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Purification and partial characterization of bacillocin 490, a novel bacteriocin produced by a thermophilic strain of *Bacillus licheniformis*

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Abstract

Background: Applications of bacteriocins as food preservatives have been so far limited, principally because of their low antimicrobial activity in foods. Nisin is the only bacteriocin of significant use, but applications are restricted principally because of its very low activity at neutral or alkaline pH. Thus the isolation of new bacteriocins active in foods is desirable.

Results: We isolated a *Bacillus licheniformis* thermophilic strain producing a bacteriocin with some novel features, named here bacillocin 490. This bacteriocin was inactivated by pronase E and proteinase K and was active against closely related *Bacillus* spp. both in aerobic and in anaerobic conditions. Bactericidal activity was kept during storage at 4°C and was remarkably stable in a wide pH range. The bacteriocin was partially purified by elution after adhesion to cells of the food-isolated strain *Bacillus smithii* and had a rather low mass (2 KDa). Antimicrobial activity against *B. smithii* was observed also when this organism was grown in water buffalo milk.

Conclusions: Bacillocin 490 is a novel candidate as a food anti-microbial agent since it displays its activity in milk, is stable to heat treatment and during storage, is active in a wide pH range and has bactericidal activity also at high temperature. These features may allow the use of bacillocin 490 during processes performed at high temperature and as a complementary antimicrobial agent of nisin against some *Bacillus* spp. in non-acidic foods. The small size suggests its use on solid foods.

Background

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by a number of different bacteria [1], whose activity is generally directed towards species that are closely related or share the same ecological niche [2,3]. Bacteriocins produced by Gram-positive bacteria have attracted much attention because of their potential use as food preservatives [4]. Many bacteriocins from Gram-positive bacteria, some of which produced by *Bacillus* spp., have been isolated and studied [5–13]. Nisin, a bacteriocin secreted by *Lactococcus lactis*, is commercially used as a food preservative in forty Countries since 1983 [14]. Like

the lactic acid bacteria (LAB), that are the most studied bacteriocin-producing microorganisms, some representatives of *Bacillus* spp., such as *B. subtilis* and *B. licheniformis*, are "generally recognized as safe" (GRAS) bacteria [15].

A wider use of bacteriocins as antimicrobial agents in milk and dairy foods has been hindered so far by the low activity of many known bacteriocins in foods. In this study we report the isolation and partial characterization of a new bacteriocin with promising properties as an antimicrobial agent against *Bacillus* spp. frequently found in non-acidic foods.

Results

Approximately 23,000 bacterial isolates deriving from dairy foods were tested for their ability to produce antimicrobial activity against Gram-positive and Gram-negative species. We found that 0.48% of tested isolates produce antimicrobial activity against *Salmonella*, while a higher percentage (1.41%) produce anti-*Listeria* activity. The proteinaceous nature of the antimicrobial products was assessed by sensitivity to protease. During the screening, we isolated a strain showing high antimicrobial activity against some adjacent colonies. The producer strain was identified as *Bacillus licheniformis* based on standard phenotypic and metabolic characterization, and 16S ribosomal RNA gene sequence, and named *Bacillus licheniformis* 490/5. The bacteriocin produced, called here bacillocin 490, was completely inactivated by 1 mg of pronase E and proteinase K ml^{-1} but not by the same amount of trypsin or chemotrypsin.

B. licheniformis 490/5 was shown to be thermophilic with optimal growth rate at 65°C. Its growth was inhibited by a commercial bacitracin preparation at concentrations that kill the sensitive species *Staphylococcus epidermidis* but not the resistant one *Escherichia coli*.

The activity range of bacillocin 490 (Table 1) was rather narrow and limited to some Gram-positive bacteria. Highest antimicrobial activity was against *B. stearothermophilus*, *B. smithii*, *B. subtilis* and *B. anthracis*. We also observed some inhibition of *B. cereus* and *B. licheniformis*, very low inhibition of *Listeria innocua* and *S. aureus* and no inhibition of *B. thuringensis* and *Streptococcus thermophilus*. This activity spectrum clearly shows that bacillocin 490 is active principally against species phylogenetically related to the producer strain.

