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New Biologically Active Hybrid Bacteriocins Constructed by Combining Regions from Various Pediocin-Like Bacteriocins: the C-Terminal Region Is Important for Determining Specificity

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The pediocin-like bacteriocins, produced by lactic acid bacteria, are bactericidal polypeptides with very similar primary structures. Peptide synthesis followed by reverse-phase and ion-exchange chromatographies yielded biologically active pediocin-like bacteriocins in amounts and with a purity sufficient for characterizing their structure and mode of action. Despite similar primary structures, the pediocin-like bacteriocins, i.e., pediocin PA-1, sakacin P, curvacin A, and leucocin A, differed in their relative toxicities against various bacterial strains. On the basis of the primary structures, the polypeptides of these bacteriocins were divided into two modules: the relatively hydrophilic and well conserved N-terminal region, and the somewhat more diverse and hydrophobic C-terminal region. By peptide synthesis, four new biologically active hybrid bacteriocins were constructed by interchanging corresponding modules from various pediocin-like bacteriocins. All of the new hybrid bacteriocin constructs had bactericidal activity. The relative sensitivity of different bacterial strains to a hybrid bacteriocin was similar to that to the bacteriocin from which the C-terminal module was derived and quite different from that to the bacteriocin from which the N-terminal was derived. Thus, the C-terminal part of the pediocin-like bacteriocins is an important determinant of the target cell specificity. The synthetic bacteriocins were more stable than natural isolates, presumably as a result of the absence of contaminating proteases. However, some of the synthetic bacteriocins lost activity, but this was detectable only after months of storage. Mass spectrometry suggested that this instability was due to oxidation of methionine residues, resulting in a 10- to 100-fold reduction in activity.

Bacteriocins are proteinaceous, antagonistic compounds produced by bacteria. Because of their potential for use as antibacterial additives in food and feed, bacteriocins produced by lactic acid bacteria (LAB) have been the subject of considerable interest, and a number of LAB bacteriocins have been characterized in recent years. Most of these LAB bacteriocins are short cationic polypeptides which seldom contain more than 60 amino acids. The pediocin-like bacteriocins form a group of LAB bacteriocins that have similar amino acid sequences. The first bacteriocins to be characterized that belong to this group were pediocin PA-1 (5, 8), sakacin P (14, 16), leucocin A (3), curvacin A (6, 7, 14, 15), and mesentericin Y105 (4). Other LAB bacteriocins that were more recently identified, such as carnobacteriocin BM1 and B2 (10) and enterocin A (1), may also be placed in this group of pediocin-like bacteriocins. The permeabilization of the cell membrane appears to be the mechanism by which the pediocin-like bacteriocins exert their bactericidal activity (2).

The extensive similarity of the amino acid sequences of the pediocin-like bacteriocins makes these bacteriocins well suited for analyzing the relationship between their target cell specificity and primary structure. The aim of this study was to determine whether the pediocin-like bacteriocins, despite their similar primary structures, differ in their relative toxicities towards various bacterial strains. Insight into which region of the bacteriocins determines target-cell specificity may then be gained by measuring the specificity of hybrid bacteriocins constructed by combining the N-terminal and C-terminal halves of various pediocin-like bacteriocins, enabling a more rational design and use of new bacteriocin constructs as antimicrobial agents.

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MATERIALS AND METHODS

Bacterial strains and media. The pediocin PA-1 producer, *Pediococcus acidilactici*, was isolated from commercial starter cultures obtained from Christian Hansen Laboratories, Copenhagen, Denmark. Natural curvacin A and sakacin P were produced by *Lactobacillus curvatus* LTH1174 and *Lactobacillus sake* LTH673, respectively. The indicator strains used in the bacteriocin assay were *L. sake* NCDO 2714 (type strain), *Lactobacillus coryneformis* subsp. *torquens* NCDO 2740, *Carnobacterium piscicola* UI49 (12, 13), and *Pediococcus acidilactici* NCDO 521. All strains, except *C. piscicola* UI49 were grown at 30°C in MRS broth (Oxoid). *C. piscicola* UI49 was grown at 30°C in M17 (Biokar Diagnostica) supplemented with glucose to a final concentration of 0.4% (wt/vol).

Bacteriocin assay. Bacteriocin activity was measured as described previously (9) with a microtiter plate assay system. A 200-μl volume of culture medium, bacteriocin fractions at twofold dilutions, and the indicator strain (A_{600} = 0.1) were added to each well of a microtiter plate. The microtiter plate cultures were incubated for 3 to 5 h at 30°C, after which growth of the indicator strain was measured spectrophotometrically at 600 nm with a microtiter plate reader. One bacteriocin unit was defined as the amount of bacteriocin that inhibited the growth of the indicator strain by 50% (50% of the turbidity of the control culture without bacteriocin).

