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## Purification and characterization of thermophilin ST-1, a novel bacteriocin produced by *Streptococcus thermophilus* ACA-DC 0001

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**Abstract** – Thermophilin ST-1 is produced by *Streptococcus thermophilus* ACA-DC 0001, a “wild” strain isolated from traditional Greek yogurt products. It exerts an inhibitory effect on lactic acid bacteria, several food spoilage and food-borne pathogenic microorganisms, and some Gram-negative phytopathogen bacteria, including *Listeria innocua* BL 86/20, *Enterococcus faecalis* EF1, *Staphylococcus aureus* ATCC 29996, *Xanthomonas campestris* BPIC 1660, *Pseudomonas syringae* BPIC 1549 and *Erwinia rubrifasciens* BPIC 1710. The crude antimicrobial compound is heat-labile (60 °C for 10 min) and sensitive to the proteolytic enzymes pronase and trypsin and high acidic and alkaline conditions, and shows a bactericidal mode of action against the indicator strain *Lactococcus lactis* ssp. *cremoris* CNRZ-117. Production of thermophilin ST-1 starts during the early growth of the producer strain and reaches a maximum titer of 2560 AU·mL<sup>-1</sup> at the end of the exponential growth. Thermophilin ST-1 was partially purified by ammonium sulfate precipitation, ion-exchange and size-exclusion chromatography. SDS-PAGE electrophoresis of purified thermophilin ST-1 showed a single protein band with a molecular mass of 30 kg·mol<sup>-1</sup>, classifying this novel bacteriocin with the large heat-labile proteins. Until now, however, the molecular mass of bacteriocins reported in the species of *S. thermophilus* was less than 10 kg·mol<sup>-1</sup> (small, heat-stable peptides). Curing experiments did not result in the loss of bacteriocin production, suggesting that the genetic determinant is probably located on the chromosome.

***Streptococcus thermophilus* / bacteriocin / thermophilin / lactic acid bacteria / antimicrobial activity / phytopathogen**

**Résumé – Purification et caractérisation de la thermophiline ST-1, une nouvelle bactériocine produite par *Streptococcus thermophilus* ACA-DC 0001.** La thermophiline ST-1 est une bactériocine produite par *Streptococcus thermophilus* ACA-DC 0001, une souche «sauvage» isolée d’un yaourt traditionnel Grec. Elle présente un large spectre d’activité contre les germes pathogènes alimentaires ainsi que contre certaines bactéries Gram-négatives phytopathogènes. Elle inhibe, non seulement, des bactéries lactiques mais également, les souches *Listeria innocua* BL 86/20, *Enterococcus faecalis* EF1, *Staphylococcus aureus* ATTC 29996, *Xanthomonas campestris* BPIC 1660, *Pseudomonas syringae* BPIC 1549 et *Erwinia rubrifasciens* BPIC 1710, ainsi que la souche indicatrice *Lactococcus lactis* ssp. *cremoris* CNRZ-117. L’extrait brut antimicrobien est sensible aux enzymes protéolytiques pronase et trypsine, à la température de 60 °C après 10 min et aux conditions très acides et très alcalines. La production de la thermophiline commence dès le début de

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la phase de croissance et atteint son niveau maximum d'activité de 2560 AU·mL<sup>-1</sup> à la fin de la phase exponentielle. Elle a été partiellement purifiée après précipitation au sulfate d'ammonium, chromatographie d'échange d'ions et ensuite chromatographie d'exclusion stérique. Sa masse moléculaire apparente est d'environ 30 kg·mol<sup>-1</sup>. L'électrophorèse SDS-PAGE de la thermophile ST-1 purifiée présentait une seule bande avec une masse moléculaire de 30 kg·mol<sup>-1</sup>, classant cette nouvelle bactériocine sous les protéines de haut poids moléculaire et thermolabiles. Jusqu'à présent, la masse moléculaire des bactériocines retrouvées dans les différentes espèces de *S. thermophilus* était inférieure à 10 kg·mol<sup>-1</sup> (petites peptides stables à haute température). De récents essais de cure ont montré une stabilité génétique de la production de la bactériocine, suggérant une localisation probable du gène codant pour la thermophile ST-1 sur le chromosome.