During growth the peak of bacteriocin production occurred in the late logaritmic phase and optimal production was obtained with resting cells at 55°C. Bacteriocin production was increased by 58% and 56% in the presence of 1% (w/v) skimmed milk and gelatin, respectively. This latter result is in agreement with previous observations that, in some cases, solid or semi-solid media (supplemented with gelling agents) increase significantly the amount of bacteriocin production [16–18].

Table 1: Activity spectrum of bacillocin 490

Microorganism	Sensitivity*
***Pseudomonas aeruginosa* ATCC 15442**	-
***Bacillus licheniformis* 5 A2**	+
***Escherichia coli* ATCC 13762**	-
Listeria innocua our isolates	+
***Staphylococcus aureus* ATCC 6538**	-
***Streptococcus faecalis* our isolates**	-
***Salmonella* non *tiphi* our isolates**	-
***Proteus mirabilis* our isolates**	-
***Staphylococcus epidermidis* our isolates**	+
***Bacillus anthracis* 7700**	+++
***Bacillus subtilis* AZ56**	++
***Bacillus cereus* 6A2**	+
***Bacillus stearothermophilus* 9A19**	+++
***Bacillus smithii* PRO/S**	+++
Bacillus thuringensis	-
***Streptococcus thermophilus* ST11**	-

* Results were identical with plates incubated both in the presence and in the absence of oxygen. See Materials and Methods for other experimental details. (-), not inhibited; (+), (++), (+++): low, high, very high inhibition, respectively.

Incubation of *B. smithii* (10^6 bacteria per ml) in the presence of bacillocin 490 (1 μg ml^{-1}) at 60°C resulted in 96% killing in 30 minutes, indicating that the bacteriocin has a bactericidal effect.

Bacillocin 490 was purified taking advantage of its ability to bind to the sensitive cells of *B. smithii*. After elution of bound proteins with phosphate buffer, pH 4.5 (see Materials and Methods), a single 2 kDa band showing bactericidal activity was obtained by 10–25% acrylamide SDS-PAGE electrophoresis (data not shown) with a very high yield (60%) and increased specific activity (from 33.7 to 237.8 AU per mg of total proteins) with a purification factor of approximately 7. The latter low value is probably explained by small amount of protein secreted and is in agreement with other bacteriocin purifications [19]. The apparent weight of the bacteriocin was determined by gel-filtration and resulted approximately 2 kDa in agreement with electrophoretic data.

Purified bacillocin 490 was partially characterized. It was equally active both in the presence and in the absence of oxygen, showed great stability in a wide pH range, since it had optimum activity at neutral and moderately basic pH and maintained some 60% of activity after incubation at

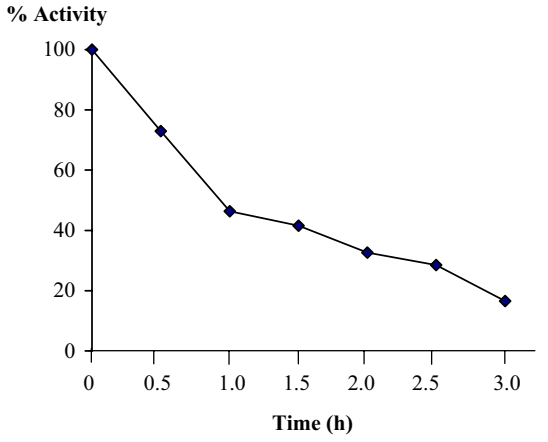

Figure 1
Thermal resistance of bacillocin 490 at 100°C. Other experimental details are in the Materials and Methods.

