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Monoclonal Antibody-Based Enzyme Immunoassay for Pediocins of *Pediococcus acidilactici*†

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Monoclonal antibody (MAb) R2-AR against pediocin RS2 was developed. Mice were immunized for 12 weeks with pediocin RS2 conjugated to a polyacrylamide gel. Two hybridoma fusions yielded an MAb that in Western blots (immunoblots) reacted only with pediocins RS2 and AcH (3 kDa) from *Pediococcus acidilactici* RS2 and H, respectively, and did not react with any other bacteriocin, including sakacin A from *Lactobacillus sake* Lb 706, leuconocin LCM1 from *Leuconostoc carnosum* LM1, nisin from *Lactococcus lactis* ATCC 11454, and pediocin A from *Pediococcus pentosaceus* FBB61. Each of the bacteriocin bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels was confirmed to be biologically active by a gel overlay test performed with sensitive indicator organisms. In dot immunoblot assays, the MAb could detect a minimum of 32,000 arbitrary units of pediocin RS2 or AcH per ml. In colony immunoblot assays, the MAb was used successfully to differentiate bac$^+$ and bac$^-$ variants of *P. acidilactici* RS2 strains.

The pediocins which are produced by *Pediococcus acidilactici* are peptides that have molecular masses of about 2,700 to 4,000 Da (4, 5, 19, 23, 25). The antimicrobial activities of pediocins are mediated through changes in the membrane potentials of indicator organisms, which in turn lead to a collapse of the proton motive force, an efflux of intracellular ions, and cell death (7, 10, 11). There is great potential for pediocins to be used as food biopreservatives to control food-borne pathogens and spoilage organisms (21, 26).

Currently, the antimicrobial activities of pediocins are measured by determining the ability of pediocin dilutions to inhibit sensitive test organisms in solid or liquid bacteriological media (26). Falahee et al. (13) described a polyclonal antibody-based enzyme immunoassay to detect nisin in artificially inoculated cheese samples. Previous attempts to develop antibodies against pediocin AcH in mice or rabbits were unsuccessful (6). In this paper I describe a successful alternative approach for developing polyclonal antibodies and monoclonal antibodies (MAb) against pediocin in which mice were used. Characterization and the use of the MAb in enzyme immunoassays to detect and quantitate pediocins from *P. acidilactici* are described.

MATERIALS AND METHODS

Cultures and bacteriocin preparation. *P. acidilactici* RS2, which produces pediocin RS2, was originally isolated from fermented foods by colony immunoblotting in my laboratory (3, 9). Pediocin AcH produced by *P. acidilactici* H (4, 5), pediocin A produced by *Pediococcus pentosaceus* FBB61 (12), and nisin produced by *Lactococcus lactis* ATCC 11454 (18) were also used in this study. All of the bacteriocins were prepared from cell-free culture supernatants according to the method described previously (8). Sakacin A (16) and leuconocin LCM1 (27) preparations produced by *Lactobacillus sake* Lb 706 and *Leuconostoc carnosum* LM1, respectively, were obtained from Bibek Ray, University of Wyoming. *Lactobacillus plantarum* NCDO 955, *Pediococcus dextrinicus* ATCC 33087, and *Enterococcus faecalis* ATCC 344 were used as sensitive indicator organisms in this study. The protein concentrations of all of the bacteriocin preparations were determined with a protein assay kit (Bio-Rad Laboratories, Richmond, Calif.).

Antigen preparation. Pediocin RS2 precipitates were loaded into several wells (30 μg per well) of gradient sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gels containing 10 to 20% acrylamide. The sample preparation and electrophoresis conditions used were similar to those described previously (4, 8). After electrophoresis the gels containing pediocin bands were stained for 15 min with Coomassie blue, destained for 45 min, and equilibrated with changes of deionized water (8). The pediocin bands in four lanes were excised from each gel, resuspended in 0.5 ml of sterile 20 mM phosphate-buffered saline (pH 7.0), and homogenized for 10 min (70% duty cycle) with a sonicator (Branson Sonic Power Co., Danbury, Conn.). Antigens were collected from the SDS-PAGE gels by using the procedure mentioned above at least four times during the course of immunization and were stored at −20°C until they were used.

