Journal of Microbiology and Biotechnology
Vol. 1, No. 3, 169-175, 1991

Plasmid Linkage of Bacteriocin Production and Sucrose Fermentation Phenotypes in *Pediococcus acidilactici* M

KIM, WANG-JUNE*, DUK-MO HA, AND BIBEK RAY[1]

Department of Food Technology, College of Engineering, Dongguk University, Seoul 100-715, Korea
[1]Food Microbiology Laboratory, Department of Animal Science, College of Agriculture
University of Wyoming, P.O. Box 3684, Laramie, WY 82071-3684, USA

Received 28 October 1991 / Accepted 30 October 1991

***Pediococcus acidilactici* strain M produced a bacteriocin which was proteinaceous, heat stable, and exhibited antimicrobial activity against lactic acid bacteria, variety of food spoilage and pathogenic bacteria. The antimicrobial activity was not caused by H_2O_2 and organic acid, and was remained between pHs of 4.0 to 9. Molecular weight of crude bacteriocin was approximately 2,500. Phenotypic assignment after plasmid cruing experiment demonstrated that a 53.7 kilobase (kb) plasmid, designated as pSUC53, was responsible for the sucrose fermentation phenotype (Suc^+) and a 11. 1 kb plasmid, designated as pBAC11, was associated with bacteriocin production phenotype (Bac^+). Neither of the two plasmids were linked to antibiotic resistance.**

The pediococci are Gram-positive homofermentative lactic acid bacteria that are widely used in the fermentation of vegetable (8), sausages (20) and Cheddar cheese (9, 23). They play an important role in food fermentation not only because it produce mainly lactic acid, thereby contribute to the preservation of foods (6), but also some strains have been found to produce bacteriocin or bacteriocin-like compounds that inhibit the proliferation of health hazardous bacteria, and other lactic acid bacteria that are competing in food fermentation (2, 3, 7, 17).

However, very few genetic studies have been conducted to understand the genetic control and involvement of plasmid in these industrially important bacteria. Understanding plasmid biology of pediococci will help to develop genetically improved strains (19). Only recently, there have been few reports concerning plasmid encoded phenotypes in some strains of *Pediococcus* (5, 12-15, 18, 24).

In this study, we report the plasmid linkage of bacteriocin production (Bac^+) and sucrose fermentation (Suc^+) phenotypes in *P. acidilactici* M along with some characteristics of the bacteriocin and its antimicrobial spectrum.

*Corresponding author

Key words: *Pediococcus acidilactici* M, plasmid curing, bacteriocin production phenotype (Bac^+), sucrose fermentation phenotype (Suc^+), strain development, physiological markers, food biopreservative

MATERIALS AND METHODS

Bacterial Strains and Media

Bacteriocin producing wild type *P. acidilactici* M and two strains of bacteriocin sensitive *P. acidilactici* (J and L) were isolated from fermented sausage and maintained in our stock culture collection. The sources of bacteria used in this studies are listed in Table 1. All lactic acid bacteria were grown and maintained in modified MRS broth (mMRS, 1% glucose, 1% proteose peptone #3, 1% casein hydrolysate, 0.5% yeast extract, 0.2% ammonium citrate, 0.2% disodium phosphate, 0.5% sodium acetate, 0.1% Tween 80, 0.01% $MgSO_4$, 0.005% $MnSO_4$, pH 6.5). The media used for the growth of non-lactic acid bacteria were: APT broth for *L. monocytogenes*; Sodium Thioglycollate agar for *C. perfringens*; TS broth for *S. aureus* and *E. coli*; Nutrient broth for *B. cereus*; and Brilliant Green broth for *S. anatum* and *S. typhimurium*. All media used in this study were Difco Laboratory made.