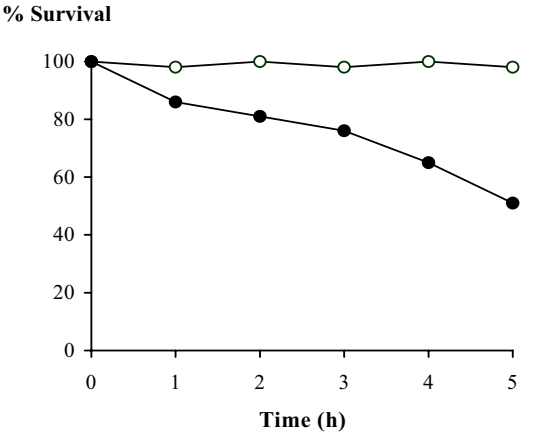

Figure 2
Survival of *Bacillus smithii* in milk at 25°C in the absence (open circles) and in the presence (bold circles) of bacillocin 490. See Materials and Methods for experimental details.

pH 3.5 and 11.0, with 95–100% of activity detected rather uniformly from pH 4.5 to 9.0. This is of great advantage from the industrial point of view, because the pH of many foods varies from very acidic to basic. In this connection it is interesting to notice that nisin, the only bacteriocin so far employed commercially as food additive against Gram-positive bacteria, acts in acidic conditions, as its maximum stability is at pH 2.0, and it is pratically not soluble at pH 8.0 [20].

Bacillocin 490 showed high stability at 100°C with 46.4% residual activity after 1 hour of exposure at this temperature (Fig. 1). It also showed remarkable stability at 4°C (no loss of activity after 12-month storage).

Bacillocin 490 was also used in preliminary experiments as an antimicrobial agent against the most sensitive species, *B. smithii*, inoculated in sterile water buffalo milk. Bacillocin 490 was not affected by protein aggregation since no difference of stability was observed when the bacteriocin was dissolved in milk and in buffer. As shown in Fig. 2, we observed 50% growth inhibition of *B. smithii*, after 5-h incubation in milk in the presence of 65 ng of bacillocin 490 ml^{-1}. This result indicates that this bacteriocin is suitable for milk-based foods.

Two other bacteriocins found in *B. licheniformis* isolates have been described recently. One, named by the authors Lichenin, displays its activity only in strictly anaerobic conditions and against anaerobic organisms [21]. The other, named BSCY2, has a higher molecular mass with respect to both bacillocin 490 and Lichenin, and is moderately heat stable [22].

Conclusions

We report here the identification, purification and partial characterization of bacillocin 490, a *B. licheniformis* bacteriocin with very promising features for industrial applications. It displayed bactericidal activity in milk as well as in synthetic media, showed remarkable stability to heat and during storage and was active up to 60°C in a wide pH range. The latter feature may allow the use of bacillocin 490 at high temperature and as a complementary antimicrobial agent of nisin against some *Bacillus* spp. in non-acidic foods. The small size of bacillocin 490 suggests its employment on solid foods.

Materials and Methods

Bacterial strains and growth conditions

Bacterial strains used are those listed in Table 1, with the addition of *B. licheniformis* 490/5, the bacteriocin producer. All strains, except *S. thermophilus*, were cultured aerobically in TY medium (16 g tryptone l^{-1}, 10 g yeast extract l^{-1}, 5 g sodium chloride l^{-1}). *S. thermophilus* was growth anaerobically using GasPack apparatus (OXOID, Basingstoke, UK) in HJL broth (3% tryptone, 1% yeast extract, 1% lactose, 0.2% beef extract, 0.5% KH_2PO_4, pH 6.5) and plated on solid M17 agar containing 1% lactose.

Solid and soft media were prepared by adding 20 g/liter and 7 g/liter of agar to the broth, respectively.

Bacillus licheniformis 490/5 and *Bacillus smithii* PRO/S were isolated from dairy products at 65°C as adjacent colonies on the same plate. Taxonomic classification of both

strains was performed by use of API 50 CHB fermentation strips (bioMérieux, Marcy l'Etoile, France) and by sequence analysis of one of the 16S rRNA genes amplified with two oligonucleotides: P1 (5'-GCGGCGTGCCT AATACATGC) and P2 (5'-CACCTTCCGATACGGCTACC), annealing to nucleotides 40 to 59 and 1532 to 1513, respectively, of *B. subtilis rrnE*.