Mouse immunization and MAb preparation. A 0.35-ml portion of a homogenized PAGE gel containing pediocin RS2 was mixed with 0.15 ml of Freund's complete adjuvant (Difco) and 0.1 ml of poly(A) · poly(U) (4 mg/ml; Sigma) (17). For the initial immunization 0.2 ml of the gel mixture was injected intraperitoneally into each of two 6-week-old female BALB/c mice. After 2 weeks, each mouse received 0.1 ml of gel mixture prepared without Freund's complete adjuvant every week for 12 weeks. On average, each mouse received about 10 to 15 μg of pediocin during each immunization. Blood was collected from each mouse by ocular vein puncture at weeks 8 and 12 and was tested for production of antibody against pediocin RS2 by performing an enzyme-linked immunosorbent assay (ELISA) and by Western blotting (immunoblotting) (6). After ensuring that there was a positive antibody titer against pediocin RS2 at week 12, I proceeded with hybridoma cell fusions. The mice received three additional booster injections on three consecutive days, 72 h prior to the start of hybridoma

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cell fusion. Both immunized mice were sacrificed, and spleen cells were collected and fused with an equal number of mouse myeloma P3/Ns1/1-Ag4-1 (NS1) cells (1). Blood was collected from these mice by heart puncture; this blood was used as a source of polyclonal antibodies, and the antibody titers were determined by performing an ELISA. Hybridoma culture supernatants (0.5 ml) were tested against pediocin RS2 by Western blotting (1). Selected hybridoma cultures were cloned by limiting dilution, and selected clones were injected intraperitoneally into pristane-primed BALB/c mice for ascites production. The MAb from ascites fluid was partially purified by ammonium sulfate precipitation (20). The isotype of the MAb was determined by using Bio-Rad Mouse Typer.

PAGE, antimicrobial activity, and immunoblotting. Crude bacteriocin preparations (pediocins RS2 and AcH, sakacin A, leuconocin LCM1, nisin, and pediocin A) were separated on SDS-PAGE gels containing 10 to 20% acrylamide (8). The pediocin RS2 and AcH preparations used in the SDS-PAGE analysis were obtained from two separate batch preparations. Both pediocin RS2 and pediocin AcH were extracted from cell-free culture supernatants after 20 or 27 h of incubation at 30°C (8). The PAGE gel was either stained with Coomassie blue R250 or tested for antimicrobial activity by the gel overlay method described previously (4, 8). The indicator organisms used for the gel overlay procedure were *Lactobacillus plantarum* NCDO 955 for pediocins RS2 and AcH, *E. faecalis* ATCC 344 for sakacin A and leuconocin LCM1, and *P. dextrinicus* ATCC 33087 for nisin and pediocin A. The bacteriocins obtained from the PAGE gels were also transferred to an Immobilon-P membrane, blocked with 5% bovine serum albumin at 37°C for 16 h, and reacted with MAb R2-AR (1:300) or polyclonal antibodies (1:200) as previously described (1, 9).

Dot immunoblotting and antimicrobial activity of pediocin. Pediocins RS2 (1.45 mg/ml) and AcH (1.62 mg/ml) from stock preparations were serially diluted with 5 mM phosphate buffer (pH 7.0), and 0.1-ml portions of each dilution were placed on Immobilon-P membranes by using a dot blot manifold apparatus (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). A 5-μl volume of each dilution was also tested for antimicrobial activity, which was expressed in arbitrary units (AU) per milliliter (5, 7). Dot immunoblotting was carried out essentially by the procedure described previously (1), except that the membranes were blocked with 5% bovine serum albumin for 3 h prior to reaction with MAb R2-AR (1:300).

Isolation of bac$^-$ mutant by colony immunoblotting. The ability of MAb R2-AR to differentiate bac$^-$ *P. acidilactici* RS2 colonies from bac$^+$ *P. acidilactici* RS2 colonies was studied by using a colony blot test. *P. acidilactici* RS2 cultures were grown in MRS broth at an elevated temperature (45°C) for 24 h and were plated onto MRS agar (2). The plates were overlaid with Immobilon-P membranes and allowed to incubate for 48 h. The membranes were removed and probed with MAb R2-AR (9). Colonies that exhibited a reaction or no reaction with MAb R2-AR in the membrane blots were picked from the corresponding plates, examined by phase-contrast microscopy, and tested for bacteriocin activity (9). The presence of a 9.4-kb plasmid in cells from *P. acidilactici* RS2 colonies having bac$^+$ and bac$^-$ phenotypes was determined by a procedure described previously (2).

