Association of a 13.6-Megadalton Plasmid in *Pediococcus pentosaceus* with Bacteriocin Activity†

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***Pediococcus pentosaceus* FBB61 and L7230, isolated from cucumber fermentations, produced a bacteriocin, designated pediocin A, which had identical activity spectra against species of *Pediococcus*, *Clostridium*, *Staphylococcus*, and *Streptococcus*. Both strains possessed a 13.6-megadalton plasmid (pMD136). Plasmid curing experiments suggested that both bacteriocin immunity and production determinants were encoded by pMD136. Use of pediocin-producing strains in food fermentations is discussed.**

In pure culture fermentations of cucumbers inoculated with *Lactobacillus plantarum* and *Pediococcus pentosaceus* FBB61, Etchells et al. (5) observed that growth of *L. plantarum* is inhibited. They stated, "The rapid acid production by the *Pediococcus* species is the only possible reason evident from our data for this initial inhibition of *L. plantarum* growth. However, *L. plantarum* is so acid-tolerant that it does not appear likely that this is the sole reason for the inhibition, and there may be other factors more important." This observation was further investigated by Fleming et al. (6), who screened pediococcal cultures from various sources for inhibitory activity against a variety of microorganisms, including lactic acid bacteria associated with cucumber fermentation. Only two strains of *P. pentosaceus* showed inhibitory activity: strain FBB61, which was originally isolated from a Michigan cucumber fermentation in 1953 (1); and L7230, which had been isolated from a North Carolina cucumber fermentation in 1972 (6). Both strains had the same inhibitory spectrum against gram-positive bacteria, and neither was active against gram-negative bacteria, yeasts, or each other. Fleming et al. (6) ruled out the possibility that the inhibitory agent was hydrogen peroxide by using catalase in their assays.

Rueckert (M.S. thesis, Michigan State University, East Lansing, 1979) characterized the chemical nature of the inhibitory agent produced by *P. pentosaceus* FBB61 as bacteriocidal, nondialyzable across a semipermeable membrane, stable to heat (100°C for 60 min) and freezing, and sensitive to pronase. These characteristics and others led Rueckert to conclude that the inhibitor is bacteriocinlike.

We present evidence that bacteriocin activity and immunity in *P. pentosaceus* FBB61 and L7230 are associated with a 13.6-megadalton (MDa) plasmid.

Strains and culture conditions. Lactic acid bacteria were maintained and grown in MRS broth (Difco Laboratories, Detroit, Mich.) at 30°C. Clostridia were propagated in cooked meat medium (Difco) at 37°C under anaerobic conditions. *Staphylococcus aureus* and *Escherichia coli* V517 were propagated in brain heart infusion broth (Difco) at 37°C. *E. coli* V517 was used as a source of reference plasmids as described by Macrina et al. (10). Other strains were also used (Table 1).

Bacteriocin sensitivity. Cultures were screened for bacteriocin sensitivity by using the agar spot test as described by Fleming et al. (6). A producer strain which was cured of bacteriocin activity and immunity but which in other respects, such as sugar fermentation patterns and specific growth rate, was identical to the producer parent was used as a control. A clear zone of 1 mm or more surrounding the spotted producer colony was considered positive inhibition as long as the control strain failed to produce a zone.

Plasmid isolation and purification. Plasmid isolation from pediococcal strains was achieved by using the general method of Klaenhammer (9). Plasmid DNA was purified with cesium chloride-ethidium bromide gradients. Samples were desalted and concentrated to 50 μl in TES buffer (50 mM NaCl, 30 mM Tris, 5 mM EDTA; pH 8.0) by using Amicon Centricon-30 microconcentrators according to the instructions of the manufacturer. Electrophoresis was conducted on 0.5% agarose, horizontal slab gels in Tris acetate buffer (11) at pH 8.0 by using a constant voltage of 75 V for 105 min. The purified plasmids of *E. coli* V517 described by Macrina et al. (10) were used as standards for molecular weight determinations.

