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Coagulin, a bacteriocin-like inhibitory substance produced by *Bacillus coagulans* I_4

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B. HYRONIMUS, C. LE MARREC AND M.C. URDACI. 1998. A protease-sensitive antibacterial substance produced by *Bacillus coagulans* I_4 strain, isolated from cattle faeces, was classified as a bacteriocin-like inhibitory substance and named coagulin. The inhibitory spectrum included *B. coagulans* and unrelated bacteria such as *Enterococcus*, *Leuconostoc*, *Oenococcus*, *Listeria* and *Pediococcus*. Coagulin was stable at 60 °C for 90 min, at a pH ranging from 4 to 8 and appeared to be unaffected by α-amylase, lipase or organic solvents (10% v/v). Coagulin exhibited a bactericidal and a bacteriolytic mode of action against indicator cells. The apparent molecular mass was estimated to be about 3–4 kDa by SDS-PAGE. The *B. coagulans* I_4 strain harbours a plasmid, pI_4, approximately 14 kb in size. Novobiocin curing experiments yielded two derivatives that no longer produced the bacteriocin-like inhibitory substance. Plasmid content of these two derivatives showed that one had lost pI_4,whereas the second harboured a deleted form of this plasmid, thus suggesting a plasmid location for the genes for coagulin production.

INTRODUCTION

Antibacterial compounds such as bacteriocins are produced by a wide number of micro-organisms. Bacteriocins isolated from lactic acid bacteria (LAB) have been the subject of a large amount of research work in recent years because of their potential use in the food industry to eliminate organisms that are responsible for food spoilage or food-related pathogenicity. Bacteriocin activity is considered to be species- specific, but some bacteriocins have proven to have a greater spectrum of activity, and thus possibly have a broader industrial application (Tagg *et al.* 1976). The best known and most fully studied bacteriocin is nisin A, which has been accepted by the World Health Organisation as a biopreservative in the food industry in over 50 countries including the EEC and the USA (Delves-Broughton *et al.* 1996). Four major classes of bacteriocins are produced by LAB: I, lantibiotics; II, small heat-stable peptides; III, large heat-labile proteins and IV, complex proteins that additionally require carbohydrate or lipid moieties for bacteriocin activity (Klaenhammer 1993). Other Gram-positive bacteria, such as *Staphylococcus aureus* (Jung and Sahl 1991), *Carnobacterium piscicola* (Stoffels *et al.* 1992), *Streptococcus pyogenes* (Jack *et al.* 1994) and *Enterococcus faecalis* (Maisnier-Patin *et al.* 1996), also produce antibacterial peptides. Within the genus *Bacillus*, bacteriocins or bacteriocin-like inhibitory substances (BLIS) have been reported from *Bacillus subtilis* (Jansen and Hirschmann 1944), *B. thuringiensis* (De Borjac *et al.* 1974), *B. stearothermophilus* (Shafia *et al.* 1966), *B. licheniformis* (Bradley 1967), *B. megaterium* (Ivanovics 1962), *B. thermoleovorans* (Novotny *et al.* 1992) and *B. cereus* (Naclerio *et al.* 1993). Spore-forming micro-organisms are responsible for food spoilage, especially *Bacillus* species. Hence *B. cereus* is considered the most important spore-forming species in pasteurized milk (Christiansson *et al.* 1989). *Bacillus coagulans* is of considerable concern during the processing of acid and acidified foods, causing flatsour spoilage especially in tomato products (Frazier 1958). In this paper, the identification of a new BLIS, named coagulin, in the culture supernatant fluid of a strain of *B. coagulans* I_4, isolated from animal faeces, is described. Coagulin exhibits the classical characteristics of most BLIS and bacteriocins, but also affects several pathogens and food-spoilage micro-organisms. Characterization of its properties and proof for the location of its genetic determinants are presented here.

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MATERIALS AND METHODS

Bacterial strains and growth conditions

Strains isolated for the screening of bacteriocin-producing micro-organisms were grown aerobically at 37 °C in MRS medium (De Man *et al*. 1960) (Difco). Other strains used for the antimicrobial spectrum of *B. coagulans* I_4 inhibitory substance, media and incubation temperatures are reported in Table 1. Solid media were prepared by adding 1·2% (w/v) granulated agar to broth media. Stock cultures were maintained at −80 °C in broth supplemented with 20% (w/v) glycerol.

