APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1994, p. 2158–2163
0099-2240/94/$04.00+0

Vol. 60, No. 6

# Detection, Purification, and Partial Characterization of Plantaricin C, a Bacteriocin Produced by a *Lactobacillus plantarum* Strain of Dairy Origin

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Received 26 October 1993/Accepted 28 February 1994

**A bacteriocin produced by *Lactobacillus plantarum* LL441 was selected from the inhibitory products of 75 mesophilic lactobacilli because of its potency and broad spectrum. It is a peptide of 3.5 kDa whose amino-terminal sequence is $NH_2$-K-K-T-K-K-N-X-S-G-D-I-. It is bactericidal and, in some cases, bacteriolytic. The peptide, called plantaricin C, retained its activity after boiling, storage, and treatment at different pHs.**

Lactic acid bacteria (LAB) are used in food fermentations, because they convert sugars into organic acids, thus improving the organoleptic and rheological properties of sugars, and also because they display inhibitory activities towards food spoilage microorganisms. Of the inhibitors produced by LAB, bacteriocins have attracted increasing interest. Bacteriocins are bactericidal proteins or peptides acting against related species (13), some of which are associated with food spoilage and foodborne illnesses (9, 15, 16). Thus, bacteriocins produced by starter cultures are excellent candidates to improve the quality and safety of various fermented foods. Up to now, nisin has been the most extensively studied bacteriocin and the only one permitted as a food preservative (4). The aim of this study was to assay a set of "wild" *Lactobacillus* strains for bacteriocin production in an attempt to find new molecular entities with improved spectra and/or stability over those previously described. As a result, we report the purification and partial characterization of plantaricin C, a bacteriocin produced by a *Lactobacillus plantarum* strain isolated from a ripening cheese.

**Screening of antimicrobial activities among wild lactobacilli.** Seventy-five *Lactobacillus* strains isolated from different sources, mainly fermentations in the absence of starter cultures (Table 1), were examined for cross-antagonistic activities, using the agar spot test essentially as described by Fleming et al. (6). To reduce acid production, MRS medium (Biokar) with a low glucose content (0.2%) was used, and to avoid formation of $H_2O_2$, anaerobic incubation was used. A total of 10 strains (nine *L. plantarum* and one *L. sake* strain) were inhibitory to some of the lactobacilli used as indicators (Table 2). When cell-free culture supernatants of these strains (adjusted to pH 6.5 and treated with catalase) were checked by the agar well diffusion assay (18), only those corresponding to three strains of *L. plantarum* (NCDO 1193, C3.8, and LL441) produced zones of inhibition. The three antagonistic activities were sensitive to proteases, indicating that they were due to bacteriocins. The bacteriocin produced by *L. plantarum* LL441, a strain isolated from Cabrales cheese, was chosen for further characterization on the basis of (i) its stability in the production medium, (ii) its broad spectrum of activity, (iii) its high potency, and (iv) the ease with which the producer organism could be grown. It was named plantaricin C.

**Plantaricin C production.** Plantaricin C production was assayed in CM (7), TGE (2), and MRS broths at 28, 30, and 37°C, aerobically and anaerobically, with and without agitation, and with different glucose concentrations. Maximal production was obtained in MRS broth containing at least 0.6% glucose; the rest of the conditions were irrelevant. Consequently, standard culture conditions were set as follows: MRS broth and aerobic incubation at 30°C and with gentle agitation. To quantify bacteriocin activity, serial twofold dilutions were assayed by the agar well diffusion test. The titer was defined as the reciprocal of the highest dilution showing definite inhibition of the indicator lawn and was expressed in arbitrary activity units per milliliter. Unless otherwise stated, *L. sake* CECT 906 was used as the indicator organism. Plantaricin C was produced during the exponential growth phase. The inhibitory activity was maximal at the beginning of the stationary phase and remained stable long after growth had ceased, even in the presence of the producer cells (Fig. 1). Stability in the production medium has been reported for pediocin SJ-1 (19) and reutericin 6 (20). In contrast, some studies have reported substantial losses of bacteriocin activity upon prolonged incubation of the cultures (1, 10).