Purification of natural pediocin PA-1, curvacin A, and sakacin P from producer strains. The natural bacteriocins were purified to homogeneity from 1- to 2-liter cultures of the producer strain by ammonium sulfate precipitation and cation-exchange, hydrophobic-interaction, and reverse-phase chromatographies, as described previously (8).

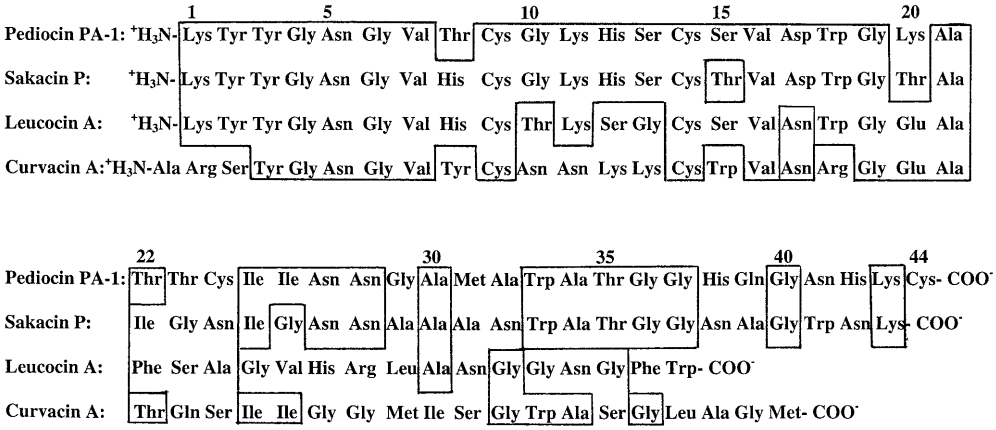

FIG. 1. Amino acid sequences of pediocin PA-1, sakacin P, leucocin A-UAL 187 (leucocin A), and curvacin A. The boxes enclose common amino acid residues. The sequence for pediocin PA-1 is from Henderson et al. (5) and Nieto Lozano et al. (8); that for sakacin P is from Tichaczek et al. (14, 16); that for leucocin A is from Hastings et al. (3); and that for curvacin A is from Holck et al. (6, 7) and Tichaczek et al. (14, 15).

Peptide synthesis and purification and analysis of the polypeptides. Peptides were synthesized by solid-phase methods with an Applied Biosystems model 430A peptide synthesizer and the standard *tert*-butoxycarbonyl synthesis protocol of the manufacturer. All of the synthesized peptides were solubilized in 0.1% trifluoroacetic acid (TFA) to a concentration of 3 to 5 mg/ml, except for pediocin PA-1 and sakacin P, which were solubilized to a concentration of about 25 mg/ml. For purification, the synthesized bacteriocins were first applied to a C_2-C_{18} reverse-phase PepRPC HR 5/5 column (Pharmacia Biotech) equilibrated with 0.1% TFA with a fast protein liquid chromatography system (Pharmacia Biotech). The bacteriocins were eluted with a 15 to 40% linear gradient of 2-propanol containing 0.1% TFA, and fractions containing bacteriocin activity were collected. Synthetic sakacin P and the hybrid bacteriocins Sak-Ped and Sak-Cur were purified further by chromatography on the C_2-C_{18} reverse-phase column (see Results). After the first reverse-phase chromatography step, all of the other synthesized bacteriocins were further purified by chromatography on a Mono S PC 1.6/5 cation-exchange column (Pharmacia Biotech) with the SMART chromatography system (Pharmacia Biotech). Ion-exchange chromatography was performed at pH 5.5 with a 20 mM phosphate buffer to which 2-propanol was added to a final concentration of 40% (vol/vol). The bacteriocins were eluted from the column with a linear NaCl gradient, and fractions containing bacteriocin activity were collected. The bacteriocins were purified further by chromatography once (for the hybrid bacteriocins Cur-Sak and Ped-Sak) or twice (for pediocin PA-1, curvacin A, and leucocin A) on a reverse-phase C_2-C_{18} μRPC SC 2.1/10 column (Pharmacia Biotech) with the SMART chromatography system. The bacteriocins were eluted with a 0 to 45% gradient of 2-propanol containing 0.1% TFA, and fractions containing bacteriocin activity were collected.

The primary structure and the purity of all of the synthetic peptides were confirmed by protein sequencing (automatic sequencer model 477A [Applied Biosystems, Foster City, Calif.] with an on-line 120-A phenylthiohydantoin amino acid analyzer), electrospray mass spectrometry analysis, capillary electrophoresis (Beckman P/ACE System 2050 capillary electrophoresis; with untreated capillary tubing, a setting at 10 kV, and 50 mM phosphate buffer [pH 2.5]), and analytical reverse-phase chromatography with μRPC C_2-C_{18} SC 2.1/10 and Sephasil C_8 SC 2.1/10 columns (Pharmacia Biotech) and the SMART chromatography system (Pharmacia Biotech).