Production of bacteriocin-like inhibitory substance

Synthesis of the antimicrobial substance was carried out in MRS medium. A 500 ml flask was seeded with 1% inoculum (10^6 cfu ml^{-1}) from an overnight culture of *B. coagulans* I_4. The culture was incubated for 12 h at 37 °C with shaking. Cells were removed by centrifugation (10 000 *g*, 30 min, 10 °C). The supernatant fluid was adjusted to pH 6·5 and filtered through Schleicher & Schuell (Ecquevilly, Yvelines, France) 0·22 μm sterile filters. This preparation, designated active crude fluid supernatant (ACFS), was stored at −20 °C.

Bacteriocin assays

Bacteriocin activity was determined by an agar-well diffusion method (Tagg and Mc Given 1971), using MRS medium plates adjusted to pH 6·5. An overnight culture of the indicator strain was used to inoculate agar growth media maintained at 45 °C, and poured into Petri dishes. After solidification, wells of 5 mm in diameter were cut into the agar plates and filled with 100 μl of ACFS dilutions. Plates were examined for the presence of an inhibition halo after 24 h of incubation. The titre was defined as the reciprocal of the highest dilution showing definite inhibition of the indicator lawn and was expressed in activity units (AU) per millilitre. Determination of the antimicrobial spectrum was carried out using the same protocol, except that the ACFS was not diluted before adding to the wells.

Sensitivity to enzymes, organic solvents, pH and temperature

Enzymes (1 mg ml^{-1}) and organic solvents (10% (v/v); see Table 2) were added to the ACFS. Enzyme-treated samples were incubated for 1 h at 37 °C and solvent-treated samples for 1 h at 30 °C before being tested for antimicrobial activity. Thermostability of the BLIS was analysed by assaying the antimicrobial activity of the ACFS after 15, 30, 60 and 90 min of incubation at 50, 60, 70, 80, 90 and 100 °C. Samples were cooled to room temperature and tested for residual activity as described above. Effects of pH on bacteriocin activity were determined by adjusting the pH of the ACFS with diluted HCl and NaOH. Samples were incubated for 2 h at 37 °C, readjusted to pH 6·5 and assayed as described above.

Direct detection of bacteriocin activity on SDS-PAGE gels

Proteins contained in the ACFS were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). At the end of the migration, the gel was removed and cut into two vertical parts. One part, containing one sample and the molecular weight standards, was stained with Coomassie brilliant blue R250 (Sigma). The other part, containing the second sample, was assayed for antimicrobial activity by the direct detection method described by Bhunia *et al.* (1987) with the modifications reported by Naclerio *et al.* (1993). Samples of this part did not contain bromophenol blue. Gels were superimposed and photographed.

Mode of action of coagulin

Cells of a log phase or stationary phase culture of *B. coagulans* CIP 6625 grown in MRS were pelleted by centrifugation, washed and resuspended in sterile 50 mmol l^{-1} sodium phosphate buffer (pH 6·2) to give 10^6 cfu ml^{-1}. Cells were exposed for a maximum of 180 min to the neutralized active supernatant fluid from *B. coagulans* I_4 (ACFS) at concentrations of 6 and 60 AU ml^{-1}. Samples were incubated at 37 °C and, at intervals, the number of surviving bacteria (cfu ml^{-1}) was determined by plate counting on MRS plates. Cell lysis was measured by incubating *B. coagulans* CIP 6625 (mid-log phase of growth) in MRS broth, with the addition of 60 AU ml^{-1} coagulin. Samples (1 ml) were taken immediately before addition and at regular intervals thereafter. Each sample was measured spectrophotometrically at 600 nm.

Inhibition of *B. coagulans* spores by coagulin in agar plates

Spore production by *B. coagulans* CIP 6625 was carried out on nutrient agar (Difco) as reported by Roberts and Hoover (1996). Samples were harvested after 10 days of incubation, resuspended in sterile physiological water and treated at 80 °C for 10 min. Aliquots were serially diluted with sterile physiological water to the initial concentration of 10^6 spores ml^{-1}. The homogeneity of the spore preparation was examined by adding a control containing vegetative cells (from an overnight culture). This control, similarly heat-treated, resulted in no growth on MRS plates after 5 d. The ACFS containing coagulin I_4 was added to tempered MRS agar to achieve coagulin I_4 concentrations of 10–100 AU ml^{-1}. The initial

Table 1 Media and temperatures for culture of the different Gram-positive and Gram-negative bacteria and inhibitory activity of *Bacillus coagulans* I_4 supernatant fluid against these bacteria