**Purification and sequence analysis of plantaricin C.** Plantaricin C formed aggregates in excess of 100 kDa in culture supernatants; these were resolved by treatment with 7 M urea or 0.1% sodium dodecyl sulfate (SDS), or simply by boiling, without apparent loss of activity. Consequently, for purification purposes, ultrafiltered MRS broth (obtained by passage of MRS broth through a filter of 10-kDa cutoff) was used, since it supported plantaricin C production to a level comparably to standard MRS broth. The purification scheme is presented in Table 3. The bacteriocin was precipitated from culture supernatants, adjusted to pH 6.5, by addition of ammonium sulfate to 55% (wt/vol) saturation. After centrifugation, the precipitate was dissolved in 25% acetonitrile in water containing 0.1% (vol/vol) trifluoroacetic acid and applied to a $C_8$ hydrophobic interaction column (Mega Bond Elut; Varian) equilibrated with the same diluent. The column was extensively washed, and the bacteriocin was eluted with 50 ml of 50% acetonitrile–0.1% trifluoroacetic acid in water. The active fractions were pooled, concentrated, and applied to a fast protein liquid chromatography Mono S cation-exchange column (Pharmacia) equili-

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TABLE 1. Strains of *Lactobacillus* spp. examined for antagonistic activities

| Origin | Organism | No. of strains |
|---|---|---|
| ATCC[a] | *L. helveticus* | 1 |
| | *L. casei* | 2 |
| Majorero cheese (Canary Islands)[b] | *L. plantarum* | 2 |
| | *L. casei* | 1 |
| NCDO[c] | *L. plantarum* | 1 |
| La Serena cheese (Cáceres)[d] | *L. plantarum* | 1 |
| | *L. acidophilus* | 1 |
| | *L. brevis* | 3 |
| Cabrales cheese (Asturias)[e] | *L. plantarum* | 4 |
| Gamonedo cheese (Asturias)[f] | *L. plantarum* | 10 |
| | *L. casei* | 12 |
| | *L. brevis* | 2 |
| Montilla-Moriles wine (Córdoba)[g] | *L. brevis* | 4 |
| | *L. hilgardii* | 6 |
| Utiel-Requena wine (Valencia)[h] | *L. plantarum* | 10 |
| CECT[i] | *L. bavaricus* | 1 |
| | *L. collinoides* | 1 |
| | *L. fermentum* | 1 |
| | *L. helveticus* | 1 |
| | *L. sake* | 1 |
| | *L. viridescens* | 1 |
| | *L. hilgardii* | 1 |
| | *L. brevis* | 4 |
| | *L. acidophilus* | 1 |
| Other sources | *Lactobacillus* spp. | 3 |

[a] ATCC, American Type Culture Collection.
[b] IFPL, Instituto del Frío-Productos Lácteos (CSIC, Madrid, Spain).
[c] NCDO, National Collection of Dairy Organisms.
[d] Supplied by Germán Larriba (Universidad de Badajoz, Badajoz, Spain).
[e] Laboratory collection.
[f] Supplied by Ana Rodríguez (Instituto de Productos Lácteos de Asturias, Villaviciosa, Spain).
[g] Supplied by Enrique Sancho (Universidad de Córdoba, Córdoba, Spain).
[h] Supplied by Federico Uruburu (Universidad de Valencia, Valencia, Spain).
[i] Colección Española.