RESULTS AND DISCUSSIONS

Immunization. In a previous study, coworkers and I reported that partially purified pediocin AcH alone or pediocin AcH conjugated with bovine serum albumin apparently could not elicit an immune response in BALB/c mice when it was injected over a 4-week period (6). In this study, I immunized BALB/c mice with crude precipitates of pediocin RS2 or pediocin RS2 bound to heat-killed *Lactobacillus plantarum* for 8 to 10 weeks; these two immunization strategies again failed to produce antibodies against pediocin. In the latter experiments *Lactobacillus plantarum* was used as carrier for pediocin, since pediocins bind strongly to the cell surfaces of gram-positive bacteria (7). When I used a third method, immunization of mice with pediocin RS2 linked to a polyacrylamide gel for 12 weeks, antipediocin antibodies were produced. Pediocin RS2 is a low-molecular-weight peptide (molecular weight, 3,000) that could not elicit an immune response alone in mice; therefore, the polyacrylamide gel matrix acted as a carrier for pediocin and induced antibody production. During the immunization procedure the serum from each mouse was monitored for antibody production. At week 8 there was very little production of antibody against pediocin RS2. Therefore, I subjected the mice to an additional 4 weeks of immunization; the serum collected after this treatment exhibited a very high antibody titer, and hybridoma cell fusion experiments were continued. The titers of polyclonal antibodies obtained after 12 weeks from each of two mice were determined to be about 1:12,800. I repeated this immunization procedure with two different groups of mice (two mice per group) and obtained polyclonal antibodies against pediocin from each mouse, with antibody titers ranging from 1:5,120 to 1:10,240. Collectively, my data demonstrated that use of pediocin linked to a polyacrylamide gel matrix effectively induced an immune response against pediocin RS2 in all six mice tested.

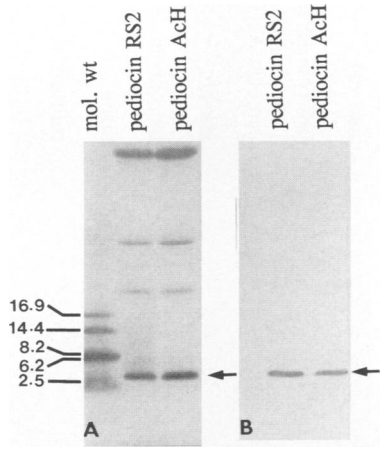
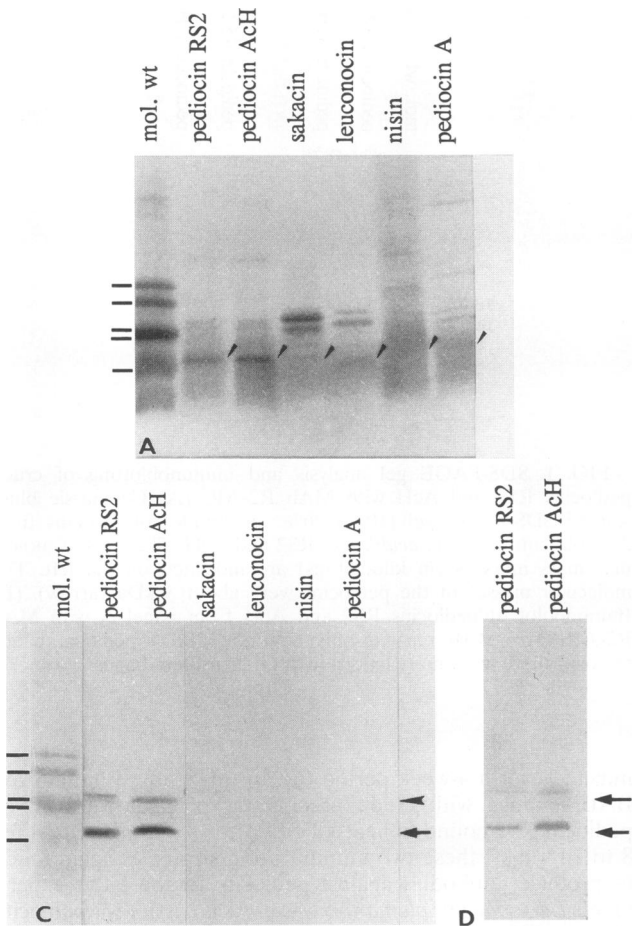