Bacteria	Media	Temperature (°C)	Inhibition
Gram-positive bacteria			
Bacillus amyloliquefaciens LMAB121	MRS	37	−
Bacillus cereus LMAB 136	BNL	37	−
Bacillus coagulans CIP 5264	MRS	37	+
Bacillus coagulans CIP 6625	MRS	37	+
Bacillus coagulans LMAB143	MRS	37	+
Bacillus licheniformis CIP 5271	BNL	37	−
Bacillus megaterium LMAB220	MRS	37	−
Bacillus subtilis ATCC 21332	BNL	37	−
Brevibacterium linens ATCC 9172	LB	30	−
Clostridium butyricum DSM 10702	RCM	37	−
Clostridium histolyticum DSM 627	RCM	37	−
Clostridium perfringens DSM 756	RCM	37	−
Corynebacterium glutamicum ATCC 21650	LB	30	−
Corynebacterium lilium ATCC 15590	LB	30	−
Enterococcus faecium LMAB737	MRS	37	+
Enterococcus faecalis CIP 76117	MRS	30	+
Lactobacillus acidophilus ATCC 4356 T	MRS	30	−
Lactobacillus brevis DSM 20054 T	MRS	30	−
Lactobacillus casei DSM 20011 T	MRS	30	−
Lactobacillus cellobiosus DSM 20055 T	MRS	30	−
Lactobacillus farciminis DSM 20184 T	MRS	30	−
Lactobacilus fermentum DSM 20052 T	MRS	30	−
Lactobacillus halotolerans DSM 20190 T	MRS	30	−
Lactobacillus plantarum ATCC 8014	MRS	30	−
Lactococcus cremoris LMAB856	MRS	30	−
Lactococcus diacetylactis LMAB857	MRS	30	−
Lactoccus lactis 1441 FOEB*	MRS	30	−
Lactococcus lactis LMAB858	MRS	30	−
Leuconostoc mesenteroides CIP 5349	MRS	30	+
Listeria innocua LMAB354	TRY	37	+
Listeria monocytogenes 4b LCHA/CNEVA	TRY	37	+
Listeria monocytogenes $\frac{1}{2}$b LCHA/CNEVA	TRY	37	+
Listeria seeligheri CIP 100100	TRY	37	+
Oenococcus oeni (a) FOEB*	MRS	30	+
Oenococcus oeni (b) FOEB*	MRS	30	+
Pediococcus pentosaceus ATCC 33316	MRS	30	−
Pediococcus pentosaceus CIP 5350	MRS	30	+
Pediococcus damnosus FOEB*	MRS	25	+
Staphylococcus aureus CIP 53156	BNL	37	−
Gram-negative bacteria			
Aeromonas hydrophila CIP 7430	BNL	37	−
Escherichia coli NM522	BNL	37	−
Klebsiella oxytoca LMAB1014	BNL	37	−
Pseudomonas fluorescens CIP 5690	BNL	37	−
Salmonella typhimurium LMAB78	TRY	30	−
Salmonella enteritidis LMAB79	TRY	30	−
Salmonella virchow LMA1025B	TRY	30	−
Yersina enterocolitica LMAB86	BNL	37	−

ATCC, American Type Culture Collection; CIP, Collection de l'Institut Pasteur, France; DSM, Deutsche Sammlung von Mikroorganismen; FOEB, Faculté d'Oenologie, Université Bordeaux I, France; LCHA/CNEVA, Laboratoire Central d'Hygiène Alimentaire/Centre National d'Etudes Vétérinaires Appliquées, Paris, France; LMAB, Laboratoire de Microbiologie Alimentaire et Biotechnologies, ISTAB, Université Bordeaux I, France; BNL, Liquid Nutrient Broth (Difco); LB, Luria Bertani broth (Miller 1972); MRS (de Man, Rogosa and Sharpe 1960) (Difco); RCM, Reinforced Clostridial Medium (Biokar Diagnostics, Beauvais, France); TRY, Tryptose broth (Difco).
+, Inhibited by *B. coagulans* I_4 supernatant fluid; −, not inhibited.
*Strains were isolated from spoiled wines.

Table 2 Factors affecting coagulin activity

Treatment	% Activity*
Enzymes†	
trypsin	0
α-chymotrypsin	0
papaïne	0
pepsin	0
pronase E	0
α-amylase	100
lipase	100
Organic solvents‡	
acetone	100
acenonitrile	100
ethyl alcohol	100
methanol	100
chloroform	100
toluene	100
Heat (15 min) at	
60 °C	100
70 °C	93
80 °C	85
90 °C	60
100 °C	30
pH	
1	75
2	87
4	100
6	100
8	100
9	62
10	25
12	0

*Coagulin activity is expressed as the % of residual activity.
†The enzyme concentration was 1 mg ml^{-1}.
‡A 10% (v/v) concentration was used.

spore concentration was pour-plated with MRS amended with coagulin I_4 (MRS + coagulin) and with MRS to determine the starting spore concentration. Plates were incubated at 37 °C for 4–5 d before counting.