brated with 0.02 M ammonium acetate buffer, pH 5.3. The column was washed until the $A_{280}$ returned to zero, and the bacteriocin was eluted with a linearly increasing NaCl gradient (0 to 1 M) in the same buffer. The activity coeluted with a single peak of $A_{280}$ at about 0.5 M NaCl (Fig. 2). The purity of plantaricin C was confirmed by polyacrylamide gel electrophoresis (PAGE), in the presence of 0.1% SDS (14), of the dialyzed fractions corresponding to this peak. The fractions showed a single band that migrated to a position corresponding to a peptide of ca. 3,500 Da (Fig. 3A). This band corresponded to a zone of inhibition of the growth of *L. sake* CECT 906 used in the bioautography experiment indicated in Fig. 3B. Further confirmation of the purity of plantaricin C was obtained through its amino-terminal sequencing by automated Edman degradation (5). The sequence obtained was as follows: $NH_2$-Lys-Lys-Thr-Lys-Lys-Asn-Xaa-Ser-Gly-Asp-Ile-, where Xaa represents an unidentified residue. After the 11th amino acid, the sequence was blocked. A simple explanation for this could be that a disulfide bond was established between the amino acid in position 12 and another one located further down in the sequence, as was observed by Hastings et al. (8). Alternatively, it might indicate that plantaricin C is a new member of the lantibiotic family, a family subjected to posttranslational modifications that result in the presence of unusual amino acids, such as lanthionine and dehydrobutyrine. Lanthionine results in blank cycles during sequencing by Edman degradation, while dehydrobutyrine produces blockage of the reaction (12). If further studies confirm this, plantaricin C would be the first lantibiotic produced by a strain of *L. plantarum*. No homology to the N-terminal sequence of plantaricin C was found in the

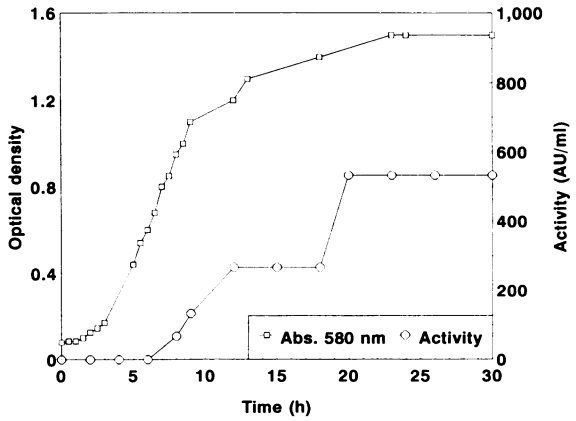


FIG. 1. Growth of *L. plantarum* LL441 and production of plantaricin C in MRS broth at 30°C without pH regulation of the cultures. At different time intervals, the optical density at 580 nm and bacteriocin activity against *L. sake* CECT 906 were determined. AU, activity units.

TABLE 2. Strains of *Lactobacillus* spp. showing antagonistic activities by cross-reactions when tested in solid medium

| Producer strain | Source | No. of sensitive strains | % Sensitive strains | Activity[a] |
|---|---|---|---|---|
| *L. plantarum* LL441 | Cabrales cheese | 30 | 56.6 | +++ |
| *L. plantarum* 300 | Gamonedo cheese | 34 | 64.1 | + |
| *L. plantarum* 166 | Gamonedo cheese | 38 | 71.1 | ++ |
| *L. plantarum* 181 | Gamonedo cheese | 35 | 66.0 | ++ |
| *L. plantarum* 185 | Gamonedo cheese | 23 | 43.4 | ++ |
| *L. plantarum* 450 | Gamonedo cheese | 38 | 71.7 | ++ |
| *L. plantarum* C3.8 | La Serena cheese | 26 | 49.0 | ++ |
| *L. plantarum* CECT 749 | Sauerkraut | 20 | 38.5 | + |
| *L. plantarum* NCDO 1193 | NCDO[b] | 43 | 81.1 | +++ |
| *L. sake* CECT 906 | Sake | 5 | 9.4 | + |

[a] Relative activity as judged from the mean values of the inhibition halos.
[b] National Collection of Dairy Organisms.

TABLE 3. Purification of plantaricin C

| Purification stage | Total $A_{280}$ | Total activity (AU)[a] | Sp act | Yield (%) | Fold purification |
|---|---|---|---|---|---|
| Supernatant | 24,000.0 | 53,400 | 2.2 | 100.0 | 1.0 |
| Ammonium sulfate precipitation (fraction 1) | 1,424.0 | 53,325 | 37.5 | 99.8 | 16.8 |
| Hydrophobic interaction (fraction 2) | 127.5 | 38,403 | 301.2 | 71.9 | 135.3 |
| Cation exchange (fraction 3) | 3.2 | 25,848 | 8,077.5 | 48.4 | 3,630.3 |

[a] Determined by the critical dilution method. AU, activity units.