Antimicrobial Spectrum of Bacteriocin

Supernatant fluid of wild type *P. acidilactici* M was prepared by centrifugation (12,000 rpm, 10 min) of over night grown culture in mMRS broth (37℃). To exclude

Table 1. Antimicrobial spectrum of bacteriocin produced by wild type *P. acidilactici* M

Test bacteria	Sensitivity[a]	Source[b]
Lb. plantarum WSO 39	+	Dr. Daeschel (Oregon St. U.)
Lb. plantarum NCDO 955	+	NCDO
Lb. plantarum ATCC 8014	+	KCTC
Lb. viridescens ATCC 12706	+	ATCC
L. mesenteroides	+	Dr. Sandine (Oregon St. U.)
L. monocytogenes	+	Dr. Fowlers (Silliker Lab.)
C. perfringens	+	Dr. Andrew (FDA)
S. aureus	+	Dr. Andrew (FDA)
P. acidilactici J	+	Our culture collection
P. acidilactici L	+	Our culture collection
B. cereus	+	Dr. Maki (U. Wyoming)
E. coli	−	Dr. Maki (U. Wyoming)
S. anatum	−	Dr. Maki (U. Wyoming)
S. typhimurium	−	Dr. Maki (U. Wyoming)

[a]Presence (+) or absence (−) of zone of inhibition when 30 μl of pH adjusted cell-free culture broth was disc assayed on lawn of test bacteria.
[b]ATCC: American Type Culture Collection, NCDO: National Collection of Dairy Organisms, KCTC: Korean Collection for Type Cultures, FDA: US Food and Drug Administration.

antagonistic effect caused by low pH, the supernatant fluid was adjusted to pH 5.5 by adding 3 N NaOH and membrane sterilization (0.22 μm, Millipore) was followed. Approximately 5×10^6 cells of over night grown test bacteria (Table 1) were mixed well with 5 ml of suitable soft agar (0.75% agar) and then poured on presolidified bottom agar (1.5% agar). Sterile paper discs (5 mm in diameter) were placed on each test bacteria lawn and 30 μl of supernatant fluid was loaded on discs. The plates were incubated over night at 30℃ and examined for the absence or presence of clear zone of inhibition (>6 mm) around disc.

Effect of Enzymes, Heat and pH Treatments on Bacteriocinogenic Activity

Protein in supernatant fluid of wild type *P. acidilactici* M was precipitated by adding 70% ammonium sulfate (2). The precipitate was collected by centrifugation (20, 000 rpm, 20 min) and residual ammonium sulfate was removed by dialysis (14,000 molecular weight cut-off) in 5 mM phosphate buffer (pH 7.0). The dialysate was lyophilized and the crude bacteriocin was reconstituted in 5 mM phosphate buffer (pH 7.0) to mak a final concentration of 10 mg/ml and was membrane sterilized.

In order to examine the chemical nature of crude bacteriocin, various proteolytic enzymes, DNase, lysozyme and catalase were dissolved in sterile 5 mM phosphate buffer (pH 7.0) to a final concentration of 200 μg/ml and finally membrane sterilized. An equal volume of crude bacteriocin and enzyme solutions were mixed, incubated for 1 h at 37℃, and boiled at 95℃ for 15 min to inactivate the enzymes. Remaining activity of crude bacteriocin was disc assayed against lawn of indicator *Lb. plantarum* WSO 39.

To determine the thermal stability of crude bacteriocin, sample was autoclaved at 121℃ for 15 min and then disc assayed against indicator.

Isolation of Spontaneous Bac^-Suc^+ and Bac^+Suc^- Segregants

For the isolation of spontaneous Bac^-Suc^+ and Bac^+Suc^- segregants, wild type *P. acidilactici* M, Bac^+Suc^+ in phenotype, which has been subcultured more than twenty times in mMRS broth at 37℃ without the presence of curing agent, was used for the following test.

In order to isolate spontaneous Bac^-Suc^+ segregants from the mixtures of wild type and Bac^+Suc^- segregants, diluted cell suspension of subcultured wild type was mixed and poured with 5 ml of melted mMRS as bottom agar (1.5% agar). Additional 5 ml mMRS soft agar (0.75 % agar), which did not contain diluted cell, was overlaid and the plates were kept at 37℃ for 16 to 18 h. Fresh indicator soft agar lawn was overlaid and incubated at 37℃ over night. Agar was inverted and colonies which did not show zone of inhibition were carefully excavated from the bottom agar and were purified by streaking on BCP-sucrose agar (1% sucrose, 1% proteose peptone #3, 0.5% yeast extract, 0.1% Tween 80, 0.005% $MnSO_4$, 0.008% bromcresol purple, pH 6.8). Yellow colonies, therefore Bac^-Suc^+ in phenotype, were purified and were designated as segregant M1.