RESULTS

Peptide synthesis of sakacin P, pediocin PA-1, curvacin A, and leucocin A yields biologically active bacteriocins. Sakacin P, pediocin PA-1, curvacin A, and leucocin A were synthesized according to their published amino acid sequences (Fig. 1) (3, 5–7, 14–16). A relatively large amount of biologically active bacteriocin, approximately 1 g with a purity of about 1 to 10%, was obtained after each synthesis.

Synthetic sakacin P was purified by passing it twice over a reverse-phase column. Purification of synthetic pediocin PA-1, curvacin A, and leucocin A was performed by cation-exchange chromatography followed by three reverse-phase chromatography runs. The resulting bacteriocin preparations were relatively homogeneous when analyzed by analytical reverse-phase chromatography and capillary electrophoresis. The purity and correct primary structures for all four peptides were confirmed by peptide sequencing (results not shown) and electrospray mass spectrometry (Table 1). The sequence analysis revealed 95 to 85% purity for pediocin PA-1 and leucocin A and 80 to 70% purity for sakacin P and curvacin A. The yield from peptide synthesis was calculated to be about 10% for sakacin P, 3% for curvacin A and leucocin A, and about 1% for pediocin PA-1.

These purified bacteriocin preparations were used to determine the specific activities of the synthetic bacteriocins. For sakacin P and pediocin PA-1, comparable specific activities were obtained for the natural and the synthetic bacteriocins (Fig. 2, natural leucocin A was not available). For curvacin A, the specific activity of the synthetic form was less than that of the natural form (Fig. 2), which may be due in part to the lower purity of the synthetic form (70 to 80%). The high specific activities of the synthetic bacteriocins confirmed that the pri-

TABLE 1. Theoretical and experimental molecular weights of bacteriocins

Bacteriocin	Theoretical mol wt[a]		Expt mol wt[b]
	Without disulfide bridges	With disulfide bridges	
Synthetic leucocin A	3,932	3,930	3,930
Synthetic sakacin P	4,437	4,435	4,434
Natural pediocin PA-1	4,628	4,624	4,623
Synthetic pediocin PA-1	4,628	4,624	4,624
Pediocin PA-1-ox-n[c]	4,644	4,640	4,640
Natural curvacin A	4,309	4,307	4,306
Synthetic curvacin A	4,309	4,307	4,306
Curvacin A-ox-n[d]	4,325	4,323	4,323
Curvacin A-ox-s[d]	4,325	4,323	4,322
Cur-Sak	4,643	4,641	4,641
Ped-Sak	4,414	4,412	4,412

[a] Theoretical molecular weights were calculated from the amino acid sequence to the nearest whole number, assuming that the cysteine residues (four in pediocin and two in the other bacteriocins) do not (without disulfide bridges) and do (with disulfide bridges) form disulfide bridges.

[b] Experimental molecular weights were determined by electrospray mass spectrometry to the nearest whole number; standard deviations are within 1.

[c] Pediocin PA-1-ox-n is a variant obtained upon storage of natural pediocin PA-1, presumably obtained by oxidation of a methionine residue.

[d] Curvacin A-ox-n and -ox-s are variants obtained upon storage of, respectively, natural and synthetic curvacin A, presumably by oxidation of a methionine residue.

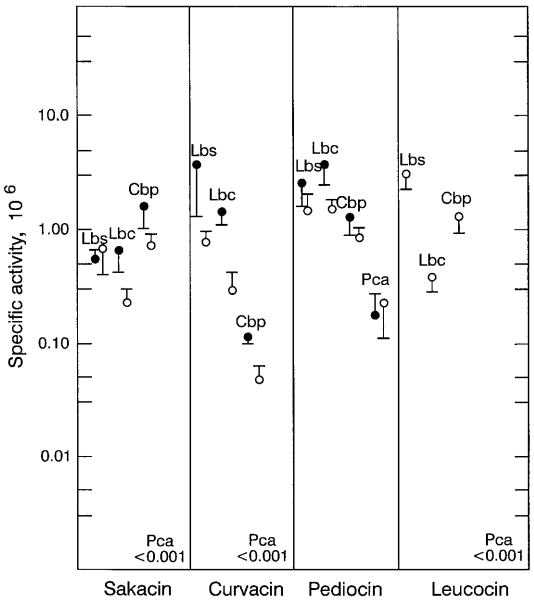

FIG. 2. Specific activities of natural (●) and synthetic (○) sakacin P, curvacin A, pediocin PA-1, and leucocin A towards indicator strains *L. sake* NCDO 2714 (Lbs), *L. coryneformis* subsp. *torquens* NCDO 2740 (Lbc), *C. piscicola* UI49 (Cbp) (12, 13), and *P. acidilactici* NCDO 521 (Pca). The specific activity was defined as bacteriocin units per milliliter per optical density at 280 nm and was measured with purified bacteriocin preparations. The bactericidal activity was measured seven times, with two to three parallel assays for each bacteriocin each time, and the bars indicate standard deviations for all seven measurements.

mary structures (Fig. 1) are sufficient to induce toxicity and thus do not depend on unidentified factors and/or chemical or structural modifications. Peptide synthesis consequently proved to be a relatively simple method to obtain pediocin-like bacteriocins in amounts required for biological, chemical, and structural characterization.