DNA isolations and gel electrophoresis

Plasmid DNA in *B. coagulans* I_4 and Bac^- variants was isolated by the rapid alkaline extraction procedure described by Maniatis *et al.* (1982), except that the lysozyme concentration was increased to 10 mg ml^{-1}. Extraction of bacterial DNA was performed as described previously (Quéré *et al.* 1997). DNA was visualized following electrophoresis in 1% (w/v) agarose gels (Eurobio, Les Ulis, France) in Tris-borate buffer (Maniatis *et al.* 1982) and staining with ethidium bromide (0·5 μg ml^{-1}; Sigma).

Plasmid curing

Experiments to produce BLIS-negative strains and to determine if BLIS production was plasmid-encoded were carried out using novobiocin (Sigma) as the curing agent. An overnight culture of *B. coagulans* I_4 diluted to 10^4 cfu ml^{-1} was inoculated into MRS broth containing novobiocin (1·6 μg ml^{-1}) and incubated at 37 °C for 48 h. Cells were plated onto MRS plates. Colonies were replicated and checked for bacteriocin production. An aliquot of an overnight culture was diluted twofold with 0·3 mol l^{-1} phosphate buffer (pH 6·5) and 5 μl of the diluted culture were spotted onto tryptose agar (Difco) containing *L. innocua* as the indicator bacteria. Colonies from spots which did not produce zones of inhibition ($BLIS^-$) were restreaked on MRS agar, and their immunity to the bacteriocin tested by spotting 5 μl of *B. coagulans* I_4 ACFS onto lawns of each of the non-producing derivatives.

RAPD or PCR-specific reaction

A PCR-specific reaction was performed to amplify the 16S ribosomal DNA gene of the *Bacillus* BLIS producer strain I_4, using the direct and reverse primers 5′-AGAGTTTGATCATGGCTCAG-3′ and 5′-GGTTACCTTGTTACGACTT-3′; this primer pair has been shown to amplify the maximum number of nucleotides of 16S ribosomal DNA from a wide variety of bacterial taxa (Weisburg *et al.* 1991). Primer OPR13 (5′-GGACGACAAG-3′) was used in a random amplified polymorphic DNA (RAPD) reaction. The PCR and RAPD reactions were performed as previously described (Quéré *et al.* 1997) using a DNA thermal cycler (Own E, Hybraid). Oligonucleotides were synthesized by Eurogentec (Seraing, Belgium).

DNA sequencing

The DNA sequences of the PCR products were determined by using the *Taq* DyeDeoxy terminator cycle sequencing kit (Perkin Elmer, Forster City, CA, USA) and the protocol recommended by the supplier. Sequencing reaction products were analysed with a model 373 A automated DNA sequencer (Applied Biosystems, Foster City, CA, USA).

RESULTS

Isolation and characterization of a spore-forming lactic acid bacterium producing an inhibitory substance

In the course of a survey of 500 spore-forming LAB for novel antimicrobial compounds, an antimicrobial substance-

producing strain was isolated from cattle faeces. This organism is a Gram- and catalase-positive bacterium, able to grow at 55 °C and to produce lactic acid using a homofermentative pathway. A preliminary identification of this strain was obtained through phenotypical and physiological characteristics (data not shown). In order to confirm the identification, a partial sequence of the 16S ribosomal DNA (500 pb from the 5′ region) was determined. A comparison of this sequence with all of the 16S rRNA sequences in the databases was performed. The highest levels of similarity were found with the sequence of *B. coagulans* (accession number D16267, 100% identity). This result was confirmed by comparisons of SDS-PAGE of whole proteins of strain I_4 with *B. coagulans* strains from various collections (data not shown). It was concluded that the micro-organism was a strain of *B. coagulans*, which we designated *B. coagulans* I_4.

Inhibitory spectrum of the bacteriocin-like inhibitory substance

Cell-free supernatant fluid of a culture of *B. coagulans* I_4 was tested for the presence of antimicrobial activity by the halo inhibition assay on agar plates. The host range was determined with a number of strains from various collections, including Gram-positive and Gram-negative bacteria. A total of 47 strains, representing 18 genera, were tested and the results are shown in Table 1. Strain I_4 was shown to prevent the growth of different Gram-positive bacteria. By using the size of the inhibition halo, it was determined that the most sensitive strains were *B. coagulans* CIP 5264, CIP 6625 and LMAB. Inhibition was also observed against *Listeria monocytogenes* 1/2b, 4b, *L. seeligheri*, *L. innocua*, *Ent. faecium* and *Ent. faecalis*, as well as the LAB *Oenococcus oeni*, *Leuconostoc mesenteroides* and *Pediococcus pentosaceus* (Table 1). The antimicrobial substance was not active against the Gram-negative strains tested. In all subsequent experiments, either *B. coagulans* CIP 6625 or *L. innocua* was used as the indicator.