For the isolation of Bac^+Suc^- segregants, subcultured wild type was diluted and surface plated on BCP-sucrose agar and incubated at 37℃ for 48 h. White colonies were picked up by tooth pick and transferred on mMRS plate by stabbing. Transferred colonies were sealed by dropping soft mMRS agar, indicator was overlaid and looked for the colonies forming zone of inhibition. Isolates, Bac^+Suc^- in phenotype, were designated as segregant M2.

Identity of segregants M1 and M2 were examined for their morphology, catalase reaction, bacteriocin production and immunity, plasmid profile, sugar fermentation and antibiotic resistance patterns.

Isolation of Bac^-Suc^- Segregants by Plasmid Curing

To eliminate Suc^+ in segregant M1, Bac^-Suc^+ in phenotype, cell has subcultured twice in the broth containing 25 μg/ml acriflavine, the highest sublethal concentration that permitted growth, and was surface plated on BCP-

sucrose agar. White colonies were purified and this strain was designated as segregant M3. The same test performed on segregants M1 and M2 were carried out to identify segregant M3.

Bacteriocin Sensitivity and Immunity Test

Crude bacteriocin solution (10 mg/ml), which did not inhibit the growth of wild type (data not shown), was disc assayed against the soft agar lawns of wild type and three segregants (M1, M2, and M3). Sensitivity (Bac^s) and immunity (Bac^r) against their own bacteriocin were judged by the presence or absence of clear zone of inhibition, respectively.

Sugar Fermentation and Antibiotic Resistance Tests

Patterns of sugar fermentation, other than sucrose, and antibiotic resistance were compared in wild type and three segregants.

Plasmid Isolation

The plasmid isolation method developed by Anderson and McKay (1) was used with some modifications. Fresh culture was inoculated (2% inoculum) into a lysis broth, mMRS broth supplemented with 20 mM D,L-threonine to improve cell lysis (4), and the broth was incubated at 37℃ until the cells reached mid-exponential phase of growth ($OD_{600\,nm}$ of 0.6). Cells were harvested, washed with TES buffer (0.1 M NaCl, 10 mM Tris, 1 mM EDTA, pH 7.5) and suspended in a sucrose buffer (6.7% sucrose, 50 mM Tris, 1 mM EDTA, pH 8.0). The lysis of cell wall with fresh lysozyme (10 mg/l in 25 mM Tris, pH 8.0) was carried in an ice bath for 30 min and then in a 37℃ water bath for 30 min. In some cases, cells in lysozyme solution were frozen at −20℃ over night and thawed at room temperature. After adding ET buffer 0.25 M EDTA, 50 mM Tris, pH 8.0) and SDS buffer (20% SDS in 50 mM Tris, 20 EDTA, pH 8.0), sample was shaken (rotary shaker, 200 rpm) until it became thick and viscous. The lysate was adjusted to pH 12.25 by adding fresh 3 N NaOH and was put on a rotary shaker (200 rpm) for 10 min. The pH of the alkaline lysate was reduced to 8.75 by adding 2 M Tris (pH 7.0) and gentle shaking was continued for further 5 min. After adding 5 M NaCl, fresh phenol solution, washed three times with STE buffer (3% NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0), was added and sample was gently shaken for 3 min. Aqueous supernatant fluid was collected by centrifugation (15,000 rpm for 10 min) and CHCl$_3$-isoamyl alcohol (24:1, v/v) extraction was followed. Plasmid was precipitated at −70℃ over night with an equal volume of isopropanol and collected by centrifugation (20,000 rpm for 30 min at 0℃). The pellet was dried and dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The plasmid profiles of wild type and its segregants were examined by agarose gel electrophoresis (0.7% agarose at 5 V/cm).