SWISS-PROT data bank. In conclusion, it seems that plantaricin C is a small peptide and quite hydrophobic, as judged by its high affinity to $C_8$ columns and its tendency to aggregate in aqueous solvents. Furthermore, it adsorbs strongly to a cation-exchange column at pH 5.3, suggesting that we are dealing with a basic peptide with an isoelectric point in the alkaline range.

**Sensitivity to heat, pH, and proteolytic enzymes.** Plantaricin C activity was completely lost upon treatment with pronase, trypsin, and α-chymotrypsin (1 mg/ml) but was not affected by other proteases, such as pepsin and proteinase K, or by α-amylase or lipase. It is very stable under a series of different conditions such as storage at room temperature, 4°C, and −20°C, treatment with organic solvents (methanol, chloroform, and acetonitrile), and heating (100°C for 60 min or 121°C for 10 min). Bacteriocin activity was found to be most stable at acid and neutral pHs. At alkaline pH, plantaricin C is not active and becomes gradually inactivated, but its activity is partially restored upon reversion to acid pH. Heat stability is shared by several LAB bacteriocins of relatively small size (9, 10, 18), although in some cases it is lost after purification (1, 3). Temperature stability is very convenient if the bacteriocin is to be used as a food preservative, because many processing procedures involve a heating step, and cold is one of the most popular preservation procedures. Furthermore, activity at neutral pH constitutes an advantage over other bacteriocins used as food preservatives and particularly over nisin, whose maximal solubility and stability are at pH 2, with these parameters decreasing significantly as the pH increases (9). This is a considerable disadvantage for its use as an additive in nonacidic foods (cheeses during ripening, canned foods, etc).

**Mode of action.** The viability of exponentially growing cultures of *L. sake* CECT 906 was reduced by 30% in 5 min and by up to 50% in 60 min after treatment with plantaricin C, although no reduction in absorbance was observed (Fig. 4A and B). This indicates a bactericidal mode of action of the bacteriocin that occurred in the absence of concomitant or subsequent cell lysis. In contrast, cultures of *L. fermentum* LMB 13554 were much more sensitive; viable counts dropped to 0.6% in the first 5 min after addition of plantaricin C, which was accompanied by a drastic lowering in the optical density, indicating a bacteriolytic mode of action in this case (Fig. 4C and D). Further incubation of the cells with the bacteriocin resulted in a further decrease of viable counts. Survivors obtained after 24 h of incubation were as sensitive as the parental strain, indicating that no selection of bacteriocin-resistant mutants had occurred. Similar experiments with the other three strains, chosen because of their high sensitivity to plantaricin C, gave the following results: complete lysis in the case of *L. delbrueckii* subsp. *bulgaricus* LMG 13551 and a high viability reduction (>99.75%), without apparent decrease in optical density, for *L. helveticus* LMG 13555 and *Leuconostoc mesenteroides* subsp. *cremoris* NCDO 543. The lytic effect of plantaricin C is not observed with most bacteriocins produced by LAB (13, 16) and could be potentially useful in accelerated food processing through the release of the enzymes contained in the starters into their substrates.

A deeper insight into the mode of action of plantaricin C was obtained through the following experiment. Exponentially

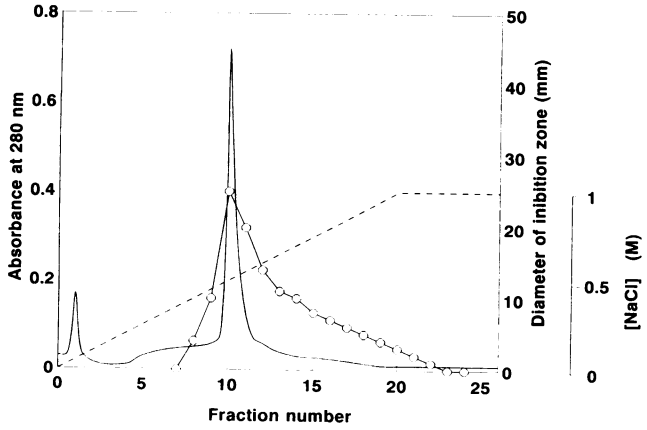


FIG. 2. Elution of plantaricin C from a Mono S cation-exchange column equilibrated with 20 mM ammonium acetate buffer (pH 5.3). Plantaricin C was eluted with a linear gradient of 0 to 1 M NaCl in the same buffer (– – –), collecting 1-ml fractions. The $A_{280}$ was monitored (——), and plantaricin C activity was determined by the agar well diffusion method (○) (18).