General properties of the antimicrobial substance

The ACFS preparation was tested for sensitivity to various enzymes, organic solvents, heat and pH values. As shown in Table 2, the antimicrobial activity was not affected by treatment with α-amylase, lipase, or any of the organic solvents used, while it was completely lost after treatment with trypsin, α-chymotrypsin, papaïne, pepsin or pronase-E (Table 2), thus suggesting a proteinaceous nature of the inhibitory compound. The inhibitory compound was stable to heat treatments. Maximal activity was observed during treatments under 60 °C, and residual activity was maintained to 30% after 15 min of incubation at 100 °C (Table 2). The inhibitory substance was also active over a wide range of pH, as full activity was retained at pH values between 3 and 8 (Table 2). Activity was more stable in acidic than basic conditions. Activity was reduced to 75% at pH 1 while it was severely altered for pH over 9, with a total loss of activity at pH 12 (Table 2).

Direct detection of antimicrobial activity after electrophoresis on SDS-PAGE gels overlayed with *L. innocua* indicated that the antimicrobial compound contained in the ACFS has a molecular mass between 3 and 4 kDa (Fig. 1). Nevertheless, SDS-PAGE did not reveal a specific stained band corresponding to the active compound.

Considering the properties of the inhibitory compound produced by *B. coagulans* I_4, i.e. a relative insensitivity to pH and temperature and the presence of an essential protein or peptide moiety, it was attributed as a BLIS, further named coagulin.

Coagulin production

Coagulin production was carried out in MRS (Difco) at 37 °C, with shaking, yielding a titre of 1200 AU ml^{-1} (Fig. 2). When the temperature was increased to 50 and 55 °C (i.e. the optimal temperatures for the growth of *B. coagulans* I_4), the titre was reduced to 300 and 10 AU ml^{-1}, respectively (data not shown). Tween-80 (present at a concentration of 1 g l^{-1} in commercial medium) has been reported to have a positive effect on bacteriocin production (Huot *et al.* 1996). Similar observations were made for coagulin production. When 0%

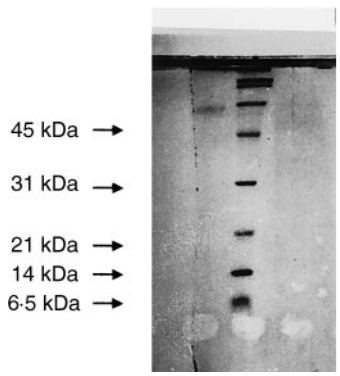

Fig. 1 Antibacterial detection sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel of coagulin. Proteins contained in the active crude fluid supernatant (ACFS) were analysed by SDS-PAGE. The gel was removed and cut into two parts. The first half, containing molecular mass markers (lane 1), was stained with Coomassie blue. The other half, containing serial dilutions of the ACFS, was overlaid with *Listeria innocua*. Both gels were superimposed and photographed. The arrow indicates the inhibition halo observed after incubation at 30 °C. Lane 1 contains the broad range molecular mass standards (200; 116·25; 97·4; 66·2; 45; 31; 21·5; 14·4 and 6·5 kDa) (Bio-Rad)

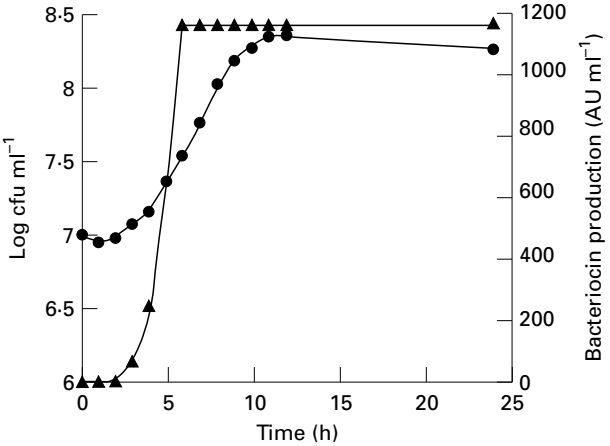

Fig. 2 Production of coagulin. Changes in viable cell number (log cfu ml^{-1}) (●) and bacteriocin titre (▲) during growth of *Bacillus coagulans* I_4 in MRS broth at 37 °C for 24 h