FIG. 1. SDS-PAGE gel analysis and immunoblotting of crude pediocins RS2 and AcH with MAb R2-AR. (A) Coomassie blue-stained SDS-PAGE gel (10 to 20% acrylamide) of proteins from 27-h-old cultures of *P. acidilactici* RS2 and AcH. The sizes of molecular mass markers (in kilodaltons) are indicated on the left. The molecular masses of the pediocins were about 3 kDa (arrow). (B) Immunoblot of pediocins RS2 and AcH from panel A with MAb R2-AR. The MAb reacted only with the 3-kDa pediocin bands (arrow); there were no reactions with other protein bands.

MAb. Approximately 230 hybridoma clones generated after two fusions were screened for production of antibody against pediocin RS2. Two clones, designated R2-A and R2-AR, were found to produce antibody against pediocin RS2. The immunoglobulins produced by these clones were immunoglobulin G1 and immunoglobulin M, respectively, and both had κ light chains. One of the two MAb, MAb R2-AR, exhibited a superior reaction with pediocin RS2 in both the ELISA and



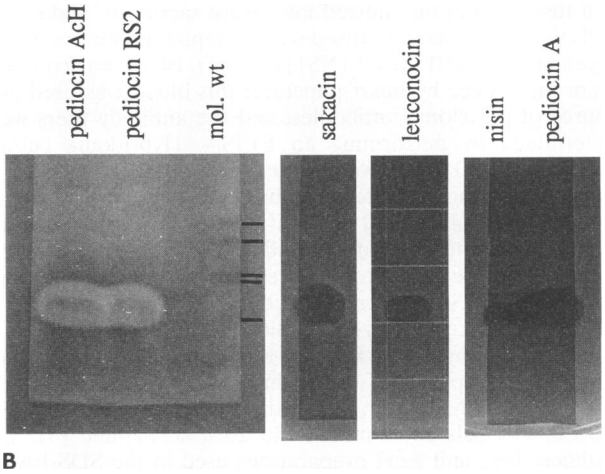


FIG. 2. SDS-PAGE gel analysis, antimicrobial activities, and immunoblotting of bacteriocins from various lactic acid bacteria. (A) Coomassie blue-stained SDS-PAGE gel containing crude pediocins RS2 and AcH which were prepared from cultures grown for 20 h. The gel also contained sakacin A, leuconocin LCM1, nisin, and pediocin A. The bacteriocin bands are indicated by arrowheads. The sizes of the molecular mass markers indicated on the left are (from top to bottom) 16.9, 14.4, 8.2, 6.2, and 2.5 kDa. (B) Antimicrobial activities of bacteriocin bands as determined by the gel overlay method. The gels containing pediocins RS2 and AcH were stained briefly (20 min) with Coomassie blue, destained, and overlaid with *Lactobacillus plantarum* NCDO 955 (8). Pediocin AcH and RS2 bands were clearly visible in the center of the zone of bacterial growth inhibition after 20 h of incubation. The molecular mass markers used (lane mol.wt) were the same as the markers used in the experiment shown in panel A. This gel segment was photographed with the light source below the gel. The gels containing sakacin A, leuconocin LCM1, nisin, and pediocin A were overlaid with either *E. faecalis* ATCC 344 or *P. dextrinicus* ATCC 33087 and were not stained with Coomassie blue (4) because the staining and destaining solutions, which contained methanol (25%) and acetic acid (10%), tended to inactivate these bacteriocins. The clear zones of inhibition produced by all of the bacteriocins corresponded to the lowermost bands in the Coomassie blue-stained gel in panel A; these gels were photographed with the light source above the gel. (C) Immunoblotting of bacteriocins pediocin RS2, pediocin AcH, sakacin A, leuconocin LCM1, nisin, and pediocin A with MAb R2-AR from panel A. MAb R2-AR reacted strongly with pediocin RS2 and AcH bands (arrow) and weakly with 6- to 8-kDa protein bands which were assumed to be prepediocin molecules (15, 24). The presence of prepediocin molecules in pediocin preparations obtained from cell-free culture supernatants depended on the age of the culture. Supernatants collected from 20-h-old cultures contained both prepediocin and pediocin molecules, while supernatants collected from 27-h-old cultures contained only pediocin molecules when the preparations were analyzed by SDS-PAGE and immunoblotting. The other bacteriocins did not exhibit any reactions with MAb R2-AR, except that there were some weak reactions with two protein bands in the 8.2- to 14.4-kDa range in sakacin A preparations. Lane mol.wt contained fast green (1%)-stained molecular mass markers similar to the markers used in the experiment shown in panel A. (D) Immunoblotting of the same pediocin RS2 and AcH preparations used for the experiment shown in panel A with mouse serum containing polyclonal antibodies against pediocin RS2. The 3-kDa pediocin RS2 and AcH bands, as well as the 6- to 8-kDa bands, reacted with polyclonal antibodies.