Screening of bacteria producing antimicrobial activities

Milk and dairy products were homogenized, diluted and plated onto various selective and non selective media (PCA, M17, MRS) in different growth conditions (varying temperature and oxygen supply). After incubation, individual clones were replicated in duplicate plates, incubated and, after appearance of colonies, one set of plates was $u.v._{254nm}$ irradiated for 15 min to kill the cells and overlaid with 5 ml of 50°C – heated soft agar suspension containing 100 µl of an exponentially growing bacterial culture (10^6 cells per ml) of an indicator strain. Plates were incubated at 37°C for 18 h and inhibition zones were measured. Corresponding producer strains were isolated from the non-irradiated set of plates.

Bacteriocin production and detection

Bacillus licheniformis 490/5 was grown at 55°C in TY broth to stationary phase, and the culture was centrifuged at 2,500×g for 10 min. 20 µl of cell-free filter-sterilized supernatant were spotted on a sterile susceptibility blank discs (OXOID Basingstoke, UK) placed onto a plate filled with 5 ml of solidified soft agar containing 10^6 cells of *Bacillus smithii* (in 100 µl) used as indicator strain for detection of bacteriocin activity. The plate was incubated at 55°C for 18 h in the presence and in the absence of oxygen (the latter condition was accomplished by incubating the plates in GasPack apparatus) and inhibition areas were measured using a GelDoc 2000 photographic apparatus (BioRad) equipped with MultyAnalyst software (BioRad). One Arbitrary Unit (AU) was defined as the amount of bacteriocin producing 1 mm^2 of inhibition area around the sterile disk.

Bacteriocin characterization

To evaluate thermal stability, partially purified bacteriocin was heated at 100°C or exposed at 4°C. Samples were collected and the activity measured at various times and reported as % of values of untreated samples.

Bacteriocin was also treated with pronase E, trypsin, α-chemotrypsin, proteinase K (all purchased from Sigma Chemical Co.). Enzymes were filter-sterilized and added to crude bacteriocin preparations at final concentration of 1 mg/ml. Following incubation at 37°C for 2 h, enzymes were denatured by heating at 100°C for 5 min. Untreated samples incubated in the same conditions were used as controls. Residual activity of samples was determined as mentioned above.

Purification

The bacteriocin was partially purified from 50 ml cultures of *Bacillus licheniformis* 490/5. Cells were grown to stationary phase in TY broth at 55°C and then collected by centrifugation (2,500 × g at 4°C, 10 min). The supernatant was filter-sterilized, ultrafiltered on 1,000 cut-off membrane to concentrate 10-fold and finally mixed with a pellet of pre-washed *B. smithii* cells from 1 liter of an 18 h culture at 55°C. The suspension was gently up-down mixed for 1 h to favour adhesion of the bacteriocin to the cell walls, and then centrifuged 5 min at 700 × g. The supernatant containing unbound proteins was discarded and cells were washed 3 times with 5 ml of 50 mM sodium phosphate buffer, pH 7.0. Finally, bound bacteriocin was eluted with the same buffer at pH 4.5.

SDS-polyacrylamide gel electrophoresis

Electrophoresis was performed by standard protocols [23]. For detection of bacteriocin activity after gel separation a previously reported method was used [9].

Gel-filtration

To determine its apparent molecular weight, the bacteriocin was loaded on a 1.3 ml G-75 Superdex (Pharmacia) gel filtration column, pre-equilibrated with the elution buffer (50 mM sodium phosphate, pH 7.0). Antimicrobial activity was assayed on each fraction.

Stability and antimicrobial activity in milk

For stability studies, equal amounts of bacillocin 490 were added to 10 ml of either 50 mM sodium phosphate buffer pH 7.0 or sterile fresh water buffalo milk and incubated at 25°C. 20 µl of samples were tested for antimicrobial activity each day.

To test the efficacy of bacteriocin against microorganisms present in milk, 10 ml of heat-sterilized fresh water buffalo milk containing 10^6 cells per ml of *Bacillus smithii* strain PRO/S were incubated 5 h at 25°C in the presence of 65 ng of bacteriocin ml^{-1}. 100 µl samples were diluted in buffer and plated on TY medium each time to detect cell survival. A sample treated in the same conditions, without bacteriocin, was used as a control. Plates were incubated at 55°C.

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