Bacteriocins of the pediocin-like family differ in their relative toxic potencies against different bacterial strains. The natural and synthetic forms of the bacteriocins had the same relative toxic potencies against target cells (Fig. 3). However, despite similar primary structures, sakacin P, pediocin PA-1, curvacin A, and leucocin A differed in their relative toxic potencies (Fig. 3). Sakacin killed *C. piscicola* UI49 more readily than *L. sake* NCDO 2714 and *L. coryneformis* subsp. *torquens* NCDO 2740, the order of sensitivity to sakacin being *C. piscicola* UI49 > *L. sake* NCDO 2714 ≥ *L. coryneformis* subsp. *torquens* NCDO 2740 ≫ *P. acidilactici* NCDO 521, where strains NCDO 2714 and NCDO 2740 had similar sensitivities and strain NCDO 521 was relatively resistant (Fig. 3). For curvacin, the situation was quite different in that curvacin killed both *L. sake* NCDO 2714 and *L. coryneformis* subsp. *torquens* NCDO 2740 much more readily than *C. piscicola* UI49, the order of sensitivity being *L. sake* NCDO 2714 > *L. coryneformis* subsp. *torquens* NCDO 2740 ≫ *C. piscicola* UI49 ≫ *P. acidilactici* NCDO 521 (Fig. 3). The sensitivity pattern obtained with pediocin was *L. coryneformis* subsp. *torquens* NCDO 2740 > *L. sake* NCDO 2714 = *C. piscicola* UI49 > *P. acidilactici* NCDO 521 (Fig. 3). For leucocin, no significant difference was detected in the sensitivity of *L. sake* NCDO 2714 and *C. piscicola* UI49, whereas *L. coryneformis* subsp. *torquens* NCDO 2740 was less sensitive, and *P. acidilactici* NCDO 521 was resistant (Fig. 3).

Only pediocin PA-1 was able to efficiently kill *P. acidilactici* NCDO 521 (Fig. 2 and 3). Pediocin PA-1 has a somewhat broader spectrum than that of sakacin P and curvacin A. We tested a total of 21 arbitrarily chosen *Lactobacillus*, *Carnobacterium*, and *Pediococcus* strains. Pediocin PA-1, sakacin P, and curvacin showed activity against 14, 7, and 6 of these strains, respectively (results not shown).

Hybrid bacteriocins constructed by combining N- and C-terminal regions of various pediocin-like bacteriocins have bactericidal activity. Hybrid bacteriocins were constructed by combining the N-terminal part of one pediocin-like bacteriocin with the C-terminal part of another. The N-terminal part starts with residue 1 and ends with Ala-21 (Ala-22 for curvacin A), which is conserved in all four bacteriocins (Fig. 1). The C-terminal part is from residue 22 (residue 23 for curvacin A) to the C terminus of the bacteriocin (Fig. 1). We synthesized the following hybrid bacteriocins: Ped-Sak, Sak-Ped, Cur-Sak, and Sak-Cur. For these hybrid bacteriocins, the N-terminal halves are the same as those in, respectively, pediocin, sakacin, curvacin, and sakacin, and the C-terminal halves are the same as those in, respectively, sakacin, pediocin, sakacin, and curvacin.

All hybrid bacteriocins had significant bactericidal activity (Fig. 4). The hybrid bacteriocin Ped-Sak, in fact, killed *L. sake* NCDO 2714, *L. coryneformis* subsp. *torquens* NCDO 2740, and *C. piscicola* UI49 with an efficiency similar to that of the parent bacteriocins, pediocin PA-1 and sakacin P (Fig. 2 and 4). The other hybrid bacteriocins were 10 to 1,000 times less potent than the parent bacteriocins (Fig. 2 and 4), but their bactericidal activity was nevertheless readily detected.