Western blots (data not shown). Therefore, I used MAb R2-AR for all of the subsequent studies.

PAGE, antimicrobial activity, and immunoblotting. Coomassie blue staining of SDS-PAGE gel lanes containing crude pediocin RS2 or AcH obtained from 27-h-old cultures revealed the presence of multiple proteins bands (Fig. 1A). Subsequent immunoblotting results indicated that only the 3-kDa pediocin RS2 and AcH bands reacted with MAb R2-AR (Fig. 1B). Immunoblotting of the same pediocin RS2 and AcH preparations with polyclonal antibodies gave similar results (data not shown). In contrast, SDS-PAGE and immunoblotting of pediocins RS2 and AcH obtained from 20-h-old cultures revealed that MAb R2-AR reacted with 3-kDa protein bands and with 6- to 8-kDa protein bands in both pediocin RS2 preparations and pediocin AcH preparations (Fig. 2A and C). Similar immunoblotting results were obtained when the same pediocin preparations from 20-h-old cultures were tested with polyclonal antibodies raised against pediocin RS2 (Fig. 2D). The 6- to 8-kDa protein bands may represent prepediocin molecules, as suggested by Henderson et al. (15) and Motlagh et al. (24). These protein bands do not represent aggregates of pediocin molecules, since they exhibited no antimicrobial activity in SDS-PAGE gels after being overlaid with sensitive indicator organisms (*Lactobacillus plantarum*). Conversely, both pediocin RS2 bands and pediocin AcH bands produced clear zones of bacterial growth inhibition (Fig. 2B). The presence of 6- to 8-kDa prepediocin molecules in pediocin preparations probably depends on the age of the *P. acidilactici* cultures prior to protein extraction (unpublished data). As mentioned above, the pediocins obtained from 20-h-old cultures contained prepediocin molecules, whereas the pediocins obtained from 27-h-old cultures did not contain prepediocin molecules.