Restriction endonuclease analysis. Plasmid preparations for restriction endonuclease analyses were buffer exchanged from TES buffer into RE buffer (10 mM Tris, 4 mM NaCl, 0.1 mM EDTA [pH 8.0]) with an Amicon Centricon-30 microconcentrator. Plasmid DNA was digested with *Pvu*II restriction enzyme (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) according to the instructions of the manufacturer. Analysis of fragments was conducted by using horizontal gel electrophoresis under the same conditions as previously described for plasmid analyses. Reference standards for restriction analysis were *Hin*dIII-digested λ phage DNA fragments (Bethesda Research Laboratories).

Curing conditions. Chemostat cultures were established with bacteriocin-producing pediococci to exert environmental pressures on cells which would favor plasmid loss (4). The conditions chosen were a growth temperature of 42°C, which was higher than optimum (38°C), limiting glucose (3 mM) in MRS broth (3), and a dilution rate of 0.2 h^{-1}. At periodic intervals, samples were removed from the growth vessel, and individual colonies were tested for loss of bacteriocin phenotype (Bac^-). Strains found to be Bac^-

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TABLE 1. Bacterial strains and their sensitivity to bacteriocins produced by *P. pentosaceus* L7230 and FBB61

Species	Source[a]	Sensitivity to strain: L7230	FBB61
Clostridium spp.			
C. botulinum 62A (type B)	P. M. Foegeding, NCSU	+	+
C. botulinum 213B (type B)	P. M. Foegeding, NCSU	+	+
C. botulinum 12885A (type A)	P. M. Foegeding, NCSU	+	+
C. perfringens NCTC 8798	P. M. Foegeding, NCSU	+	+
C. sporogenes	University of Tennessee	+	+
Lactobacillus spp.			
L. brevis LB50	Our culture collection	+	+
L. plantarum WSO	Our culture collection	+	+
Pediococcus spp.			
P. acidilactici 33314	ATCC	+	+
P. dextrinicus 33087	ATCC	+	+
P. pentosaceus L7230	Our culture collection	−	−
P. pentosaceus FBB61	Our culture collection	−	−
P. pentosaceus FBB61-2	This study	+	+
P. pentosaceus FBB61-8	This study	+	+
P. pentosaceus PC39	Our culture collection	+	+
P. pentosaceus B1325	NRRL	+	+
P. pentosaceus B11465	NRRL	+	+
P. pentosaceus 25745	ATCC	+	+
P. pentosaceus 33316	ATCC	+	+
Strain "lactocel 75"	Microlife Technics, Sarasota, FL	+	+
Staphylococcus spp.			
S. aureus MD-9	University of Tennessee	+	+
S. aureus 138-CPS	ABC	+	+
S. aureus 146-CPS	ABC	+	+
S. aureus 153-CPS	ABC	+	+
S. lactis 11454[b]	ATCC	+	+

[a] Abbreviations: NCSU, North Carolina State University; ATCC, American Type Culture Collection (Rockville, Md.); NRRL, Northern Regional Research Laboratory (Peoria, Ill.), ABC, ABC Research Corp. (Gainesville, Fla.).

[b] Whereas *S. lactis* 11454 was sensitive to *P. pentosaceus* FBB61 and L7230 bacteriocins, the latter strains were sensitive to *S. lactis* 11454 (nisin producer). This indicated that the pediocin was not nisin.

were subjected to plasmid analysis along with Bac^+ strains as controls.

Curing studies. Bac^- variants were isolated from *P. pentosaceus* FBB61, but not from L7230, when cultivated in chemostat culture under plasmid curing conditions. After 10 days of culture, 17% of the FBB61 sample isolates were Bac^-, and at 14 days, 53% were Bac^-. Bac^- variants could not be isolated from cultures grown as batch cultures in tubes. The Bac^+ phenotype was routinely determined by the agar spot test (Fig. 1). Loss of Bac^+ in chemostat isolates was concurrent with loss of immunity to the bacteriocin, indicated by a sensitivity to producer strains. Strain FBB61-2 was no longer able to inhibit PC39 and became sensitive to FBB61 and L7230. Two Bac^- cultures, FBB61-2 and FBB61-8, were examined for reversion to Bac^+. A total of approximately 37,000 and 29,000 colonies from each culture, respectively, were tested and were found to remain Bac^-.