The C-terminal region is an important determinant of antibacterial specificity. The relative toxic potencies of the hybrid bacteriocins against *L. sake* NCDO 2714, *L. coryneformis* subsp. *torquens* NCDO 2740, *C. piscicola* UI49, and *P. acidilactici* NCDO 521 were determined and compared with that of the parent bacteriocins. Ped-Sak showed a specificity pattern similar to that of sakacin P but not to that of pediocin PA-1, in that Ped-Sak killed *C. piscicola* UI49 more readily than it killed *L. sake* NCDO 2714 and *L. coryneformis* subsp. *torquens* NCDO 2740, the order of sensitivity to Ped-Sak being *C. piscicola* UI49 > *L. coryneformis* subsp. *torquens* NCDO 2740 ≥ *L. sake* NCDO 2714 ≫ *P. acidilactici* NCDO 521 (Fig. 5). Sak-Ped, however, was similar to pediocin PA-1 in that Sak-Ped killed *L. coryneformis* subsp. *torquens* NCDO 2740 more readily than it killed *C. piscicola* UI49 and *L. sake* NCDO 2714, the order of sensitivity to Sak-Ped being *L. coryneformis* subsp.

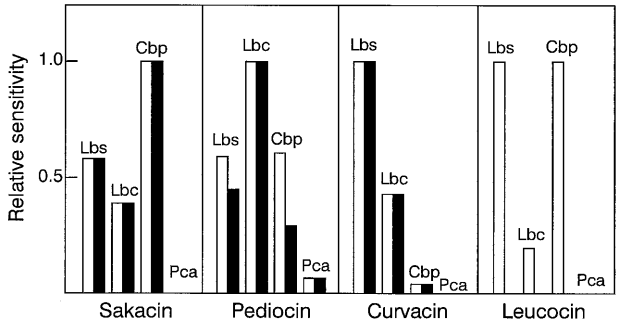

FIG. 3. The relative sensitivities of the indicator strains *L. sake* NCDO 2714 (Lbs), *L. coryneformis* subsp. *torquens* NCDO 2740 (Lbc), *C. piscicola* UI49 (Cbp), and *P. acidilactici* NCDO 521 (Pca) to synthetic (white bars) and natural (black bars) sakacin P, pediocin PA-1, curvacin A, and leucocin A. The relative sensitivity towards a bacteriocin was defined as 1.0 for the strain most readily killed by that bacteriocin. The relative sensitivity of a less sensitive strain was then defined as the amount of bacteriocin needed to kill the most sensitive strain divided by that needed to kill the less sensitive strain. The results represent the averaged data from three experiments, with seven to eight parallel assays for each bacteriocin in each experiment, and the standard error of the mean was ≤0.2.

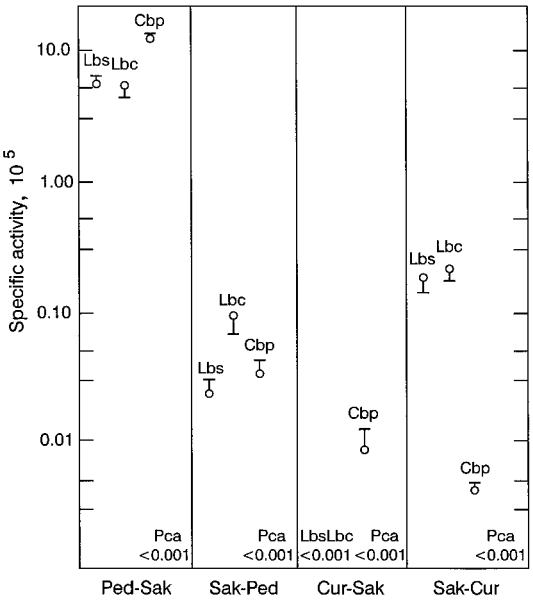

FIG. 4. Specific activity of the hybrid bacteriocins towards indicator strains *L. sake* NCDO 2714 (Lbs), *L. coryneformis* subsp. *torquens* NCDO 2740 (Lbc), *C. piscicola* UI49 (Cbp), and *P. acidilactici* NCDO 521 (Pca). The indicated hybrid bacteriocins are defined in the text; for number of measurements and definition of specific activity, see the legend to Fig. 2.

torquens NCDO 2740 > *L. sake* NCDO 2714 = *C. piscicola* UI49 ⩾ *P. acidilactici* NCDO 521 (Fig. 5).

The same trend, i.e., that the specificity of hybrid bacteriocins was similar to that of the bacteriocin from which the C-terminal region was derived, was also seen for Cur-Sak and Sak-Cur. Cur-Sak was active against *C. piscicola* UI49, the strain which was also most sensitive to sakacin P, but showed very low activity against the other strains, the order of sensitivity being *C. piscicola* UI49 ⩾ *L. sake* NCDO 2714 ≥ *L. coryneformis* subsp. *torquens* NCDO 2740 ⩾ *P. acidilactici* NCDO 521 (Fig. 5). Sak-Cur, on the other hand, killed *C. piscicola* UI49 much less readily than it killed *L. sake* NCDO 2714 and *L. coryneformis* subsp. *torquens* NCDO 2740 (Fig. 5) and was thus similar to curvacin A. Thus, the specificity pattern appeared to largely follow the C-terminal region of the hybrid bacteriocins.

