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Partial purification and characterization of the bacteriocin produced by *Propionibacterium jensenii* B1264

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Abstract — Thirteen dairy propionibacteria were screened for catalase-insensitive, protease-sensitive inhibition of various Gram-positive and Gram-negative bacteria. Production of bacteriocin-like agents was identified in 8 of these cultures. The bacteriocin produced by *Propionibacterium jensenii* B1264 inhibited closely related propionibacteria and lactic acid bacteria and was bactericidal to *Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797. It was produced in sodium lactate broth cultures during stationary phase; maximum activity was detected after 10 d of growth but only after 10-fold concentration of culture supernatants. The bacteriocin was precipated by ammonium sulphate at 70 % saturation. The ammonium sulphate-concentrated, dialyzed bacteriocin was stable to 0.1–1.0 mol·L^{-1} NaCl, 0.1–2.0 % SDS, 4 mol·L^{-1} urea, 100 °C (60 min), pH 2–10 and organic solvents (methanol, ethanol and isopropanol at 0–50 %). Batch anion exchange chromatography (pH 6.5) of the bacteriocin yielded 101 % activity and 15-fold increased purity. Flat bed analytical isoelectric focussing showed that the isoelectric point was between pH 3 and 3.5. SDS-PAGE yielded an estimated molecular mass between 6 and 9 kDa. The evidence suggests that this anionic bacteriocin is unique.

***Propionibacterium jensenii* / bacteriocin / propionibacteria**

Résumé — Purification partielle et caractérisation de la bactériocine produite par *Propionibacterium jensenii* B1264. Treize bactéries propioniques ont été examinées pour leur capacité catalase-insensible et protéase-sensible à inhiber différentes bactéries gram-positives ou gram-négatives. La production d'agents de type bactériocine a été identifiée dans huit des cultures. La bactériocine produite par *Propionibacterium jensenii* B1264 avait une activité inhibitrice sur les propionibactéries

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proches et des bactéries lactiques et une activité bactéricide envers *Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797. Elle était produite en bouillon de lactate de sodium pendant la phase stationnaire ; l'activité maximale était détectée après 10 j mais seulement avec une concentration par dix de surnageants. La bactériocine était précipitée au sulphate d'ammonium à une saturation à 70 %. La bactériocine concentrée au sulfate d'ammonium et dialysée était stable de 0,1–1,0 mol·L^{-1} de NaCl, 0,1 % à 2,0 % SDS, 4 mol·L^{-1} urée, 100 °C (60 min), de pH 2 à 10 et dans les solvants organiques (méthanol, éthanol et isopropanol à 0 à 50 %). La chromatographie d'échange d'anions en batch (pH 6,5) de la bactériocine permettait d'obtenir une activité et une pureté accrue par un facteur de quinze. La focalisation isoélectrique analytique en couche mince donnait un point isoélectrique entre pH 3 et 3,5. La masse moléculaire obtenue par SDS-PAGE se situait entre 6 et 9 kDa. Les données suggèrent que cette bactériocine anionique est unique. © Inra/Elsevier, Paris.

***Propionibacterium jensenii* / bactériocine / propionibactérie**

1. INTRODUCTION

Dairy or classical propionibacteria include *Propionibacterium freudenreichii*, *P. thoenii*, *P. acidipropionici* and *P. jensenii*, and have been isolated from cheese, milk products, soil, olives and silage fermentations [3]. Their industrial importance lies in their production of Swiss cheese flavor and 'eyes', vitamin B_{12} and inhibitors, including propionic acid and bacteriocins (antimicrobial proteins).

Only two dairy propionibacteria bacteriocins have been characterized to date. Propionicin PLG-1 is produced by *P. thoenii* P127 [11, 12] and inhibits Gram-positive bacteria including *P. thoenii*, *P. acidipropionici*, *P. jensenii* and lactic acid bacteria, Gram-negative bacteria (*Pseudomonas*, *Vibrio,* and *Campylobacter* spp. *and Escherichia coli*), and selected yeasts and molds [11]. It is sensitive to protease, pronase E, pepsin, trypsin, and α-chymotrypsin, insensitive to catalase, and lipase but stable from pH 3 to 9 and to temperatures ≤ 85 °C. The bacteriocin is produced in late stationary phase in agar and broth cultures [12]. Its production has been examined in both batch [9, 12] and fed-batch [16] fermentations; five times more activity is produced in cultures grown at pH 7.0 in a 3:1 beet molasses:corn steep liquor medium than in sodium lactate broth. Propionicin PLG-1 is bactericidal to *P. acidipropionici* P5 without causing cell lysis; it kills 99.6 % of indicator cells within 60 min [12]. Lyon and Glatz [12] purified propionicin PLG-1 to homogeneity by sequential ammonium sulphate precipitations first at 40 % and then at 60 %, ion exchange chromatography on carboxymethyl cellulose and preparative isoelectric focussing. SDS-PAGE analysis of the purified bacteriocin yielded a single 10-kDa band. In preparation for N-terminal sequencing, Paik and Glatz [15] obtained larger quantities of propionicin PLG-1 by ammonium sulphate precipitation, ion exchange chromatography, and reversed-phase high-performance liquid chromatography on a C_{18} column. Composition analysis yielded a molecular mass of 9 328 Da and a high proportion (42 %) of hydrophobic amino acids [15].