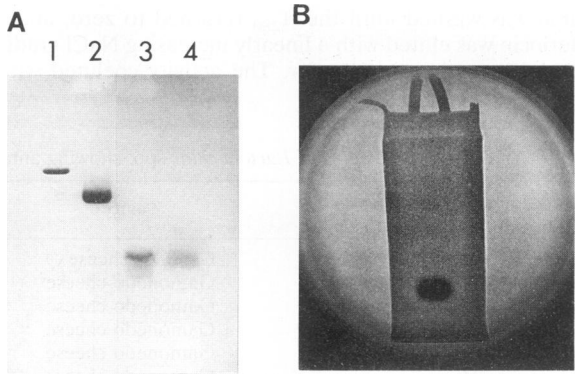


FIG. 3. SDS-PAGE of plantaricin C and detection of antimicrobial activity. (A) Coomassie blue-stained gel: lane 1, trypsin inhibitor (20,100); lane 2, cytochrome *c* (12,500); lane 3, purified plantaricin C after cation-exchange chromatography; lane 4, insulin β chain (3,400). (B) Washed gel overlaid with MRS seeded with *L. sake* CECT 906 and incubated overnight.

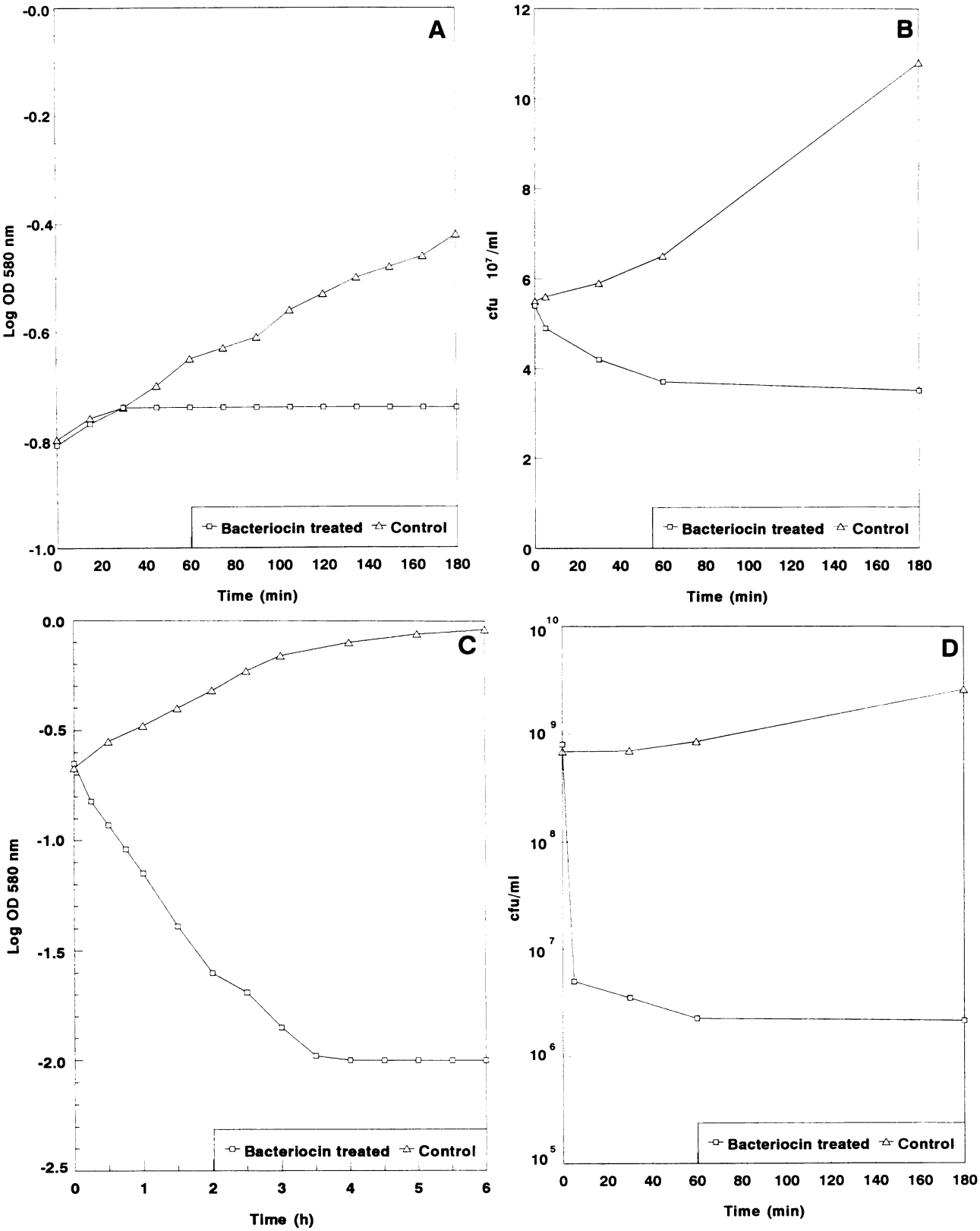


FIG. 4. Activity of plantaricin C against *L. sake* CECT 906 (A and B) and *L. fermentum* LMG 13554 (C and D). Partially purified bacteriocin was added to cultures of the indicator strains, at a final concentration of 20 activity units per ml, at time zero. (A and C) $A_{580}$; (B and D) viable count (CFU per milliliter). OD, optical density.

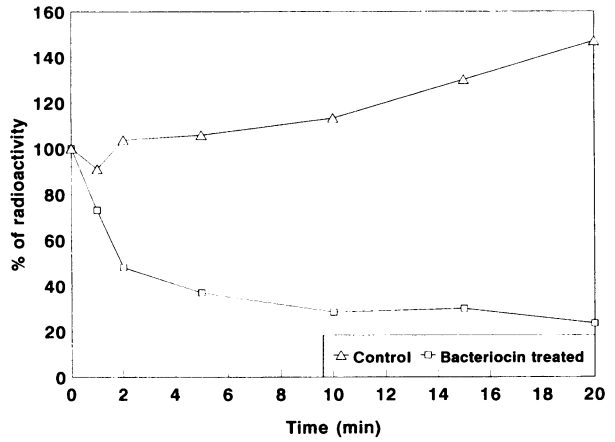


FIG. 5. Effect of plantaricin C on plasma membrane integrity. Cells of *L. sake* CECT 906, which had incorporated [$^3$H]uridine after inhibition of RNA synthesis, were treated with plantaricin C (20 activity units per ml), and the release of the labelled compound was monitored.

growing cultures of *L. sake* CECT 906 were treated with 50 μg of rifampin per ml (this concentration abolished RNA synthesis completely) followed by the addition, 5 min later, of 1 μCi of [$^3$H]uridine (1.3 to 1.8 TBq) per ml. After 15 min of incubation, a plateau in the incorporated radioactivity was reached. Plantaricin C addition to the cultures resulted in fast and complete leakage of uridine, suggesting that the primary effect of the bacteriocin on sensitive cells could be the formation of pores in the plasma membrane (Fig. 5). This result explained previous data on inhibition of DNA and RNA syntheses upon addition of the bacteriocin to sensitive cultures (data not shown). Since this mode of action does not imply bacterial lysis, and since the lytic effect, when observed, is extremely fast, it is possible that in some cases a secondary effect of plantaricin C might be the deregulation of the autolytic system of the sensitive cells.