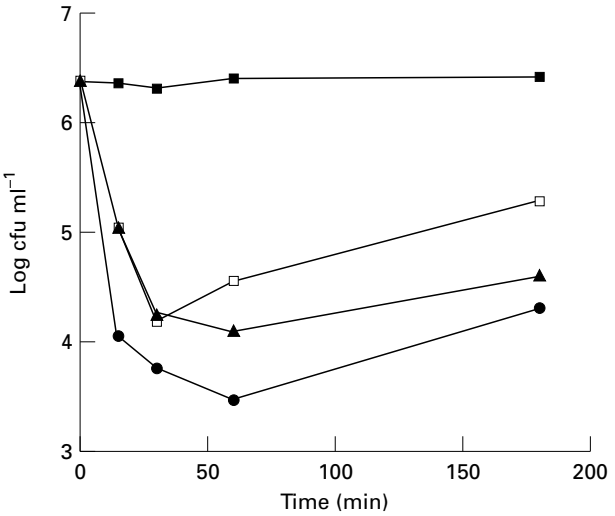

Fig. 3 Bactericidal effect of coagulin on non-growing cells of *Bacillus coagulans* CIP 6625 suspended in 50 mmol l^{-1} phosphate buffer, pH 6·2. Logarithmic phase cells added with coagulin (□), 6 AU ml^{-1} and (●), 60 AU ml^{-1}; (▲), stationary phase cells added with coagulin 60 AU ml^{-1}; (■), control containing logarithmic phase cells, no coagulin added

Tween-80 MRS broth was prepared in our laboratory, the coagulin titre was reduced to 750 AU ml^{-1} (data not shown). No stimulation of antimicrobial activity production was observed after addition of Tween-80 to MRS medium, whatever the concentration added in the range 0·1–1% (v/v). Kinetics of coagulin production studied in commercial MRS, at 37 °C, are presented in Fig. 2. Coagulin synthesis and/or secretion started at the beginning of logarithmic growth, with maximum activity corresponding to 1200 AU ml^{-1} occurring after the first 6 h (approximately 8·5 log cfu ml^{-1}). No further modification in coagulin titre was observed during the next 20 h of incubation (Fig. 2).

Bactericidal action and effect on *B. coagulans*

The neutralized culture supernatant fluid was added to the indicator strain *B. coagulans* CIP 6625, resuspended in phosphate buffer (pH 6·5). The addition of coagulin (6 and 60 AU ml^{-1}) to mid-log phase cells resulted in diminished viable counts of *B. coagulans* CIP 6625. We observed 100-fold and 1000-fold reductions, respectively, after 1 h incubation at 37 °C, suggesting a dose–response effect of coagulin on *B. coagulans* (Fig. 3). Increased coagulin concentration showed no enhanced effect (data not shown). Incubation of stationary phase indicator in the presence of the bacteriocin (60 AU ml^{-1}) resulted in a smaller decline in cell numbers, with a 100-fold reduction after 1 h incubation at 37 °C (Fig. 3). From these results, we concluded that coagulin exhibits a bactericidal mode of action on *B. coagulans*. After a longer period of time, slight increases in the number of viable cells were observed (Fig. 3). Surviving bacteria isolated after exposure to coagulin for 180 min were shown to be resistant to the BLIS by the agar-well diffusion method. This suggests the presence of a subpopulation of spontaneous variants resistant to this antimicrobial substance.

We next investigated the effect of the presence of coagulin on the survival of spores of *B. coagulans* CIP 6625. Coagulin concentrations of 10–100 AU ml^{-1} were used to obtain countable colonies for an initial spore concentration of 10^6 spores ml^{-1} (Fig. 4). An approximately 4 $\log_{10}$ reduction was

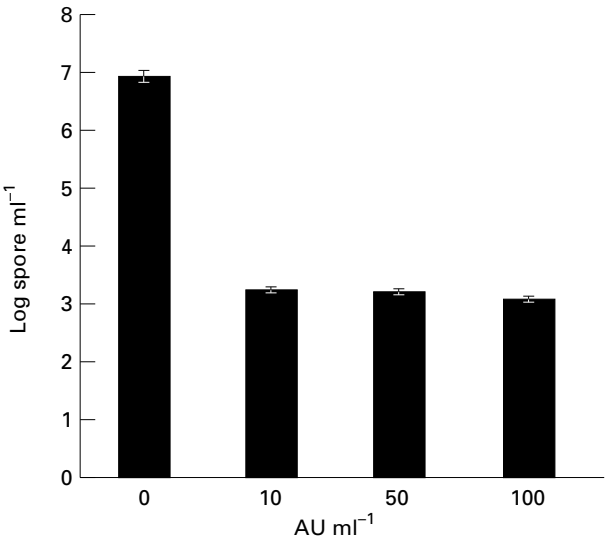

Fig. 4 Effects of coagulin in the plating medium on the colony formation of *Bacillus coagulans* CIP 6625