Jenseniin G is a bacteriocin produced by *P. thoenii* (*jensenii*) P126 [6]. It inhibits dairy propionibacteria including *P. jensenii* and *P. acidipropionici*, *Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797, *Lb. helveticus*, *Lactococcus lactis* subsp. *cremoris* and *lactis* [6], several strains of *Lb. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* [19] and *Clostridium botulinum* types A, B, and E spores (unpublished data). It is sensitive to proteinase K, pronase E, type 14 protease and insensitive to catalase. Unlike propionicin PLG-1 [11],

jenseniin G is stable at 100 °C for 15 min and to pH values between 3 and 12. Jenseniin G is bacteriostatic to *P. acidipropionici* P5 and bactericidal to *Lb. delbrueckii* subsp. *lactis* ATCC 4797. Activity is detected only in 50-to 100-fold concentrated 10-d old culture supernatants [6].

Because only two bacteriocins have been confirmatively identified in dairy propionibacteria, we examined 13 additional cultures for catalase-insensitive and protease-sensitive inhibition. One culture *P. jensenii* B1264 was selected for further study. Characteristics identified for the responsible antagonist were consistent with its classification as a bacteriocin.

2. MATERIALS AND METHODS

2.1. Bacterial cultures and media

Propionibacterium species B1262, B1263, B1264, B1265, B1267 and B1268 were obtained from the All Union Collection of Microorganisms, Pushchino, Moscow Region, Russia. *P. thoenii* P127, *P. thoenii* (*jensenii*) P126 and *P. acidipropionici* P5 were graciously provided by Dr B.A. Glatz, Iowa State University, Ames, USA. *Lactobacillus plantarum* strains 965, MOP 3 and MOP 7 were provided by Dr M. Daeschel, North Carolina State University, Raleigh, USA. *Lb. plantarum* CSCC 2901 was obtained from Commonwealth Scientific and Industrial Research Organisms, Victoria, Australia. *P. shermanii* strains MNS and KFA were obtained from Dr V.L. Crow, New Zealand Dairy Research Institute. *P. acidipropionici* CRL 756 and *P. freudenreichii* subsp. *shermanii* CRL 757 were obtained from Dr G. Oliver, National University of Tucuman, Argentina. *P. jensenii* ATCC 4868, *P. freudenreichii* ATCC 6207 and all other cultures were from the Clemson University Food Microbiology Culture Collection, USA.

Dairy propionibacteria were propagated (1 % v/v inoculum) in sodium lactate broth (NLB), consisting of 1 % w/v trypticase soy broth without dextrose (BBL Microbiology Systems, Cockeysville, MD, USA), 1 % w/v yeast extract (Difco Laboratories, Detroit, MI, USA) and 1 % sodium lactate syrup (60 % w/w, Fisher Scientific Co., Pittsburgh, PA, USA) in distilled water [7] at 32 °C under flowing CO_2 (0.4 $L \cdot h^{-1}$). Sodium lactate agar (NLA) and soft NLA were prepared by adding 1.8 % w/v and 0.75 % w/v agar (BBL) to NLB, respectively. *Lb. delbrueckii* subsp. *lactis* ATCC 4797 and other lactobacilli were propagated in Lactobacilli MRS broth (MRS; Difco) for 16 h at 37 °C under flowing CO_2 (0.4 $L \cdot h^{-1}$). Indicator lactococci were propagated at 32 °C in M-17 broth (BBL). Agar media other than NLA contained 1.5 % w/v agar; soft agars contained 0.75 % agar w/v. Populations of lactobacilli were determined by plating on MRS agar (37 °C, 48 h); propionibacteria were enumerated by plating on NLA (32 °C, 5 d). Frozen stock cultures were maintained at –70 °C in the appropriate growth medium containing 10 % glycerol. Working cultures were prepared from stock cultures by propagating twice in the appropriate growth medium. Lactobacilli [14] and propionibacteria [4] were identified by biochemical tests and purity was assessed by streaking and microscopic observation.

2.2. Bacteriocin activity and detection assays

Activity was determined by a critical point dilution method [1]. Pre-poured MRS agar plates were overlaid with 5 mL of soft MRS agar, seeded with 10 µL of a 16 to 24 h MRS broth culture of *Lb. delbrueckii* subsp. *lactis* ATCC 4797 (final population, 10^5 to 10^6 cfu·L^{-1}) and 10 µL aliquots of serial two-fold dilutions of bacteriocin were applied. Activity was defined as the reciprocal of the highest dilution showing zones of inhibition and was expressed in activity units (AU) per millilitre [8]. Activity was also detected by spotting 10-µL aliquots of putative bacteriocin preparations on the lawns.