**Spectrum of inhibitory activity.** The antagonistic effect of plantaricin C on various gram-positive and gram-negative bacteria (Table 4) was tested. This bacteriocin shows a reasonably wide spectrum of activity that includes not only LAB but also a range of other gram-positive microorganisms. Among the sensitive strains, there are natural competitors of *L. plantarum* (lactobacilli, leuconostocs, pediococci, and *Streptococcus thermophilus*) as well as some food spoilage bacteria (*Enterococcus faecalis*, *Propionibacterium* spp., and *Clostridium tyrobutyricum*). Inhibition of spore outgrowth was not tested for *Bacillus* and *Clostridium* spp.; rather, just inhibition of growth of vegetative cells was tested. None of the gram-negative bacteria tested were inhibited by the bacteriocin. From the standpoint of its inhibitory spectrum, plantaricin C appears to take an intermediate position between the lantibiotic nisin, which inhibits most gram-positive bacteria (9), and several bacteriocins from *Lactobacillus* spp. such as lactacin B (1), helveticin J (11), and caseicin 80 (17), whose activity spectra are rather narrow and include only strains belonging to the genus.

In conclusion, plantaricin C is a new bacteriocin that is very resistant to environmental conditions, which suggests the lack of a tertiary structure, and that is active against a wide range of gram-positive bacteria. Presumably, it opens pores in plasma membranes, which offers some potential applicability in food preservation, although this must be tested through pilot fermentations under controlled conditions.

TABLE 4. Inhibitory spectrum of plantaricin C

| Indicator strain[a] | Activity (diam, mm)[b] |
|---|---|
| *Lactobacillus acidophilus* LMG 13550 | 10 |
| *Lactobacillus bulgaricus* LMG 13551 | 23 |
| *Lactobacillus casei* LMG 13552 | 0 |
| *Lactobacillus curvatus* LMG 13553 | 8 |
| *Lactobacillus fermentum* LMG 13554 | 20 |
| *Lactobacillus helveticus* LMG 13555 | 20 |
| *Lactobacillus plantarum* LMG 13556 | 0 |
| *Lactobacillus reuteri* LMG 13557 | 8 |
| *Lactobacillus sake* CECT 906 | 22 |
| *Lactobacillus sake* LMG 13558 | 0 |
| *Lactobacillus salivarius* LMG 13559 | 14 |
| *Pediococcus pentosaceus* LMG 13560 | 10 |
| *Pediococcus pentosaceus* LMG 13561 | 8 |
| *Leuconostoc cremoris* LMG 13562 | 8 |
| *Leuconostoc cremoris* NCDO 543 | 16 |
| *Propionibacterium acidipropionici* LMG 13572 | 8 |
| *Propionibacterium* sp. LMG 13573 | 0 |
| *Propionibacterium* sp. LMG 13574 | 0 |
| *Lactococcus cremoris* LMG 13563 | 18 |
| *Streptococcus thermophilus* LMG 13564 | 18 |
| *Streptococcus thermophilus* LMG 13565 | 18 |
| *Enterococcus faecalis* LMG 13566 | 10 |
| *Staphylococcus carnosus* LMG 13567 | 10 |
| *Listeria innocua* LMG 13568 | 0 |
| *Bacillus cereus* LMG 13569 | 0 |
| *Bacillus subtilis* OG 1[c] | 12 |
| *Bacillus subtilis* BD 630[c] | 10 |
| *Clostridium sporogenes* LMG 13570 | 13 |
| *Clostridium tyrobutyricum* CT 3.5[d] | 23 |
| *Escherichia coli* ESS[d] | 0 |
| *Pseudomonas aeruginosa* PAO[d] | 0 |
| *Serratia marcescens* 10171[d] | 0 |
| *Enterobacter aerogenes* 196[d] | 0 |
| *Salmonella paratyphi* 777[d] | 0 |
| *Citrobacter* spp.[d] | 0 |
| *Klebsiella* spp.[d] | 0 |

[a] The bacteria referenced as LMG belong to the culture collection of the University of Ghent, Ghent, Belgium.
[b] Diameter of the inhibition zones caused by 7 μg of purified plantaricin C.
[c] Supplied by G. Venema (University of Groningen, Groningen, The Netherlands).
[d] Laboratory collection.

We thank C. L. Otín for the N-terminal sequencing of plantaricin C.
This work was supported by grant BIOT-CT91-0263 of the BRIDGE Programme from the European Community and grant ALI90-0233 from DGICYT of the Spanish Ministry of Education. B.G. and B.M. were the recipients of a predoctoral and a postdoctoral fellowship, respectively, from the Spanish Ministry of Education. P.A. was the recipient of a grant from F.I.C.Y.T.

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