Determination of Molecular Weight of Crude Bacteriocin and Assay for Antimicrobial Activity in SDS-PAGE

Same amount (40 μl) of crude bactericin solution was applied in two separate lanes of gradient (10~25%) SDS-PAGE slab gel with a molecular weight standard (MW-SDS-17, Sigma). After electrophoresis, one half, containing the sample and molecular weight standard, was stained with Coomassie Blue, and unstained the other half was washed in deionized water for 24 h. The unstained gel was placed on a sterile petri dish and 10 ml of soft agar containing approximately 5×10^6 cells of indicator bacteria was overlaid on it. The plate was incubated at 30℃ over night and observed for a zone of inhibition. The molecular weight of the band which showed zone of inhibition was determined by comparing the position with the other part of gel which has stained by Coomassie Blue.

RESULTS

Antimicrobial Spectrum and Properties of Bacteriocin

The antimicrobial spectrum of bacteriocin produced by wild type *P. acidilactici* M against several test bacteria is presented in Table 1. The bacteriocin was inhibitory to Gram-positive bacteria including food spoilage bacteria and pathogens, leuconostoc, lactobacilli and other strains of *P. acidilactici*. However, tested Gram-negative bacteria were not antagonized by bacteriocin.

Table 2. Effect of enzymes, heat and pH treatments on activity of crude bacteriocin produced by wild type *P. acidilactici* M

Treatments	Retention of activity[a]
Autoclaving (121℃, 15 min)	+
Catalase	+
DNase	+
Lysozyme	+
pH 4 to 9	+
pH 10	−
Trypsin	−
Papain	−
Ficin	−
Protease (XIV)	−
α-Chymotrypsin	−

[a]Presence (+) or absence (−) of zone of inhibition when 30 μl of treated crude bacteriocin was disc assayed against lawn of *Lb. plantarum* WSO 39 as indicator.

The activity of crude bacteriocin was retained even after autoclaving (121℃ for 15 min) and adjustment of pHs from 4 to 9, and treatment with catalase, DNase and lysozyme, However, treatment with trypsin, papain, ficin, protease, α-chymotrypsin, and exposure at pH 10 destroyed bacteriocinogenic activity.

Occurrence of Segregants by Spontaneous and Plasmid Curing

The frequency for the appearance of three segregants are listed in Table 3. From the population of wild type, which fermented sucrose and produced bacteriocin, 3.01 % lost the Suc^+ but not the Bac^+ (segregant M2) and 0.12% lost the Bac^+ but not the Suc^+ (segregant M1) by spontaneous segregation of plasmid (Table 3). After treatment of segregant M1 with acriflavine (25 μg/ml), 3.17% lost the Suc^+ (segregant M3).

Phenotypic Assignment of Plasmids

The relevant phenotype and plasmid profiles of the wild type and its segregants are listed in Table 3 and Figs. 1-3. The wild type, which exerted Bac^+Bac^r and Suc^+, harbored two plasmids of 53.7 and 11. 1 kb in size (Fig. 1, lane D). The segregant M1, which was missing the 11.1 kb plasmid but not the 53.7 kb plasmid (Fig. 1, lane B), exhibited Bac^-Bac^r and Suc^+. In contrast, the segregant M2, which has lost the 53.7 kb plasmid but not the 11.1 kb plasmid (Fig. 1, lane C), demonstrated Bac^+Bac^r but Suc^-. The plasmid-free segregant M3 (Fig. 1, lane A) exerted Bac Bac^s and Suc^-. From these data we could confirm that the 53.7 kb plasmid (pSUC 53) was associated with the Suc^+ and the 11.1 kb plasmid (pBAC11) was responsible for the Bac^+.

However, the attempts to link single plasmid to Bac^r and Bac^s were unsuccessful because the segregant M1, that was missing pBAC11, was Bac^- but Bac^r was still remained. In contrst, segregant M3 which was obtained by elimination of pSUC53 in segregant M2, showed

Table 3. Plasmid content and relevant phenotype of wild type and its segregants of *P. acidilactici* M

Strains	Phenotype[a]	Frequency[b]	Plasmid (kb)	Comments
M	Bac^+Suc^+ Bac_r		53.7, 11.1	wild type
M1	Bac^-Suc^+ Bac^r	0.12%	53.7	spontaneous segregant
M2	Bac^+Suc^- Bac^r	3.01%	11.1	spontaneous segregant
M3	Bac Suc Bac^s	3.17%	none	plasmid cured segregant (obtained from segregant M1)

[a] Bac^+: bacteriocin producing, Bac^-: bacteriocin non-producing, Bac^r: immune to bacteriocin, Bac^s: bacteriocin sensitive, Suc^+: sucrose fermenting, and Suc^-: sucrose non-fermenting.