2.3. Preparation of crude bacteriocin

P. jensenii B1264 (1 % inoculum) was added to NLB (16 L) and held at 32 °C for 10 d under flowing CO_2 (0.4 $L \cdot h^{-1}$). Cells were removed by centrifugation at 6 300 *g* for 30 min at 4 °C followed by filtration through a 0.45-mm pore-size membrane filter (Gelman, Ann Arbor, MI, USA). Ammonium sulphate was added slowly with constant stirring to filtered supernatants to 70 % or 80 % saturation and held overnight at 4 °C. Pelleted proteins were centrifuged at 19 600 *g* for 30 min at 4 °C, resuspended in 25 mL 0.1 mol·L^{-1} sodium phosphate buffer (pH 6.4)

and dialyzed against the same buffer (4 changes, 4 L each, 12 h per dialysis) in 3 500 molecular-weight-cut-off (MWCO) dialysis tubing (Spectra-Por number 3, Spectrum Medical Industries, Los Angeles, CA, USA).

2.4. Stability tests

Urea was added to crude bacteriocin (pH 6.5) or partially purified bacteriocin in sodium phosphate buffer (0.1 mol·L^{-1}, pH 6.0) to 4 mol·L^{-1} final concentration and held for 0, 30, 60, 120, 180 or 240 min at 20 to 25 °C. Crude bacteriocin (pH 6.5) was treated by holding in a water-bath at 100 °C for 0, 15, 30, 45, 60 or 120 min. SDS was added to crude bacteriocin (pH 6.5) or partially purified bacteriocin in sodium phosphate buffer (0.1 mol·L^{-1}, pH 6.0) to final concentrations of 0, 0.1, 0.4, 0.6, 0.8, 1.0 and 2.0 %. Activity of preparations was detected using lawns of the indicator *Lb. delbrueckii* subsp. *lactis* ATCC 4797.

2.5. Analytical isoelectric focussing

Analytical isoelectric focussing [5] was conducted using a pH 3 to 7 gradient agarose gel (FMC BioProducts, Rockland, ME, USA). Focussing was at 10 W constant power, 1 500 V maximum for 45 min. Focussed gels were halved. One half was overlaid with soft MRS agar containing the indicator strain, *Lb. delbrueckii* subsp. *lactis* ATCC 4797, incubated for 24 h and examined for inhibition. The other half was silver-stained per the manufacturer's instructions (Bio-Rad Laboratories, Richmond, CA, USA).

2.6. Batch anion exchange chromatography

The anion exchanger, Bio-Rad Macro-Prep Q (typically 6-mL packed wet resin) was prepared by washing five times with 0.1 mol·L^{-1} sodium phosphate buffer (pH 6.0), centrifuging at 6 300 *g* for 10 min and decanting as supernatants. Crude jenseniin P (typically 1 mL, 800 AU·mL^{-1}) was dialyzed (24 h at 4 °C) in 3 500 MWCO tubing against the same buffer, mixed with the resin and held with gentle stirring for 30 min. The resin and adsorbed protein were washed repeatedly (5 to 6 times) with 0.1 mol·L^{-1} sodium phosphate buffer containing 0.1 mol·L^{-1} NaCl. All washings were combined, concentrated by speed vacuum and assayed for activity and protein.

2.7. SDS-PAGE analysis and protein assays

Electrophoresis was performed on 4 to 20 % gradient SDS-PAGE gels (Bio-Rad Ready gels) using the protocol of Laemmli [10] at constant current (200 mA) for 60 min. Half of the gel was washed in distilled water for 60 min and overlaid with soft MRS agar (10 mL^{-1}) containing the indicator strain, *Lb. delbrueckii* subsp. *lactis* ATCC 4797. The other half was fixed and silver-stained. Protein was determined by the Bradford method [2] per the manufacturer's instructions (Bio-Rad) using bovine serum albumin as standard or by ultraviolet absorption at 280 nm [17] using serum immunoglobulin G as standard.

3. RESULTS

3.1. Identification of bacteriocin activity

Thirteen dairy propionibacteria were examined for bacteriocin production. Eight cultures, *Propionibacterium* strains B1262, B1263, B1264, B1265, B1267 and B1268, *P. jensenii* ATCC 4868 and *P. freudenreichii* ATCC 6207 showed catalase-insensitive, protease-sensitive antagonism. Inhibitory activity was limited to lactobacilli, lactococci, and some propionibacteria (*table I*). *P. acidipropionici* ATCC 25562 and *P. freudenreichii* subsp. *shermanii* strains CRL 756, CRL 757, MNS and KFA did not elicit protease-sensitive inhibition of the indicator strains. No propionibacteria produced bacteriocin-like activity against 15 Gram-positive and 14 Gram-negative bacteria including staphylococci, *Bacillus cereus*, *Listeria monocytogenes*, *Escherichia coli*, *Salmonella typhimurium* and *Vibrio parahaemolyticus*.