Formation of S-S bridges. All pediocin-like bacteriocins that have been characterized have two cysteine residues in the N-terminal half of the peptide, at positions 9 and 14 in pediocin PA-1, sakacin P, and leucocin A and at positions 10 and 15 in curvacin A (Fig. 1). Pediocin PA-1 has, in addition, two cysteine residues in the C-terminal half, at positions 24 and 44 (Fig. 1). Mass spectrometric analysis of various pediocin-like bacteriocins revealed that the cysteine residues were joined by disulfide bridges; the molecular weights obtained were consistent with the cysteine residues being oxidized (Table 1). These intrapeptide disulfide bridges formed spontaneously in the synthetic bacteriocins as well as in the hybrids (Table 1).

Stability of the pediocin-like bacteriocins. Natural sakacin P lost activity during purification and storage probably as a result of small contaminating amounts of an extracellular bacterial protease (data not shown). This was not a problem with the synthetic bacteriocins. Synthetic leucocin A was stable for at least 12 months when stored at 4°C in 20 to 30% propanol–0.1% TFA in H_2O. Synthetic sakacin P was also much more stable than natural sakacin P, although storage of synthetic sakacin P at 4°C in 20 to 30% propanol–0.1% TFA in H_2O for 6 months did result in approximately 30% reduction in specific activity. At the same time, an altered form of sakacin P, in addition to unaltered sakacin P, could be detected upon reverse-phase chromatography (data not presented). This altered form eluted ahead of unaltered sakacin P upon reverse-phase chromatography and migrated after it upon capillary electrophoresis (data not shown), suggesting that the altered form was more hydrophilic and had more negatively (or fewer positively) charged residues than unaltered sakacin P. Moreover, the altered form had a molecular mass 3.0 ± 1.7 Da greater than that of unaltered sakacin P as judged by electrospray mass spectrometry analysis, and its specific activity was less than one-third the specific activity of unaltered sakacin P (data not shown).

After storage in 30% 2-propanol–0.1% TFA in H_2O at 4°C for 8 months, about 50% of natural and synthetic curvacin A and pediocin PA-1 had been converted to less-active and inactive forms which eluted ahead of the original active form upon reverse-phase chromatography (data not shown). Pediocin PA-1 was transformed to a form designated pediocin PA-1-ox, which had a molecular weight 16 greater than that of pediocin PA-1 (Table 1) and a 100- to 200-fold-reduced specific activity. Pediocin PA-1 and pediocin PA-1-ox comigrated upon capillary electrophoresis (data not shown), suggesting that they had identical charges. Curvacin A transformed to three different forms upon storage, one of which we designated curvacin A-ox, and this form had a molecular weight that was 16 greater than that of curvacin A (Table 1) and a 10-fold-reduced specific activity. The other forms were inactive at the concentrations tested.

DISCUSSION

Peptide synthesis followed by purification by reverse-phase and ion-exchange chromatographies proved to be a relatively simple procedure to obtain biologically active pediocin-like bacteriocins in amounts and with a purity sufficient for structural studies, such as nuclear magnetic resonance-spectroscopy. Approximately 1 g with a purity of about 1 to 10% was obtained after each synthesis. The synthesized bacteriocin could be relatively easily purified by reverse-phase and ion-exchange chromatographies, which for some of the synthetic bacteriocins resulted in more than 90% purity as judged by

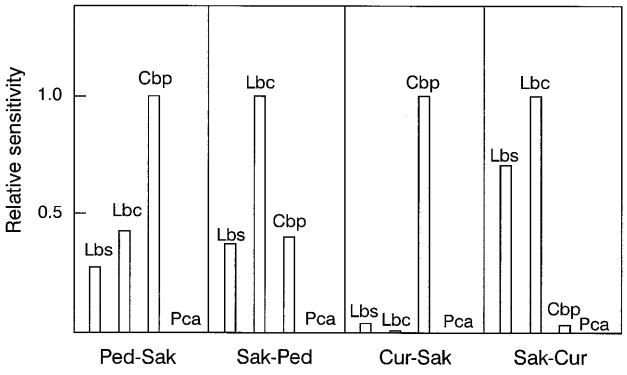

FIG. 5. The relative sensitivities of the indicator strains *L. sake* NCDO 2714 (Lbs), *L. coryneformis* subsp. *torquens* NCDO 2740 (Lbc), *C. piscicola* UI49 (Cbp), and *P. acidilactici* NCDO 521 (Pca) to the hybrid bacteriocins. The indicated hybrid bacteriocins are defined in the text. For definition of relative sensitivity and specification of number of experiments and parallel assays in each experiment, see the legend to Fig. 3.