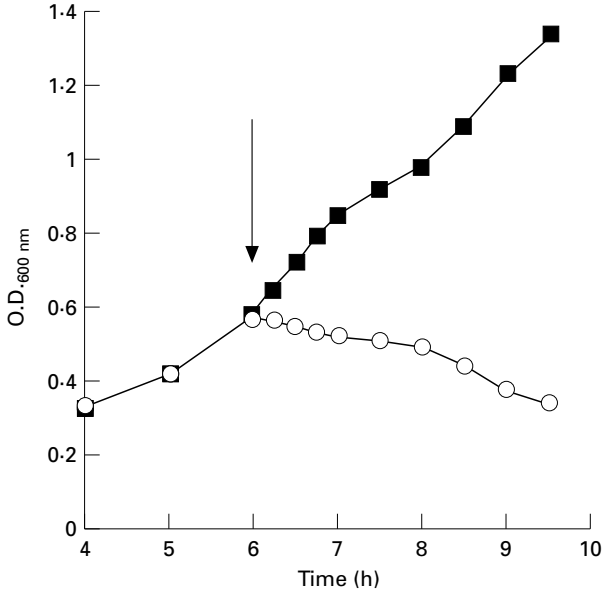

Fig. 5 Bacteriolytic effect of coagulin on *Bacillus coagulans* CIP 6625 growing in MRS broth. ■, Control (without bacteriocin); ○, assay with 60 AU ml^{-1} of coagulin added during the exponential growth (arrow)

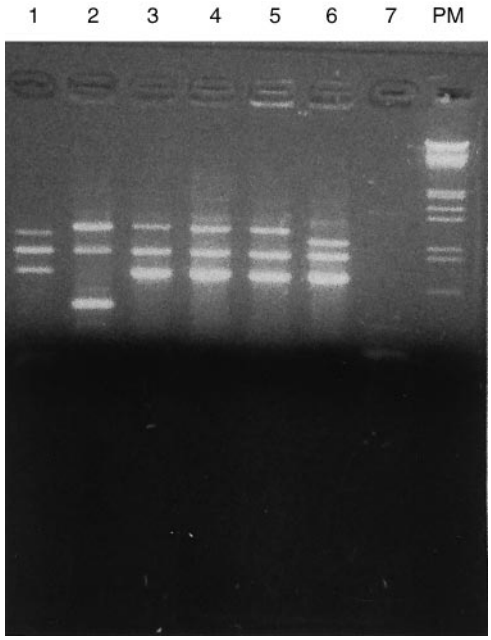

Fig. 6 Band patterns obtained after RAPD amplifications of *Bacillus coagulans* strains with the oligonucleotide OPA13. Lanes: 1, *B. coagulans* LMAB; 2, *B. coagulans* CIP 5264; 3 and 4, $BLIS^-$ derivatives; 5, *B. coagulans* I_4, 6, *B. coagulans* CIP 6625; 7, negative control; PM, molecular weight marker (double *Hin*d III-*Eco*RI digest of λ DNA)

observed when spores were plated in MRS containing 10 AU ml^{-1}. As coagulin concentration increased to 50 and 100 AU ml^{-1}, no further decrease in colony formation was observed. Bactericidal mode of action of coagulin on *B. coagulans* was accompanied by cell lysis as observed in Fig. 5.

Genetic determinants for coagulin production

DNA analysis of *B. coagulans* I_4 demonstrated the presence of a single plasmid of about 14 kb, named pI_4 (data not shown). *Bacillus coagulans* I_4 was grown in the presence of novobiocin to induce plasmid loss. Out of 640 colonies analysed, two stable non-producing ($BLIS^-$) derivatives were obtained. They were confirmed as *B. coagulans* I_4 by RAPD (Fig. 6). Amplifications carried out using primer OPR13 yielded distinct profiles for the four *B. coagulans* strains tested: LMAB, CIP 5264, I_4 and CIP 6625 (Fig. 6, lanes 1, 2, 5 and 6, respectively). $BLIS^-$ derivatives (lanes 3 and 4) exhibited a profile clearly identical to that of strain I_4. Similar results were obtained after SDS-PAGE analysis of whole protein content (data not shown). Analysis of the plasmid content of the $BLIS^-$ derivatives showed that one had lost pI_4. Southern experiments and preliminary restriction analysis revealed that the second strain harboured a homologous plasmid resulting from a deletion event occurring in pI_4 (data not shown). From these results, we suggest a plasmid location for the genetic determinants encoding coagulin production. Both derivatives were sensitive to coagulin. This observation may indicate that determinants for immunity are also likely to reside on the plasmid.