An unusual pattern of inhibition was noted for the antagonists produced by *P. jensenii* B1264, *P. jensenii* ATCC 4868 and *P. freudenreichii* ATCC 6207 in NLA

Table I. Bacteriocin-like inhibition by dairy propionibacteria.

Tableau I. Inhibition de type bactériocine par les bactéries propioniques laitières.

Indicator strains[b]	Putative producer propionibacteria[a]							
	B1264	B1262	B1263	B1265	B1267	B1268	ATCC 4868	ATCC 6207
Propionibacterium jensenii B1264	yes[c]	no	yes	no	no	no		
P. acidipropionici P5	yes	no	yes	no	yes	no	no	yes*
P. thoenii (*jensenii*) P126	yes	no	yes	yes	no	no	no	no
P. thoenii P127	yes	no	yes	no	no	no	no	no
Lactobacillus acidophilus ATCC 4356	yes	no	no	no	no	no		
Lb. casei ATCC 393	no	yes	no	yes	no	no	yes*	yes*
Lb. casei 7469	no	no	no	yes	no	no		
Lb. delbrueckii subsp. *delbrueckii* ATCC 9649	yes	no	yes	no	yes	yes	yes*	yes*
Lb. delbrueckii subsp. *bulgaricus* NCDO 1489	yes	yes	yes	no	no	no	no	no
Lb. delbrueckii subsp. *lactis* ATCC 4797	yes	no	yes	no	no	no	yes*	yes*
Lb. helveticus ATCC 15009	yes**	no	no	yes	yes	no	yes*	yes*
Lb. plantarum CSCC 2901	yes**	no	no	no	no	no		
Lb. plantarum PI 549	yes**	no	no	no	no	no		
Lactococcus lactis subsp. *lactis* C2	yes**	no	yes	no	no	no		

[a] Protease-sensitive, catalase-insensitive inhibition was assessed by a deferred agar spot assay. Producer cultures were spotted on NLA plates and incubated for 5 d at 32 °C under flowing CO_2 (0.4L·h^{-1}). Plates were overlaid with soft agars seeded with 10 µL of indicator culture (~10^7 CFU·mL^{-1}) and incubated appropriately for development of lawns. [b] Abbreviations: ATCC, American Type Culture Collection, Rockville, MD, USA; NCDO, National Collection of Dairy Organisms (currently National Collection of Food Bacteria), AFRC Institute of Food Research, Reading, England; YIT, Yakult Institute for Microbiology Research, Tokyo, Japan; PI, Presque Isle Cultures, Presque Isle, PA, USA. [c] Legend: yes, bacteriocin-like inhibition; yes*, bacteriocin-like inhibition that increased after protease treatment; yes**, bacteriocin-like inhibition, but only after protease treatment; no, no bacteriocin-like inhibition.

[a] L'inhibition protéase-sensible et catalase-insensible était testée indépendamment sur agar. Les cultures productrices étaient mises en évidence sur plaque d'agar de lactate de sodium et incubées 5 j à 32 °C sous flux de CO_2 (0,4 L.h^{-1}). Les plaques étaient recouvertes d'agar mou ensemencé avec 10 µL de culture indicatrice (~10^7 UFC.mL^{-1}) et incubées de façon appropriée pour le développement des tapis bactériens. [b] Abbréviations : voir légende anglaise. [c] Légende : yes, inhibition de type bactériocine ; yes*, inhibition de type bactériocine augmentant après traitement à la protéase ; yes**, inhibition de type bactériocine mais uniquement après traitement à la protéase ; no, pas d'inhibition de type bactériocine.

cultures (*table I*). Application of pronase E and type XIV proteinase near *P. jensenii* B1264 resulted in crescent-shaped inhibition zones (*figure 1*) at the application sites and broadened its inhibitory spectrum to *Lactococcus lactis* subsp. *lactis* C2, *Lactobacillus helveticus* ATCC 15009, *Lb. plantarum* CSCC 2901 and *Lb. plantarum* PI 549. In the absence of protease, agar cultures of *P. jensenii* ATCC 4868 inhibited indicator lactobacilli and agar cultures of *P. freudenreichii* ATCC 6207 inhibited propionibacteria and lactobacilli. Protease treatment alone did not affect the lawns; thus, inhibition was not due to the enzyme. Combination of the soluble inhibitors produced by *P. jensenii* ATCC 4868 and *P. freudenreichii* ATCC 6207 with pronase immediately before their application to indicator lawns increased inhibitory activity. Longer treatment of soluble inhibitors with protease (≥ 2 min) before applying them to indicator lawns completely destroyed the activity. The expanded spectrum could be due to limited proteolysis of either the antagonists or other bacterial products diffusing through the agar medium. The protease-sensitive antagonist produced by *P. jensenii* B1264 had the broadest inhibitory spectrum and was examined further.