[b] (Number of segregants on plate/Total cells on plate)×100.

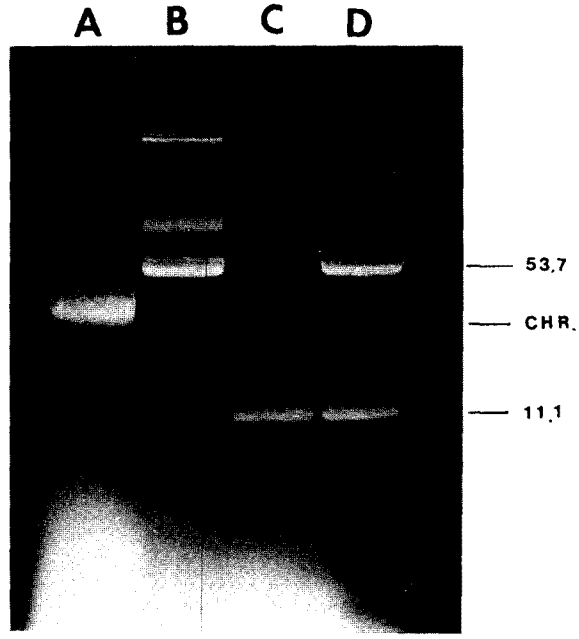

Fig. 1. Agarose gel electrophoresis showing plasmid profiles of wild type and segregants of *P. acidilactici* M.

(A) segregant M3 ($Bac^-Bac^sSuc^-$), (B) segregant M1 (Bac Bac^rSuc^+), (C) segregant M2 ($Bac^+Bac^rSuc^-$) and (D) wild type ($Bac^+Bac^rSuc^+$). Molecular weight of each plasmid is described in kilobase and "CHR." indicates chromosomal DNA.

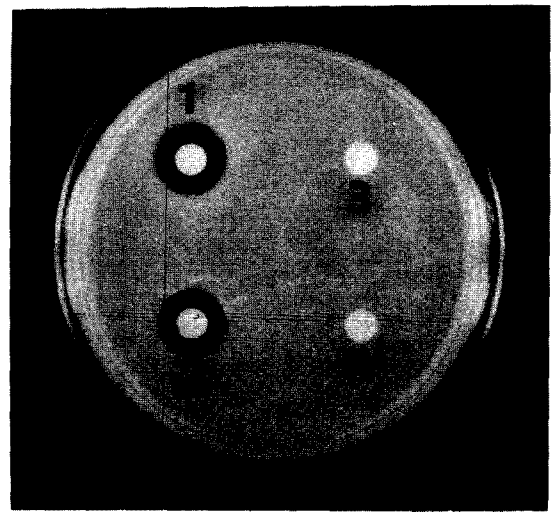

Fig. 2. Bacteriocinogenic activity of cell-free culture broth prepared from wild type and segregants of *P. acidilactici* M against lawn of indicator *Lb. plantarum* WSO 39.

(1) wild type, (Bac^+Suc^+); (2) segregant M2, (Bac^+Suc^-); (3) segregant M1, (Bac^-Suc^+); (4) segregant M3, (Bac^-Suc^-).

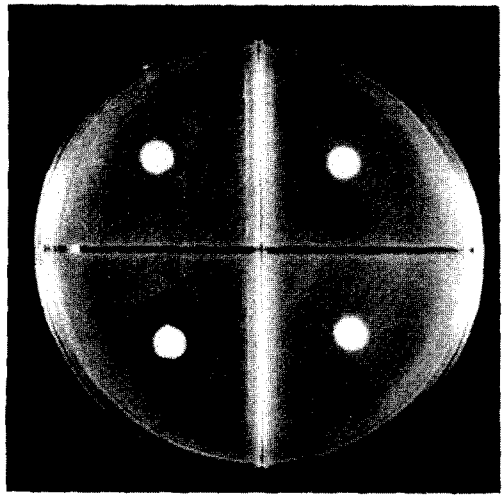

Fig. 3. Bacteriocin immunity and sensitivity of wild type and segregants of *P. acidilactici* M.
(1) wild type (Bac$^+$Bac^{r}Suc$^+$), (2) segregant M1 (Bac$^-$Bac^r-Suc$^+$), (3) segregant M2 (Bac$^+$Bac^{r}Suc$^-$), and (4) segregant M3 (Bac$^-$Bac^{s}Suc$^-$).