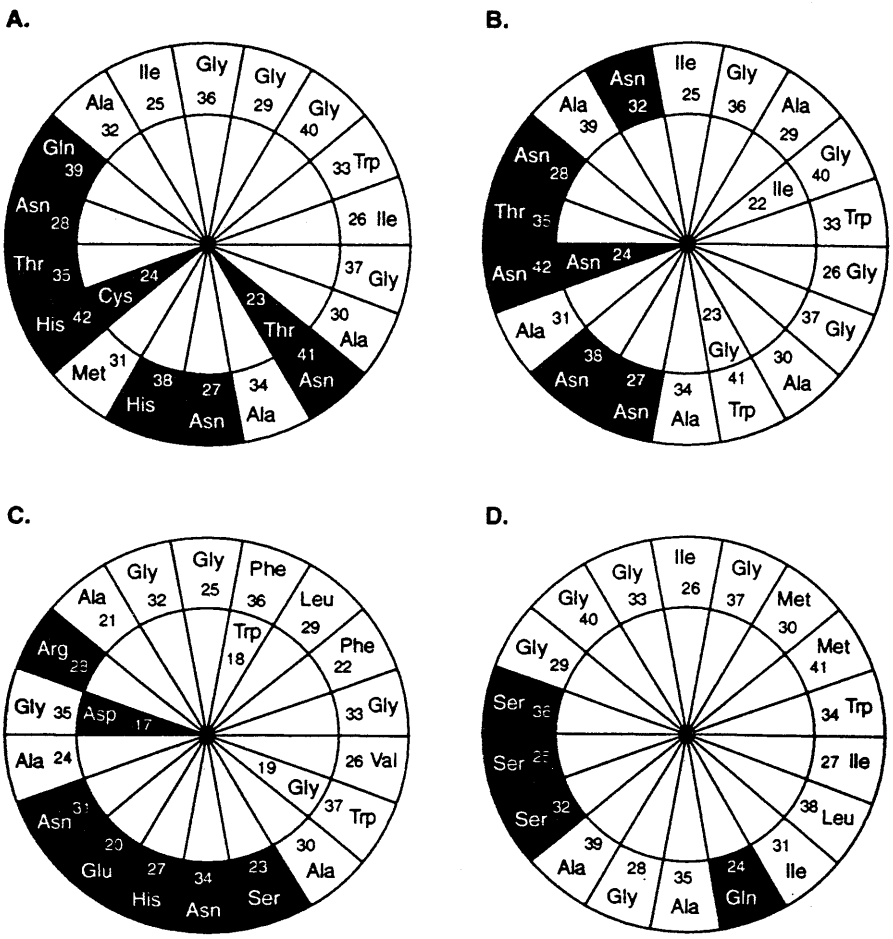

FIG. 6. Edmundson α-helical wheel representation of the C-terminal part of pediocin PA-1, from residues 23 to 42 (A), sakacin P, from residues 22 to 42 (B), leucocin A, from residues 17 to 37 (C), and curvacin A, from residues 24 to 41 (D). The black areas indicate polar residues, and the white areas indicate nonpolar residues.

amino acid sequencing. Other methods for obtaining large amounts of bacteriocins for structural and mode-of-action studies have disadvantages. Purifying the natural bacteriocins from producing strains in sufficient amounts is often time-consuming because of the low amounts produced. Moreover, it is our experience that a bacterial strain often produces several different bacteriocins or bacteriocin-like peptides which are difficult to separate completely from each other, which in turn makes it difficult to use natural isolates of a bacteriocin to determine its specificity or mode of action. Finally, natural bacteriocins are often more unstable than synthetic bacteriocins because of extracellular bacterial proteases. Synthetic leucocin A was stable for at least 12 months at 4°C, whereas purified natural leucocin A was reported to be quite unstable (3). Synthetic sakacin P was somewhat less stable than synthetic leucocin A but much more stable than natural sakacin P. The latter lost activity both during purification and after a few weeks of storage, while there was only about a 30% reduction in specific activity of the former after 6 months of storage. The reduction in activity of synthetic sakacin P after 6 months appeared to be due to the formation of a more hydrophilic peptide with an increased negative (or decreased positive) charge and an increase in the molecular mass by 3 ± 1.7 Da. Deamidation of one or more asparagine residues to aspartate residues in sakacin P may account for this. It is of interest to note that sakacin P contains asparagine residues at positions 24, 32, 38, and 42 that are not found in the other three bacteriocins (Fig. 1).