3.2. Production and mode of action

Batch cultures of *P. jensenii* B1264 were examined for inhibitory activity. Activity was detected only during late stationary phase producer growth and then only in

Figure 1. Protease potentiation of inhibitory activity of *Propionibacterium jensenii* B1264. The enzymes pronase E (**P**; 15 µL, 10 mg·mL^{-1}) and catalase (**C**; 15 µL, 10 mg·mL^{-1}) were applied to the left of 5-d, sodium lactate agar producer cultures. No enzyme was applied at the producer culture designated 'control'. Cultures were overlaid with *Lactococcus lactis* C2 (10 µL overnight culture per 5 mL soft tempered M-17) and held overnight at 32 °C under flowing CO_2 for development of the lawn.

Figure 1. Activation par la protéase de l'activité inhibitrice de *Propionibacterium jensenii* B1264. Les enzymes pronase E (**P** ; 15 µL, 10 mg·mL^{-1}) et catalase (**C** ; 15 µL, 10 mg·mL^{-1}) ont été déposées sur la partie gauche des plaques d'agar au lactate de sodium de cultures productrices à 5 j. Aucune enzyme n'était déposée pour la culture productrice désignée par « control ». Les cultures étaient recouvertes avec *Lactococcus lactis* C2 (10 µL d'une culture d'une nuit sur 5 mL de milieu M17 ramolli) et maintenu 1 nuit à 32 °C sous flux de CO_2 pour le développement des tapis bactériens.

2-fold to 16-fold concentrated culture filtrates. There was some evidence that cell lysis was occurring and that the bacteriocin could be released from the intracellular environment but this has not been explored further as yet. Maximum production (200 AU·mL^{-1}) was observed at day 10 and decreased thereafter (*figure 2*). Most bacteriocins are bactericidal to sensitive cells [18]. Treatment of cells of *Lb. delbrueckii* subsp. *lactis* ATCC 4797 cells with the antagonist produced by *P. jensenii* B1264 reduced the viability of the lactobacilli by 90 % within 60 min (*figure 3*). Turbidity dropped by 0.1 absorbance units after 75 min; therefore, lysis occurred only after indicator cell death. The data suggest a bactericidal mode of action.

3.3. Stability

Effects of various enzymes, solvents and other treatments on inhibitory activity in ammonium sulfate-precipitated *P. jensenii* B1264 culture supernates were assessed. Treatment with proteinase K, pronase E or type XIV protease eliminated activity. This data provided further support that the inhibitory activity was a protein or peptide. Treatment with trypsin, α-chymotrypsin, type VI-A protease, lysozyme or catalase did not affect activity (*table II*). Adjusting inhibitory preparations to pH values ranging from 2.2 to 10.2 or treatments with 0.1 to 1.0 mol·L^{-1} NaCl for up to 225 min did not affect activity. Inhibitory activity was stable to heating at 100 °C for 0 to 60 min, treat-

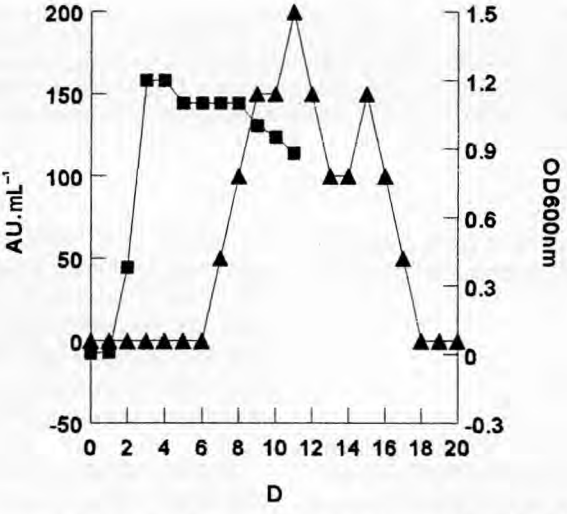

Figure 2. Bacteriocin production by *Propionibacterium jensenii* B1264. *P. jensenii* B1264 was propagated in 1 L sodium lactate broth cultures at 32 °C for 20 d under flowing CO_2 (0.4 L·h^{-1}). At daily intervals, 10 mL sample aliquots were removed and examined for turbidity by determining optical density on a Spectronic 20 at 600 nm (■, $OD_{600\ nm}$). The sample then was centrifuged (1 300 *g* for 15 min) and supernatants were filtered through 0.45 µm filters and assayed for activity (▲, AU·mL^{-1}) against *Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797.

Figure 2. Production de bactériocine par *Propionibacterium jensenii* B1264. *P. jensenii* B1264 était cultivé dans 1 L de bouillon de culture au lactate de sodium à 32 °C pendant 20 j sous flux de CO_2 (0,4 L·h^{-1}). À intervalles journaliers, des échantillons de 10 mL étaient prélevés et leur turbidité était déterminée par densité optique sur un Spectronic 20 à 600 nm (■, DO_{600} nm). L'échantillon était ensuite centrifugé (1300 *g*-15 min) et les surnageants étaient filtrés sur filtres de 0,45 µm et testés pour leur activité (▲, UA·mL^{-1}) sur *Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797.