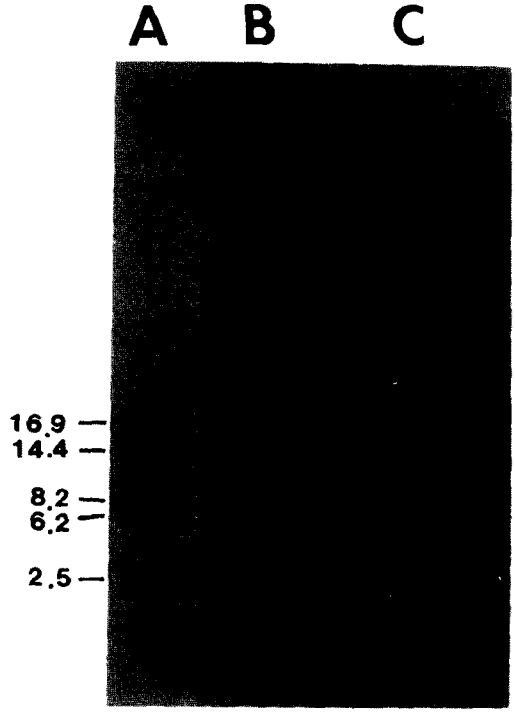

Fig 4. Molecular weight of crude bacteriocin and its activity in SDS-PAGE gel.
(A) low-molecular weight standards (MW-SDS-17, Sigma); (B) crude bacteriocin; (C) unstained portion of gel showing bacteriocinogenic activity against lawn of indicator *Lb. plantarum* WSO 39. Molecular weight of each band is described in kilodalton.

Bac$^-$ and Bac^s (Figs. 1-3).

Sugar Fermentation and Antibiotic Resistance Test

There was no differences in sugar fermentation, except sucrose, and antibiotic resistance patterns in wild type and segregants. The wild type (Bac$^+$Suc$^+$) and segregant M1 (Bac$^-$Suc$^+$) fermented sucrose, and segregant M2 (Bac$^+$Suc$^-$) and M3 (Bac$^-$Suc$^-$) did not ferment sucrose.

Determination of Molecular Weight of Crude Bacteriocin

In the SDS-PAGE gel stained by Coomassie Blue, there were four bands appeared with molecular weight ranging from approximately 50,000 to 2,500 (Fig. 4). From unstained SDS-PAGE gel, which was overlaid with indicator bacteria to detect the band associated with bacteriocinogenic activity, only one band showed zone of inhibition. Measuring electrophoretic distance, this band was at the same position with the lowermost band in the gel stained by Coomassie Blue that had a molecular weight of approximately 2,500.

DISCUSSION

The concurrent segregation of the 53.7 kb plasmid and Suc$^+$ either by spontaneous and/or by plasmid curing methods clearly indicated that Suc$^+$ is located in the 53.7 kb plasmid (pSUC53). Similarly, simultaneous loss of the 11.1 kb plasmid and the Bac$^+$ strongly demonstrated that the Bac$^+$ is encoded in the 11.1 kb plasmid (pBAC11). In addition, exhibiting Suc$^-$Bac$^-$ of the plasmid-free segregant M3, obtained upon eliminating pSUC 53 by plasmid curing, confirmed the conclusion. This was similar to the report of Gonzalez and Kunka (13) who reported a 6.2 megadalton (Mdal) plasmid was associated with Bac$^+$ and a 23 Mdal plasmid was associated with Suc$^+$ in *P. acidilactici* PAC1.0. Also, they depicted sucrose hydrolase activity was associated with a 30 Mdal plasmid in *P. pentosaceus* PPE1.0 (12). The plasmid linkage of Bac$^+$ in several other strains of *P. acidilactici* (13-15) and *P. pentosaceus* (5, 14, 15) have also been reported. The data from all of these reports are indicating that the number of plasmids (2 to 3 plasmids), size and their relevant phenotypes (i.e. plasmids encoding Bac$^+$ and Suc$^+$ ranged 8.8 kb to 21 kb and 35.6 kb to 53.7 kb, respectively) are similar in tested strains of *P. acidilactici* (13, 15, 18) and *P. pentosaceus* (5, 12, 15) even though their origins are different.