DISCUSSION

A strain of *B. coagulans* producing an extracellular inhibitory substance was isolated during a screening of sporulating micro-organisms from various animal faeces. Preliminary characterizations were carried out by using a neutralized ACFS prepared from a *B. coagulans* I_4 culture. The antibacterial activity was lost upon incubation of the ACFS with proteolytic enzymes, implying the presence of an essential protein or peptide moiety. This protein/peptide exhibited bactericidal activity against several indicator cells. Consequently, and according to the criteria outlined by Jack *et al.* (1995), we attributed the antagonistic effects of strain I_4 to a BLIS contained in the ACFS, further named coagulin. To our knowledge, this is the first report of an antimicrobial compound produced by *B. coagulans*.

Direct detection of antibacterial activity on SDS-PAGE suggested that inhibitory activity was due to a low molecular weight (about 3000–4000 Da) peptide. Nevertheless, SDS-PAGE did not reveal a specific band corresponding to the active coagulin. Similarly, other bacteriocins were found to be difficult to stain for unknown reasons (Héchard *et al.* 1992;

Van Laack *et al.* 1992; Villani *et al.* 1995). Low molecular weight has also been reported for other bacteriocins, including cerein, 9 kDa, produced by *B. cereus* (Naclerio *et al.* 1993). Some other properties make coagulin resemble BLIS and bacteriocins produced by other Gram-positive micro-organisms. Production during the log phase of growth, relative insensitivity to pH and temperature and bactericidal action against actively growing cells were similar to those of other described antimicrobial peptides (Jack *et al.* 1995). In the case of coagulin, the bactericidal mode of action was observed to cause concomitant cell lysis on sensitive indicator cells. In *Staph. simulans*, cell lysis by Pep5 and nisin has been proposed to result from the influence of the cationic properties of the antimicrobial peptides on the activity of autolytic enzymes (Bierbaum and Sahl 1991). Coagulin was demonstrated to have a significant and specific effect on *B. coagulans* spores, suggesting an effect on resting spores and/or on their germination.

Coagulin was shown to be broadly active, which is also typical of Gram-positive BLIS and bacteriocins (Jack *et al.* 1995) and particularly of antimicrobial peptides produced by *Bacillus* species. Among those, only cerein exhibits a narrow inhibitory spectrum, restricted to strains related to its producer, *B. cereus* (Naclerio *et al.* 1993). Contrary to the thermoleovorins S2 and N9 produced by *B. thermoleovorans,* which inhibit *Salmonella typhimurium* (Novotny and Perry 1992), no Gram-negative bacteria proved sensitive to coagulin.

In Gram-positive bacteria, the genetic determinants for bacteriocin production can be either plasmid- or chromosomally-encoded (Klaenhammer 1993). Some, like nisin, have been shown to be transposon-associated (Horn *et al.* 1991). In Bacillaceae, plasmid and chromosomal locations have been proposed. The determinants for subtilin, produced by *B. subtilis*, have been shown to be chromosomally encoded (Banerjee and Hansen 1988). Megacins (Rostas *et al.* 1980; Stahl 1989) and thermocin (Stahl 1991), produced by *B. megaterium* and *B. stearothermophilus*, respectively, are plasmid-encoded. *Bacillus coagulans* I_4 was shown to harbour a 14-kbp plasmid, pI_4. Evidence for plasmid-encoded coagulin was obtained by plasmid curing, suggesting that coagulin production and immunity are associated with the 14-kbp plasmid.

A number of BLIS and bacteriocins, especially from LAB, have been shown to inhibit certain spoilage and pathogenic micro-organisms. The classic example of a commercially successful naturally produced inhibitory agent is nisin, produced by *Lactococcus lactis* ssp. *lactis*, which has received the GRAS (generally recognized as safe) status (Federal Register 1988). Like nisin, coagulin is active against the food-borne pathogen *L. monocytogenes*. The ability to inhibit this pathogen without interfering with other LAB contained in starters, such as *Lactococcus* or *Lactobacillus*, appears to offer some advantages over existing preservatives. In addition, coagulin displays antibacterial effects on contaminants. Hence, *Ped. damnosus* strains isolated from spoiled wines also proved sensitive to the BLIS produced by strain I_4. Other applications of coagulin can be found in the canned foods industry, where *B. coagulans* is a prominent contaminant in the processing of acid and acidified products. Recently, Roberts and Hoover (1996) suggested that nisin could have a synergistic effect when used in conjunction with pressurization, mild heat treatment and low pH. Coagulin could represent an interesting alternative.

In conclusion, we have demonstrated the presence of a BLIS produced by *B. coagulans*, inhibiting pathogens and food-spoilage bacteria. Work is currently in progress to characterize the plasmid-encoding determinants.

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