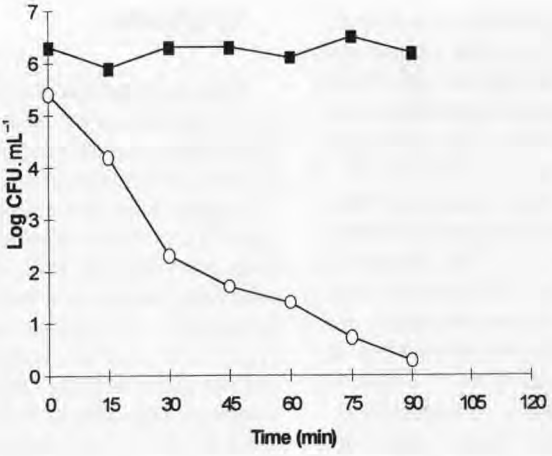

Figure 3. Lethal action of *Propionibacterium jensenii* B1264 bacteriocin against *Lactobacillus delbrueckii* subsp. *lactis*. Crude bacteriocin (○) 4 800 total AU; 119.4 AU·g^{-1} protein) or 0.05 mol·L^{-1} phosphate buffer, pH 6.8 (●), was added to washed cells of a 20-h MRS broth culture of *Lb. delbrueckii* subsp. *lactis* ATCC 4797 in the same buffer and held at 37 °C for 0, 15, 30, 60 or 90 min. Culture populations were determined at each interval.

Figure 3. Action létale de la bactériocine de *Propionibacterium jensenii* B1264 sur *Lactobacillus delbrueckii* subsp. *lactis*. La bactériocine brute (○) 4800 UA totale ; 119,4 UA·g^{-1} de protéine) ou 0,05 mol·L^{-1} de tampon phosphate, pH 6,8 (●), était ajoutés à des cellules lavées d'une culture sur bouilllon MRS de 20 h de *Lb. delbrueckii* subsp. *lactis* ATCC 4797 dans le même tampon et maintenue à 37 °C pendant 0, 15, 30, 60 ou 90 min. Des dénombrements étaient réalisés à chacun de ces temps.

ment with 4 mol·L^{-1} urea for 6 h or addition of SDS at final concentrations of 0.1 to 2.0 %.

3.4. Purification

Ammonium sulphate (80 % saturation) initially was used to concentrate and precipitate the inhibitor produced by *P. jensenii* B1264. To optimize the concentration step, ammonium sulphate was added to separate cell-free supernatants, and pellets were examined for bacteriocin activity. Ammonium sulphate at all concentrations (40, 45, 50, 55, 60, 65, 70 and 80 % saturation) precipitated activity (80, 160, 400, 2 400, 3 000, 3 200, 3 800 and 3 800 total AU, respectively). Lowest protein and maximum activity precipitated at 70 % saturation. This concentration of ammonium sulphate was used for further experiments. Resuspension of ammonium sulphate pellets from 16-L cultures into 20 mL sodium phosphate buffer typically yielded preparations containing 800 AU·mL^{-1} to 3 200 AU·mL^{-1}. Ammonium sulphate-precipitated preparations are hereafter called crude bacteriocin.

In preparation for ion exchange chromatography (IEC), crude bacteriocin was examined by analytical isoelectric focussing (IEF) on a pH 3 to 7 gradient gel (*figure 4*). The inhibitor remained active throughout focussing, allowing for detection in focussed gels. Inhibition corresponded to a silver-stained protein that focussed between pH 3 and 3.5, indicating that the inhibitor has an isoelectric point (pI) between pH 3.0 and 3.5. The inhibitor was subjected to batch anion exchange chromatography at pH 6; activity was adsorbed to separate batches

Table II. Enzyme effects on inhibitory activity against *Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797.

Tableau II. Effet des enzymes sur l'activité inhibitrice envers *Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797.

Enzyme[a]	Enzyme treated inhibitor	Untreated inhibitor control	Colony of B1264
Pronase type XIV	no[b]	yes	no
Pronase E	no	yes	no
Protease K	no	yes	no
α-Chymotrypsin	yes	yes	yes
Protease type VI-A	yes	yes	yes
Trypsin	yes	yes	yes
Lysozyme	yes	yes	yes
Catalase	yes	yes	yes
Distilled water	yes	yes	yes

[a] Enzymes (15 µL, 10 mg·mL^{-1}) were spotted beside 15 µL crude bacteriocin on NLA (119.4 AU·g^{-1}·protein; dried for 1 h at 22 °C) or beside colonies of *P. jensenii* B1264 (5 d) and held for 1 h at 25 °C or 37 °C. Plates were then overlaid with soft agar seeded with the sensitive indicator, *Lb. delbrueckii* subsp. *lactis* ATCC 4797, and incubated for development of the lawn. [b] Legend: yes, retains activity after treatment; no, loses activity after treatment.