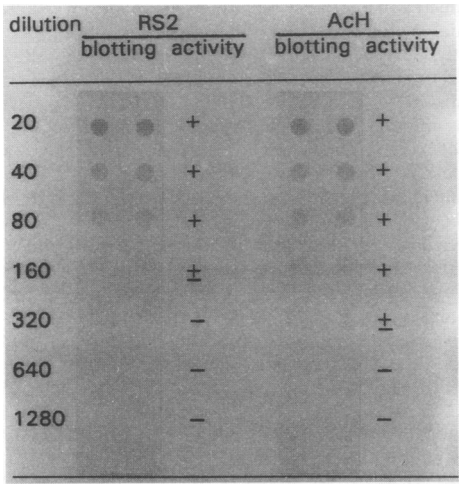


FIG. 3. Dot immunoblotting and antimicrobial activities of pediocins RS2 and AcH. Crude pediocins RS2 (1.45 mg/ml) and AcH (1.62 mg/ml) from 20-h-old culture supernatants were serially diluted in 5 mM phosphate buffer (pH 7.0). Then 0.1-ml portions of each dilution were tested by immunoblotting in duplicate spots with MAb R2-AR, and 5-μl portions of each dilution were also tested for antimicrobial activity. The MAb gave detectable spots with 160-fold-diluted preparations of pediocin RS2 or AcH (32,000 AU/ml). Both pediocins exhibited antimicrobial activity against *Lactobacillus plantarum* at this dilution; however, pediocin AcH exhibited slightly higher levels of antimicrobial activity. +, inhibition; ±, weak inhibition; −, no inhibition. Proteins prepared from culture supernatants of bac$^-$ mutants of *P. acidilactici* RS2 were used as negative controls in this study and did not exhibit any reactions with MAb R2-AR (data not shown).

SDS-PAGE analyses of other bacteriocins, such as sakacin A from *Lactobacillus sake* Lb 706 (16), leuconocin LCM1 from *Leuconostoc carnosum* LM1 (27), nisin from *Lactococcus lactis* ATCC 11454 (18), and pediocin A from *P. pentosaceus* FBB61 (12), revealed that multiple protein bands were present after Coomassie blue staining (Fig. 2A). Each of these bacteriocins produced a clear zone of inhibition in an overlay of indicator organisms at approximately 3 kDa when the preparation was compared with a Coomassie blue-stained gel (Fig. 2B). Immunoblotting results indicated that none of these bacteriocins reacted with MAb R2-AR (Fig. 2C). Only two protein bands, in the 8.2- to 14.4-kDa range, from sakacin A preparations exhibited weak nonspecific reactions with MAb R2-AR (Fig. 2C). The immunoblotting experiments were repeated at least three times with preparations of sakacin A, leuconocin LCM1, nisin, and pediocin A that were two to four times more concentrated, and I did not detect any reaction with MAb R2-AR with any of these bacteriocins. Although the amino acid sequence of sakacin A exhibits a high degree of homology with the amino acid sequence of pediocin AcH in the N-terminal half of the peptide (10, 14, 16, 22), I did not observe any cross-reaction with MAb R2-AR. These results indicated that the epitope recognized by MAb R2-AR is probably in the C-terminal half of the peptide, since there are greater differences in the amino acid sequences in that region of pediocin AcH and sakacin A (10, 16, 22, 24). While the amino acid sequences of leuconocin LCM1 and pediocin A are not available, the MAb did not react with either of these bacteriocins, suggesting that these two bacteriocins lack the epitope recognized by MAb R2-AR. The amino acid sequence and structure of nisin have been described previously (18, 21). The complete amino acid sequences of nisin and pediocin AcH are