Both the natural and synthetic forms of curvacin A and pediocin PA-1 were transformed during storage to less active forms. These altered forms were somewhat more hydrophilic, as judged by reverse-phase chromatography, but their charge was unaltered as judged by capillary electrophoresis. The altered form of pediocin PA-1, termed pediocin PA-1-ox, had a molecular weight that was 16 greater than that of the unaltered form, and the specific activity was reduced by a factor of about 100. It is likely that the sulfur atom of methionine in pediocin PA-1 has become oxidized to sulfoxide in pediocin PA-1-ox, thus increasing the molecular weight of the polypeptide by 16 and at the same time making it somewhat more hydrophilic without altering its charge. The same is probably true for curvacin A, which was transformed into three different forms during storage, as would be expected because curvacin contains 2 methionine residues. Only one of these altered forms, curvacin A-ox, was active at concentrations assayed. Its specific activity was 1/10 of that of unaltered curvacin A, and its molecular mass was 16 Da greater. The molecular masses of the two other forms were not analyzed. We found similar alterations in other bacteriocins that contain methionine residues but not in bacteriocins lacking methionine. A similar observation was reported by Quadri et al. (10). The molecular mass of the methionine-containing bacteriocin, carnobacteriocin BM1

(and fragments of the bacteriocin containing the methionine residue), increased by 16 Da upon storage, and the altered form was less active by a factor of 4 to 8 (10). A similar alteration was not observed with carnobacteriocin B2, which lacks methionine (10). It is, consequently, preferable that methionine residues are not present in bacteriocins and new bacteriocin constructs designed for use as antibacterial additives.

The mass spectrometry analysis of the bacteriocins showed that all cysteine residues in sakacin P, curvacin A, leucocin A, pediocin PA-1, and the hybrid bacteriocins form disulfide bonds and that they form spontaneously in the synthetic peptides. Disulfide bonds between cysteine residues 9 and 14 in leucocin A and between residues 9 and 14 and 24 and 44 in pediocin PA-1 have been reported previously (3, 5). The corresponding cysteine residues also form a disulfide bond in carnobacteriocin BM1 but, surprisingly, not in carnobacteriocin B2 (10). Reduction of the disulfide bond in leucocin A suggested that it may not be essential for bacteriocin activity (3), despite the fact that two cysteine residues are present in the N-terminal half of all pediocin-like bacteriocins that have been characterized.

The fact that the bacteriocins produced by peptide synthesis were biologically active and had specific activities comparable to those of the natural forms indicates that the primary structures determined earlier for these bacteriocins are sufficient to induce toxicity. This is not a trivial point, since the bacteriocins may have had unidentified modifications required for toxicity. For instance, D-alanine was recently identified in the LAB bacteriocin lactocin S (11), and this type of modification is not readily detected by amino acid sequencing.

As expected, natural and synthetic bacteriocins had the same relative toxicities against various bacterial strains tested. The four pediocin-like bacteriocins, however, differed markedly in their relative toxicities against various bacterial strains, despite similar primary structures. The primary structures of these bacteriocins suggest that their polypeptide chains might be divided into at least two functional modules, the relatively well conserved N-terminal half of the peptide and the somewhat more diverse C-terminal half, which might be expected to be important in determining target cell specificity. This, in turn, suggests that new biologically active bacteriocin constructs may be obtained by interchanging corresponding modules from various pediocin-like bacteriocins and that the target cell specificity of the new constructs will be similar to that of the bacteriocin from which the C-terminal module was derived. Construction and characterization of hybrid bacteriocins where the first 21 to 22 N-terminal residues of one bacteriocin were combined with the remaining C-terminal region of another bacteriocin support this notion. All hybrid bacteriocins constructed had significant bactericidal activity: Ped-Sak, in fact, killed the target cells with similar efficiency as did the parental bacteriocins, pediocin PA-1 and sakacin P, whereas Sak-Ped, Cur-Sak, and Sak-Cur were 10 to 1,000 times less potent than the parent bacteriocins. Moreover, the relative sensitivities of the different target strains to the hybrid bacteriocins were similar to that to the bacteriocin from which the C-terminal half was derived and different from that to the bacteriocin from which the N-terminal half was derived. Thus, the C-terminal half is an important determinant of the target cell specificity of the pediocin-like bacteriocins and must directly or indirectly interact with an entity on the target cell. This entity might simply be a cell surface binding site recognized by the C-terminal part of the bacteriocin; increased binding to the cell surface could result in higher concentrations of bacteriocins in the vicinity of the membrane, in turn leading to greater pore formation and increased cytotoxicity. Alternatively, the entity might be a membrane component which directly or indirectly interacts with the C-terminal part of the bacteriocins and thereby makes the membrane more susceptible to permeabilization. The latter alternative is more likely because the C-terminal half is most probably the part of these bacteriocins which interacts with the membrane, judging from the primary structures. Whereas the N-terminal part is largely hydrophilic, the C-terminal part is relatively hydrophobic and becomes amphiphilic if it adapts an alpha-helical secondary structure (Fig. 6). The amphiphilic character is especially dominant for leucocin A, sakacin P, and pediocin PA-1, whereas curvacin A has a more dominant hydrophobic character. It would thus appear that the C-terminal part is both the membrane-interacting module of these bacteriocins and the main determinant of target cell specificity.

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