[a] Les enzymes (15 µL, 10 mg·mL^{-1}) étaient déposées près de 15 µL de bactériocine brute sur agar au lactate de sodium (119,4 UA·g^{-1} de protéine ; séché 1 h à 22 °C) ou près de colonies de *P. jensenii* B1264 (5 j) et maintenus 1 h à 25 °C ou 37 °C. Les boîtes étaient alors recouvertes d'agar contenant la souche indicatrice sensible, *Lb. delbrueckii* subsp. *lactis* ATCC 4797, et incubée pour le développement du tapis bactérien. [b] Légende : yes, activité conservée après traitement ; no, activité perdue après traitement.

of Bio-Rad Macroprep Q resin. Sodium phosphate buffer containing 0.1, 0.3, 0.5, 0.7 or 1 mol·L^{-1} sodium chloride was applied to the resin and eluted fractions were assayed for protein and inhibitory activity. All salt concentrations eluted equal activity; an increase in salt concentration eluted increased protein. Therefore, 0.1 mol·L^{-1} sodium chloride was selected. Application of 0.1 mol·L^{-1} sodium chloride eluted all activity and resulted in 101 % recovery and 15-fold purification.

The IEC-purified inhibitor was subjected to two-dimensional SDS-PAGE/IEF. Activity migrated on SDS-PAGE gels in one inhibitory zone corresponding to three silver-stained protein bands (*figure 5*). Bands of approximately 6 kDa and 9 kDa migrated at a location corresponding to the centre of the inhibitory zone; an 11-kDa band migrated near the zone periphery. Because diffusion is not unidirectional, activity was most likely not associated with the 11-kDa band; thus it was most likely associated with either or both of the 6- or 9-kDa bands. The portion of the gel corresponding to the inhibition zone was focussed on a pH 3 to 10 gradient IEF gel. Focussing yielded an inhibition zone corresponding to a single silver-staining band migrating between pH 3 and 3.5 confirming the pI (data not shown). A faint band focussing near pH 4.5 was not associated with activity. The results suggested that bacteriocin activity produced by *P. jensenii* B1264 may be due to one agent.

4. DISCUSSION

Eight out of thirteen propionibacteria produced bacteriocins, i.e. protease-sensitive, catalase-insensitive agents that inhibited

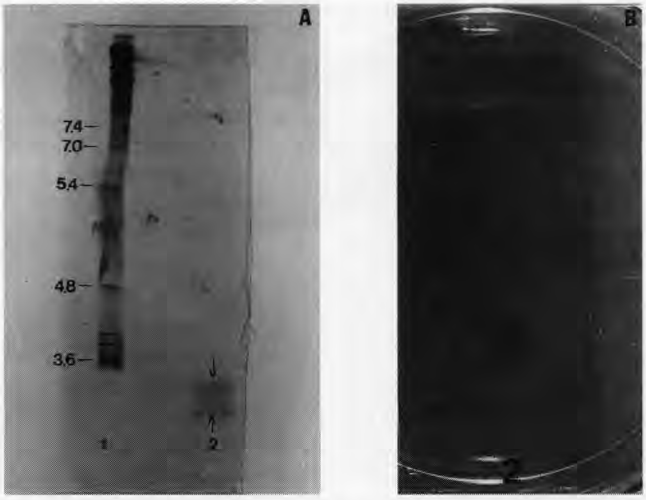

Figure 4. Analytical isoelectric focussing of bacteriocin. Crude bacteriocin (25 µL, 6 400 AU·mL^{-1}, 1.9 mg protein per mL) was focussed on a pH 3 to 7 gradient agarose isoelectric focussing (IEF) gel for 45 min at 1 500 V. **A.** Silver-stained gel; **B.** gel overlaid with *Lb. delbrueckii* subsp. *lactis* ATCC 4797 for detection of activity. Lane 1, IEF standards; lane 2, crude bacteriocin.

Figure 4. Analyse par focalisation isoélectrique de bactériocine. La bactériocine brute (25 µL, 6800 UA·mg^{-1}, 1,9 mg de protéine par mL) était observée sur 1 gel d'agarose pour focalisation isoélectrique (IEF) de gradient de pH de 3 à 7, pendant 45 min à 1500 V. **A.** gel coloré à l'argent. **B.** gel recouvert de *Lb. delbrueckii* subsp. *lactis* ATCC 4797 pour détection de l'activité. ligne 1, standards IEF ; ligne 2, bactériocine brute.

closely related species. Bacteriocin production may be as common in propionibacteria as it is in other species.