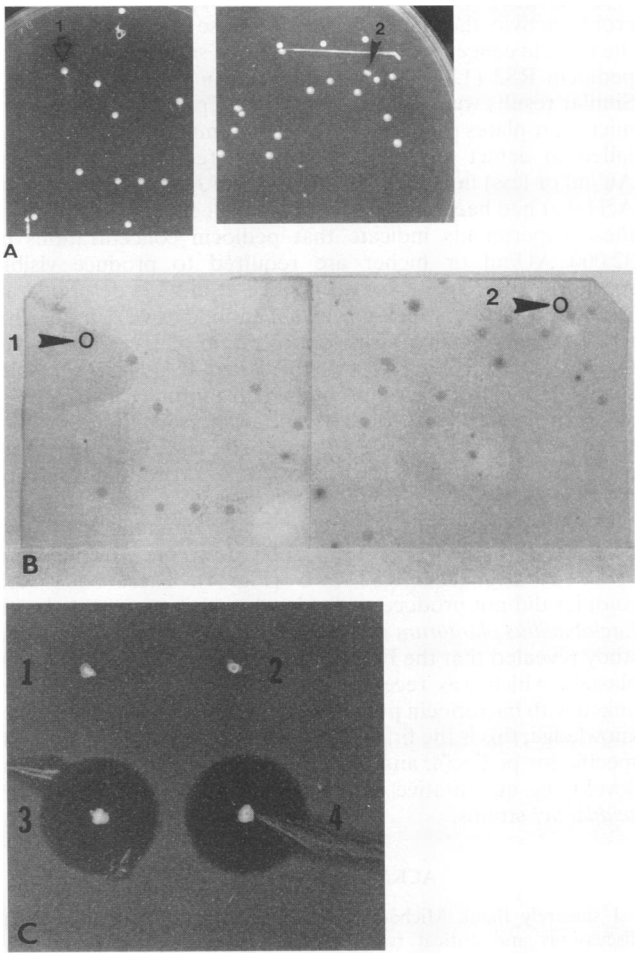


FIG. 4. Use of MAb R2-AR to differentiate bacteriocin-negative (bac$^-$) *P. acidilactici* RS2 colonies from bacteriocin-positive (bac$^+$) *P. acidilactici* RS2 colonies by colony immunoblotting. (A) Cells of *P. acidilactici* RS2 were grown at 45°C for 24 h to randomly cure the plasmid encoding pediocin production, serially diluted, and plated onto MRS agar plates. The plates were then overlaid with Immobilon-P membranes (Millipore) and incubated for 48 h at 30°C. (B) Immunoblotting of colonies from panel A with MAb R2-AR. The colonies inside circles 1 and 2 did not react with the MAb (arrowheads). (C) Colonies 1 and 2, which did not react with MAb R2-AR (see panel B) were collected from the plates shown in panel A and were tested for pediocin production with a lawn consisting of *Lactobacillus plantarum* NCDO 955. Inhibition zones are shown for colonies 1 and 2, which are bac$^-$ variants, and colonies 3 and 4, which were randomly picked from the plates shown in panel A and are bac$^+$ *P. acidilactici* RS2 colonies.

not at all homologous, except at position 10, where both bacteriocins have a glycine residue (18, 24). Therefore, it is unlikely that MAb R2-AR would react with nisin. Collectively, the Western blotting results indicate that some region in the C-terminal half of the pediocin peptide acts as the epitope for MAb R2-AR and that this epitope is not present in the other bacteriocins tested in this study.

Dot immunoblotting and antimicrobial activity. In dot immunoblotting assays MAb R2-AR reacted with pediocin RS2 (1.45 mg/ml) or pediocin AcH (1.62 mg/ml) that was diluted 160-fold (equivalent to 32,000 AU/ml). Such diluted samples also exhibited antimicrobial activity against *Lactobacillus plan-*

tarum (Fig. 3). Pediocin AcH exhibited slightly higher antimicrobial activity than pediocin RS2, because of the differences in the protein concentrations of the original stock preparations of pediocin RS2 (1.45 mg/ml) and pediocin AcH (1.62 mg/ml). Similar results were obtained in ELISAs performed in 96-well microtiter plates (data not shown). Dot immunoblotting assays failed to detect more dilute pediocin (concentration, 1,600 AU/ml or less) in culture supernatants of *P. acidilactici* RS2 or AcH that had been grown overnight. Collectively, the results of these experiments indicate that pediocin concentrations of 32,000 AU/ml or higher are required to produce visible reactions with MAb R2-AR. Falahee et al. (13) described an ELISA for nisin in which polyclonal antibodies were used. This ELISA could detect a nisin concentration of 1.9×10^2 IU/ml (equivalent to 1.9×10^4 AU/ml) (26). The ELISA results correlate well with results obtained in antimicrobial activity assays in which *Micrococcus flavus* was used. Similarly, the results of my dot blot assay for pediocins RS2 and AcH performed with MAb R2-AR correlate well with the results of the bioassays.

Colony immunoblot assays. In colony immunoblot assays performed with MAb R2-AR, I isolated two bac$^-$ colonies out of 10^6 *P. acidilactici* RS2 CFU (Fig. 4). These bac$^-$ RS2 colonies did not produce pediocin when they were tested with *Lactobacillus plantarum* cell lawns (Fig. 4C). A plasmid profile study revealed that the bac$^-$ strains did not contain the 9.4-kb plasmid which was recently shown to be necessary for and linked with bacteriocin production (data not shown) (2). To my knowledge, this is the first report of the development of a MAb specific for pediocin, and this antibody could be very useful in developing quantitative assays for pediocin production by *P. acidilactici* strains.

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