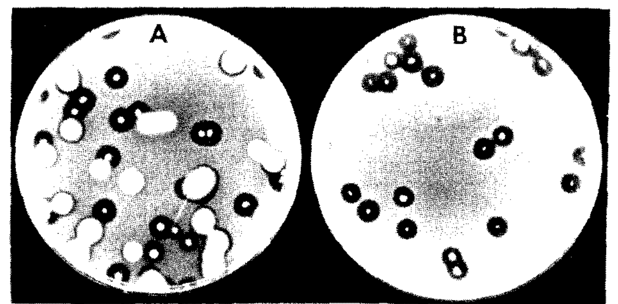

Fig. 2. Bacteriocin activity of colonies from the wild strain and non-mucoid variants of *Lb. plantarum* 27.

(A) mixture of wild strain (Bac^+ Muc^+) and non-mucoid but Bac^+ variants (Bac^+ Muc^-), (B) purified Bac^+ Muc^- variant of *Lb. plantarum* 27 against lawn of *P. acidilactici* A as an indicator.

when over night grown wild strain was transferred by pipetting, viscous long strand was observed. The ropy wild strain also produced a bacteriocin that inhibited growth of indicator strain *P. acidilactici* A as shown by the clear zone around each colony (Fig. 2A). To determine possible plasmid linkage of bacteriocin production (Bac^+) and mucoid (Muc^+) phenotypes, the wild strain was subjected to plasmid curing by several chemical and physical agents. When treated wild strain was examined for variants on these two phenotypes, some colonies were found to be non-mucoid (Fig. 2A). On successive subculturings, these variants remained non-mucoid, either in broth or in agar plate (Fig. 1B). These variants were also found to produce bacteriocin against *P. acidilactici* A (Fig. 2B). Therefore, the phenotypes of the wild strain and the non-mucoid variants are Bac^+ Muc^+ and Bac^+ Muc^-, respectively. The relative rates for the appearance of Muc^- and Bac^- (loss of bacteriocin produc-

Table 1. Effect of chemical and physical plasmid curing agents on producing Bac⁻ and Muc⁻ variants from Bac⁺ Muc⁺ wild strain

Curing agents	Cured variants (%)[a]	
	Bac⁻	Muc⁻
Acriflavine (40 μg/ml)	0	0
Acridine orange (40 μg/ml)	0	0.012
Ethidium bromide (40 μg/ml)	0	2.32
Novobiocin (40 μg/ml)	0	32.2
High temperature (40℃)	0	20.5

[a] (Number of variants/Number of total colonies) ×100. Approximately 1,000 colonies were examined for bacteriocin (Bac⁺) and mucin production (Muc⁺) phenotypes on each treatment.

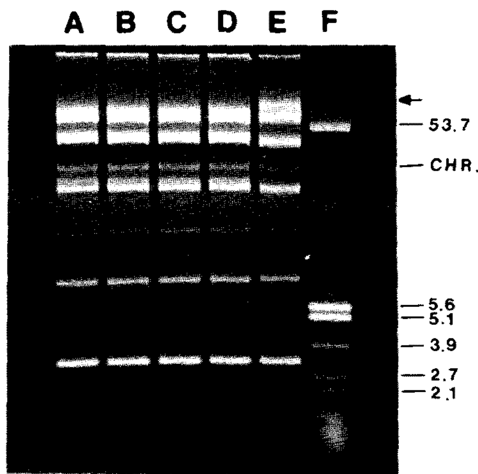

Fig. 3. Plasmid profiles of wild strain and non-mucoid variants of *Lb. plantarum 27*.
(A) to (D) Bac⁺ Muc⁻ variants (missing pMUC62); (E) wild strain (harboring pMUC62); (F) *E. coli* V517 (molecular weight standard). Molecular weight of plasmids are described in kilobase. Arrow on right indicates 62.5 kb plasmid, pMUC62, and CHR indicates chromosomal DNA.

tion) variants, following treatments with the curing agents, were listed in Table 1. Depending upon the curing agents, occurrence of Muc⁻ variants was at a rate of 0% to 32%. Novobiocin treatment showed the highest rate while acriflavine did not produce any Muc⁻ variants. However, none of the five curing agents produced any Bac⁻ variant. Many subsequent curing attempts, both with wild strain and Muc⁻ variants, did not produce any Bac⁻ variants.

A comparison between the plasmid profiles of the wild and Bac⁺ Muc⁻ variants on agarose gel showed that the wild strain harbored about 8 to 9 plasmids ranging approximately from 3.1 kb to 62.5 kb in size (Fig. 3, lane E). The Bac⁺ Muc⁻ variants harbored all the plasmids of the wild strain except the 62.5 kb plasmid

Table 2. Activity of crude bacteriocin produced by wild strain and Muc⁻ variant of *Lb. plantarum* 27 after various treatments

Treatments	Inhibition[a] by bacteriocin from	
	Wild strain	Muc⁻ variants
Autoclaving (121℃, 15 min)	+	+
Catalase	+	+
DNase	+	+
Lysozyme	+	+
Trypsin	−	−
Papain	−	−
Ficin	−	−
Protease (XIV)	−	−
α-Chymotrypsin	−	−
pH 4.0 to 9.0	+	+
pH 10.0	−	−

[a] Presence (+) or absence (−) of zone of inhibition when 30 μl of crude bacteriocin was seeded on paper disc on the soft agar lawn of *P. acidilactici* A as bacteriocin sensitive indicator.