The antagonist produced by *P. jensenii* B1264 was selected for further study and its partial purification and characterization is described. The inhibitor is proteinaceous in nature, has a narrow inhibitory spectrum and a bactericidal mode of action and thus meets the classical criterion for bacteriocins [18]. Its activity spectrum differs from those of propionicin PLG-1 and jenseniin G. Unlike propionicin PLG-1 [11], it inhibits *P. thoenii* P127 and does not inhibit Gram-negative species. Unlike jenseniin G [6], it inhibits the jenseniin G producer P. *thoenii (jensenii)* P126, *Lb. acidophilus* ATCC 4356 and *Lb. casei* ATCC 7469. It is more heat-stable (100 °C for 60 min) than propionicin PLG-1 (≤ 85 °C) [12] and jenseniin G (100 °C, 15 min) [6]. In contrast to jenseniin G (pI between 11 and 11.5; unpublished data), propionicin PLG-1 (pI of 8.8) [12] and most other characterized bacteriocins, it is an acidic bacteriocin with a pI between 3 and 3.5. It is smaller (estimated molecular mass of 6 to 9 kDa) than propionicin PLG-1 (9 328 Da) [15]. The bacteriocin shares some characteristics with the two previously identified bacteriocins from dairy propionibacteria. Like propionicin PLG-1 (pH 3–9) [11] and jenseniin G (pH 3–12; unpublished data), it is stable to a wide range of pH (pH 3–12). Like propionicin PLG-1 [9, 12] and jenseniin G [6], it is produced in late stationary phase producer cultures and the culture supernatant must be concentrated to detect activity.

Although the bacteriocin was not purified to homogeneity, two purification steps were successful in separating it from most contaminating proteins. Ammonium sulfate

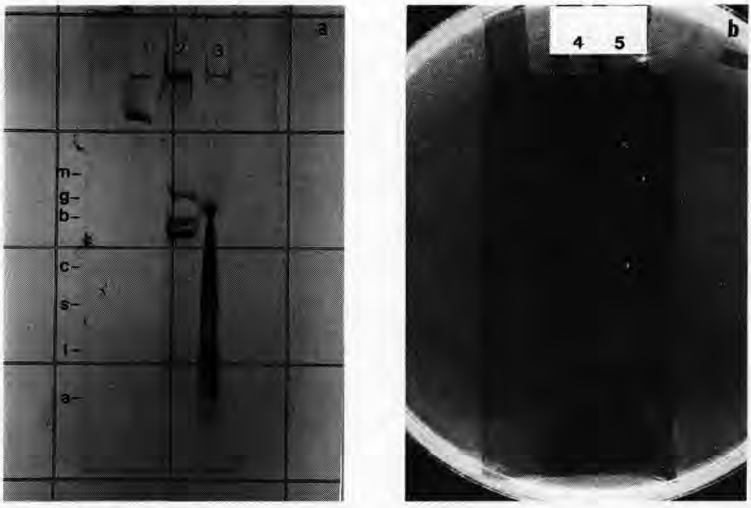

Figure 5. SDS-PAGE of crude and partially-purified bacteriocin. **a**. Silver-stained SDS-PAGE gel. **b**. Gel overlaid with *Lb. delbrueckii* subsp. *lactis* ATCC 4797 for detection of activity. Lane 1, pre-stained Bio-Rad Kaleidoscope Standards (m, myosin, 204 kDa; g, β-galactosidase, 121 kDa; b, BSA, 78 kDa; c, carbonic anhydrase, 39.5 kDa; s, soybean trypsin inhibitor 30.7, kDa; l, lysozyme, 19.7 kDa; a, aprotinin, 7.7 kDa). Lanes 3 and 5, 15 µL crude bacteriocin (6 400 AU·mL^{-1}, 1.9 mg protein per mL); lanes 2 and 4, 15 µL IEC-purified bacteriocin (800 AU·mL^{-1}, 0.1 mg protein per mL).

Figure 5. Analyse par SDS-PAGE de bactériocine brute ou partiellement purifiée. **a**. Gel SDS-PAGE coloré à l'argent. **b**. Gel recouvert de *Lb. delbrueckii* subsp. *lactis* ATCC 4797 pour détection de l'activité. ligne 1, standards Bio-Rad Kaleidoscope pré-colorés (m, myosine, 204 kDa ; g, β-galactosidase, 121 kDa ; b, BSA, 78 kDa ; c, anhydrase carbonique, 39,5 kDA ; s, inhibiteur trypsique de soja, 30,7 kDa ; l, lysozyme, 19,7 kDa ; a, apoprotéine, 7,7 kDa). lignes 3 et 5, 15 µL de bactériocine brute (6 400 UA·mL^{-1}, 1,9 mg de protéine par mL) ; lignes 2 et 4, 15 µL de bactériocine purifiée par IEF (800 UA·mL^{-1}, 0,1 mg de protéine par mL).

precipitation followed by batch anion exchange chromatography was optimized to give maximum recovery and greatest purity. Large quantities of the bacteriocin are needed for further purification and other studies. Because 20- to 50-fold concentrated supernatants are required to detect activity in producer cultures, it is produced in minute concentrations. It will be necessary to identify factors supporting its maximum production by *P. jensenii* B1264. Bacteriocins from dairy propionibacteria typically have been examined for use as food preservatives [13, 19]. The narrow inhibitory spectrum of this bacteriocin limits its application in foods. Its stability to temperature, solvents, pH, salts, and detergents are useful characteristics in industrial applications. Its use for pharmaceutical purposes is currently being explored.

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