It was unsuccessful to locate specific genetic determinant encoding bacteriocin immunity (Bac^r) in *P. acidilctici* M. If the allele encoding Bac$^+$ and Bac^r were on the same plasmid (pBAC11), both segregants M1 and M3

should be Bac^- and Bac^s. However, only segregant M3, obtained by eliminating Suc^+ in segregant M1, was Bac^- and Bac^s, whereas segregant M1 was Bac^- but Bac^r (Fig. 3). The comparable result has published by Gonzalez and Kunka (13). At this point, however, we could speculate the connection of Suc^+ to Bac^+ and/or Bac^r from number of genetic studies concerned on nisin production (Nis^+) and immunity (Nis^+) in lactococci (10, 11, 16, 21, 25). Those reports indicated that Suc^+, Nis^+ and Nis^r were simultaneously expressed, and were lost upon plasmid curing. To the contrary, Ray *et al.* (18, 19) and Daeschel (5) have reported that Bac^+ and Bac^r are encoded by the same plasmid in *P. acidilactici* and *P. pentosaceus*, respectively. Therefore, it seems both Bac^+ and Bac^r are strain specific in strains of *P. acidilactici* and *P. pentosaceus*.

The antimicrobial activity of crude bacteriocin was lost after proteolytic enzyme treatment. However, catalase, DNase and lysozyme treatments and exposing crude bacteriocin to pHs 4~9 did not cause loss of activity. This suggested that bacteriocin of *P. acidilactici* M was proteinaceous in nature and the antimicrobial activity was not due to either lactic acid, hydrogen peroxide or bacteriophage.

The molecular weight of crude bacteriocin was approximately 2,500. Gonzalez and Kunka (13) and Bhunia *et al.* (2) have reported that the molecular weight of the bacteriocins from *P. acidilactici* PAC1.0 and *P. acidilacticii* H were approximately 16,000 and 2,700, respectively.

The crude bacteriocin showed relatively broad antimicrobial spectrum including some food spoilage and pathogenic bacteria (i.e. *L. monocytogenes, S. aureus, Lb. viridescens, B. cereus* and *C. perfringens*). This will enable bacteriocin of *P. acidilactici* M an excellent candidate as food biopreservative in extending shelf-life of foods. In addition, crude bacteriocin was inhibitory to group of lactic acid bacteria including two other strains of *P. acidilactici* (J and L), three strains of *Lb. plantarum*, and *L. mesenteroides*. Therefore, bacteriocin produced by *P. acidilactici* M could be classified as a true bacteriocin due to its characteristics (22).

There is a tremendous demands in the construction of recombinant vector plasmid and developing gene transfer systems (19) in strains of *P. acidilactici* for strain development. In the development of cloning vectors applicable in food fermentation process, conventional antibiotic selection markers may be somewhat unacceptable due to possible introduction of the drug resistance plasmid to human. Additionally, many strains of *P. acidilactici* are directly used in the manufacture of fermented foods in most of the countries not only because they give beneficial effects on products but also they are generally regarded as safe (GRAS). Therefore, construction of cloning vector with physiological markers as an alternative, rather than antibiotic resistance ones, and transferring it by efficient gene transfer systems could be critical to fulfil that demands.

These studies have shown there are two resident plasmids in *P. acidilactici* M encoding two important phenotypes (Suc^+ and Bac^+) and neither of them are linked to antibiotic resistance. These two novel physiological markers could be a valuable candidate for the construction of recombinant plasmid, and thus, for future strain development and genetic engineering studies of *P. acidilactici* as well (W.J. Kim and B. Ray. 1991. Plasmid transfers by conjugation and electroporation in *Pediococcus acidilactici*. *J. Appl. Bacteriol.*).

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