Plasmid association of bacteriocin activity and immunity. Plasmid analysis of strains L7230 and FBB61 revealed the presence of a 13.6-MDa plasmid in both Bac^+ strains (Fig. 2A, lanes a and c). FBB61 also possessed a 5.0-MDa plasmid. These plasmids are designated pMD136 and pMD50, respectively. Strain FBB61-2, which was cured of the Bac^+ phenotype and immunity to the bacteriocin, was missing pMD136 but not pMD50 (Fig. 2A, lane b). All of the Bac^- derivatives of chemostat culture of FBB61 (a total of 23) lacked pMD136 yet retained pMD50.

Restriction enzyme analysis of plasmid DNA. Evidence for relatedness of pMD136 in L7230 and FBB61 was obtained by restriction enzyme analysis of plasmid DNA obtained from these two strains. L7230 contains only pMD136, which upon enzyme digestion with *Pvu*II yielded two fragments (Fig. 2B, lane a). FBB61, which contains pMD136 and pMD50, revealed five fragments, two of which had mobility patterns (Fig. 2B, lane c) identical to those of the two L7230 fragments (Fig. 2B, lane a). The other three FBB61 fragments were identical in size to those which were the digest of pMD50 only (Fig. 2B, lane b). Thus, *Pvu*II restriction of pMD136 in L7230 and FBB61 yields two fragments with identical mobility characteristics. The data indicate that pMD136 is similar in L7230 and FBB61 and suggest that pMD136 is present and encodes Bac^+ production and immunity in L7230. However, further analysis is required to establish whether or not pMD136 is responsible for Bac^+ production and immunity in L7230.

Activity spectrum. Fleming et al. (6) demonstrated that the inhibitory agent of L7230 and FBB61 was active against a broad spectrum of gram-positive bacteria, including pediococci, lactobacilli, leuconostocs, streptococci, micrococci,

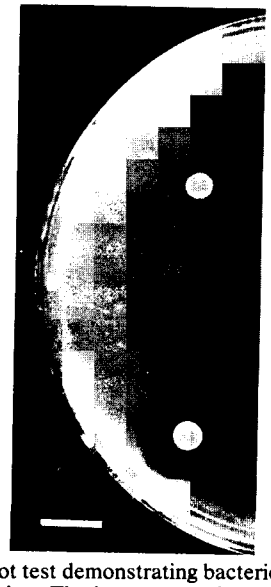

FIG. 1. Agar spot test demonstrating bacteriocin activity as seen by a zone of inhibition. The bottom spot is *P. pentosaceus* FBB61 (Bac^+). The top spot is FBB61-2 (Bac^-), which was isolated from a chemostat culture. The indicator microorganism is *L. plantarum* WSO. Bar, 1 cm.