Table 3. Antimicrobial spectrum of bacteriocin produced by wild strain and Muc⁻ variant of *Lb. plantarum* 27

Test bacteria	Inhibition[a] by bacteriocin from	
	Wild	Muc⁻ variants
Pediococcus acidilactici M	+	+
Pediococcus acidilactici H	+	+
Pediococcus sp. from meat	+	+
Lactobacillus plantarum NCDO 955	+	+
Lactobacillus sp. from meat	+	+
Staphylococcus aureus 485	+	+
Escherichia coli (enterotoxigenic)	−	−
Salmonella typhimurium	−	−

[a] Presence (+) and absence (−) of zone of inhibition when 30 μl of bacteriocin preparation (cell-free supernatant fluid) was seeded on paper disc on the soft agar lawn of test bacteria.

(Fig. 3, lanes A to D, see arrow).

The crude bacteriocin of *Lb. plantarum* 27, prepared from both Bac⁺ Muc⁺ wild strain and Bac⁺ Muc⁻ variants by ammonium sulfate precipitation and dialysis of culture supernatant fluids, were examined for several physical and chemical properties (Table 2). After either autoclaving or treatments with catalase, DNase and lysozyme, the crude bacteriocin retained the antimicrobial activity. However, treatments with five different proteolytic enzymes resulted in its loss of activity. Exposure of the crude bacteriocin at pHs from 4 to 9 did not inacti-

vate the antimicrobial activity whild the activity was lost at pH 10.

The bacteriocin of *Lb. plantarum* 27 showed antimicrobial spectrum against several strains of pediococci and lactobacilli including some strains isolated from spoiled refrigerated vacuum packaged meat as well as against a strain of *Staphylococcus aureus* (Table 3). However, Gram-negative bacteria tested, *E. coli* and *Salmonella typhimurium*, were not inhibited by bacteriocin of *Lb. plantarum* 27.

DISCUSSION

The mucin production phenotype in *Lb. plantarum* 27 is probably linked to a 62.5 kb plasmid (pMUC62). Treatments of the wild strain with plasmid curing agents consistently produced Muc^- variant at relatively high frequency, specially by growing the cells in the presence of novobiocin or at 40℃. In addition, plasmid profiles of non-mucoid variants showed that pMUC62 was missing while wild strain showed its presence. However, we were not always successful in detecting this plasmid on agarose gel from the wild strain. This could partly be due to difficulties in obtaining good lysis of the mucoid wild strain, possible low copy number of plasmid pMUC 62 and relatively large size of the plasmid (1). Association of plasmid encoding mucin production phenotype in several species of *Lactococcus* have been well documented (23, 24, 26, 32, 33). However, plasmid-linked mucin production phenotype in *Lb. plantarum* has not been reported yet.

From the attempts to eliminate bacteriocin production phenotype, by growing wild strain in conditions where plasmid is unstabilized, we were unsuccessful to obtain any variants that lost its ability to produce bacteriocin. The variants obtained were only non-mucoid but it were still producing bacteriocin. These data indicate that bacteriocin production phenotype might be linked to chromosomal DNA in *Lb. plantarum* 27. Almost all the report regarding bacteriocin phenotype in several species of *Lactobacillus* have mentioned the same results (5, 17, 30, 31). Only one report have mentioned defective or incomplete mobilization of plasmids that may be encoding bacteriocin phenotype in *Lb. acidophilus* 88 (25). However, that report couldn't clearly demonstrate the plasmid linkage of bacteriocin production in *Lb. acidophilus* 88.

The narrow antagonistic effect against closely related lactic acid bacteria and loss of antimicrobial activity upon proteolytic enzyme treatments indicate that bacteriocin produced by *Lb. plantarum* 27 should be a true bacteriocin (29). Insensitivity of bacteriocin to catalase, DNase and heat, showing activity at pH 7.0 strongly indicate H_2O_2, bacteriophages and acid were not involved in antimicrobial activity exerted by *Lb. plantarum* 27. Similar results have been reported on the properties of bacteriocin produced by many species of *Lactobacillus* (6, 19, 30).

The bacteriocin inhibited growth of several lactic acid bacteria including some psychrotrophs that were isolated from spoiled meat which was kept in vacuum package. Thus, the bacteriocin produced by *Lb. plantarum* 27 could be used to extend shelf-life of foods that are known to be spoiled by these organisms (11). In addition, this bacteriocin also inhibited the growth of a strain of *Staphylococcus aureus* use of *Lb. plantarum* 27 in sausage fermentation will have, along with its acid production, an added advantage of controlling *S. aureus* related food intoxication.

In addition to bacteriocin production, the wild strain produced mucoid substance which may contribute to unique texture of dry and semi-dry sausages. In Europe, strains of *Lactococcus* producing mucoid materials have traditionally been used to produce some distinct fermented milk (23). Wild strain of *Lb. plantarum* 27 with Bac^+ Muc^+ phenotypes thus could be used as a desirable culture bacteria in the production of fermented sausages. However, there should be more studies concerning antigenic properties of bacteriocin (10) and mucin (21), mode of action (11) and chemical nature of bacteriocin (9) produced by *Lb. plantarum* 27.

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