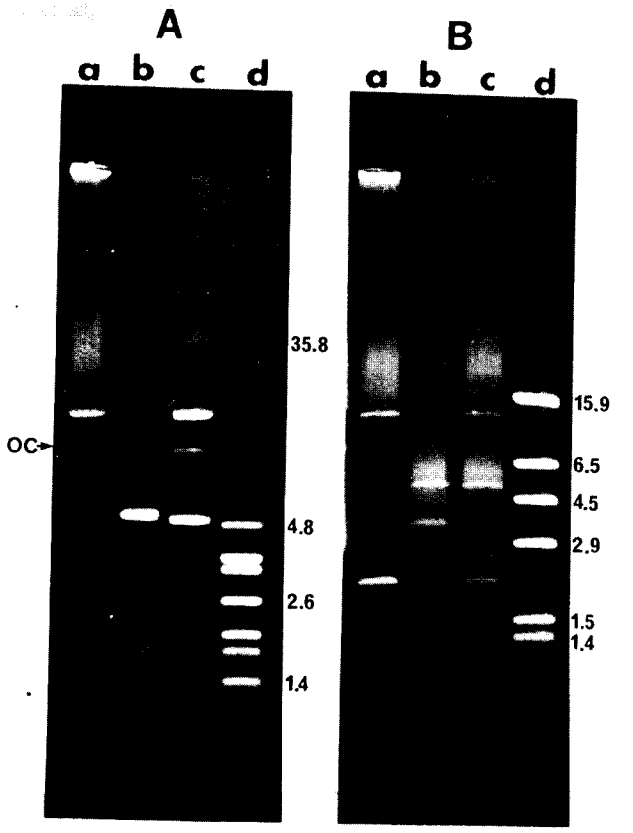

FIG. 2. Analyses of cesium chloride-ethidium bromide-purified plasmid DNA. (A) Agarose gel electrophoresis from *P. pentosaceus* L7230 Bac$^+$ (a), *P. pentosaceus* FBB61-2 Bac$^-$ (b), *P. pentosaceus* FBB61 Bac$^+$ (c), and *E. coli* V517 reference mobility plasmids (d). OC, Open circular form of pMD50. (B) *Pvu*II restriction enzyme analysis from *P. pentosaceus* L7230 Bac$^+$ (a), *P. pentosaceus* FBB61-2 Bac$^-$ (b), *P. pentosaceus* FBB61 Bac$^+$ (c), and linear DNA reference mobility fragments from *Hin*dIII-digested λ phage DNA (d).

staphylococci, and bacilli, but not against gram-negative bacteria or yeasts. We have extended this survey to include additional pediococcal strains as well as pathogenic and toxigenic gram-positive bacteria (Table 1). All the bacteria tested were sensitive to L7230 and FBB61, but not to FBB61-2, as determined by the agar spot test. The fact that both strains had identical activity spectra suggests strong homology between the bacteriocins of the two strains.

The criteria for establishing whether an inhibitory substance is a bacteriocin is well defined for gram-negative bacteria but not for gram-positive bacteria (14). Tagg et al. (14) suggest that the term bacteriocin, when used to describe inhibitory agents produced by gram-positive bacteria, should meet the minimum criteria of (i) being a biologically active protein and (ii) possessing a bacteriocidal mode of action. These requirements, having been satisfied by the work of Rueckert (M.S. thesis) and the evidence obtained in this study, and suggesting that inhibitor production and host cell immunity are plasmid-borne genetic determinants, lead us to propose that the inhibitor is a bacteriocin and that hereafter it be designated as pediocin A.

Resident plasmids have been observed in *P. pentosaceus* and *Pediococcus acidilactici*; however, their phenotypic traits have not been established (7). Our results present the first evidence of which we are aware for plasmid linkage of bacteriocin activity and immunity in *P. pentosaceus*. Curing the producer strain FBB61 of bacteriocin activity resulted in loss of immunity to the bacteriocin and in the loss of a resident 13.6-MDa plasmid. These findings suggest that the bacteriocin activity and immunity have genetic determinants that reside on the same plasmid. This phenotypic arrangement has been well documented in colicin-producing strains of *E. coli* where each Col plasmid confers immunity to the particular type of colicin it encodes (8). Neve et al. (12) recently were able to conjugally transfer bacteriocin activity and immunity in *Streptococcus cremoris* to *Streptococcus lactis* subsp. *diacetylactis*. A 39.6-MDa plasmid was linked to both activity and immunity.

Some bacteriocins of gram-positive bacteria, in contrast to gram-negative bacteria, have broad activity spectra against other gram-positive bacteria (14). This appears to be especially true of pediocin A, to which every gram-positive bacterium tested in this study proved to be sensitive. Although not every genus was represented in this study, it was noteworthy that bacteria that are commonly incriminated in food poisoning were sensitive to pediocin A. Fermented meat products are often involved in staphylococcal food poisoning outbreaks (13). Starter cultures of *Pediococcus* sp. which possess pediocin A activity may be useful in controlling staphylococcus contamination in fermented meats. Strains of selected lactic acid bacteria possessing pediocin A activity may also find use in vegetable fermentations where competing naturally occurring lactic acid bacteria make the achievement of pure culture fermentations when using starters difficult (2). Since pediocin A appears to be a plasmid-encoded trait, genetic transfer to selected strains of lactic acid bacteria with superior fermentation characteristics may result in desirable new strains capable of predominating over the natural flora by direct antagonism. Intergeneric and intrageneric conjugal transfer of plasmids in *Pediococcus* species has been demonstrated previously (7).

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