***Streptococcus thermophilus* / bactériocine / thermophile / bactérie lactique / activité antimicrobienne / phytopathogène**

## 1. INTRODUCTION

Due to the general tendency to decrease the use of chemical additives in foodstuffs, current research is mainly focused on the use of naturally-occurring metabolites produced by selected bacteria to inhibit the growth of undesirable microorganisms. Among the different types of antagonistic compounds produced by lactic acid bacteria, bacteriocins have gained increasing interest as biological food preservatives. Bacteriocins are proteinaceous compounds that inhibit mainly closely related species. Their inhibitory spectrum is restricted to Gram-positive bacteria, but several bacteriocins produced by lactic acid bacteria are also active against food spoilage and food-borne pathogenic microorganisms including *Bacillus cereus*, *Clostridium botulinum*, *C. perfringens*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli*, *Enterobacteria*, etc. [2, 3, 23, 28, 31]. The above bacteriocins are the most important with regard to food preservation and the development of probiotics. However, the production of bacteriocins that are only active against other lactic acid bacteria could also play a role in the development of starter cultures, since these strains may be used to suppress the growth of non-starter lactic acid bacteria. In addition, Visser et al. [37], have shown that a variety of lactic acid bacteria, isolated from plant surfaces and plant-associated products, were

antagonistic to the test strains of the Gram-negative phytopathogens *Xanthomonas campestris*, *Pseudomonas syringae* and *Erwinia carotovora*.

Numerous reports on bacteriocins from lactic acid bacteria (LAB) are available, and the literature has been reviewed extensively in recent years [10, 12, 20, 26, 32]. However, most papers deal with bacteriocins produced by various lactococci, pediococci, leuconostoc and enterococci, and relatively little is known about bacteriocins from *Streptococcus thermophilus* species.

*Streptococcus thermophilus* is an important dairy starter culture, which is involved in the large-scale production of yogurt and certain cheese varieties, including Feta cheese, in association with other thermophilic strains [33]. The ability of various *S. thermophilus* strains to produce several antibacterial agents has been studied in the past [1, 5, 18, 24, 36, 38]. The aim of this study was to purify and characterize a new bacteriocin produced by the "wild" strain *S. thermophilus* ACA-DC 0001, isolated from traditional Greek yogurt.

## 2. MATERIALS AND METHODS

### 2.1. Bacterial cultures and media

The producer strain *S. thermophilus* ACA-DC 0001 originated from the culture

collection of the Agricultural University of Athens (ACA-DC), and was isolated from traditional yogurt using sheep milk. The sensitive strains originated from the EC-BRIDGE T-Project on the Biotechnology of Lactic Acid Bacteria [30], the culture collection of the Agricultural University of Athens (ACA-DC), the Greek Pasteur Institute and the Collection of the Benaki Phytopathological Institute of Athens (BPIC). All strains were maintained as frozen stocks at  $-80^{\circ}\text{C}$  by the "Protect Bacterial Preservers" TS/70-A system (STC Co., Lancashire, UK).

The liquid media used were Elliker broth (Biokar, Beauvais, France) for the producer strain *S. thermophilus* ACA-DC 0001, M17 broth (Biokar) for lactococci and streptococci, MRS broth (Biokar) for lactobacilli, BHI broth (Biokar) for enterococci, listeria and staphylococci, RCM broth (Biokar) for clostridia and nutrient broth (Biokar) for phytopathogenic bacteria.

## 2.2. Culture extract preparation and bacteriocin assays

To demonstrate the presence of antimicrobial activity in the culture broths of *S. thermophilus* ACA-DC 0001, an agar well diffusion test (ADT) was used based on the assay method described by Tagg et al. [29]. Before testing, cells were removed by centrifugation and the supernatant was neutralized with  $1.0\text{ mol}\cdot\text{L}^{-1}$  NaOH and filter sterilized with a  $0.22\text{ }\mu\text{m}$ , Millex-GV filter (Millipore Co., Bedford, MA, USA). The resulting material is referred to as "crude bacteriocin". Wells 6 mm in diameter were cut into the agar media, containing about  $10^6\text{ cfu}\cdot\text{mL}^{-1}$  of the sensitive strain, by using a cork borer and then  $50\text{ }\mu\text{L}$  of crude bacteriocin were placed into each well. The plates were placed at  $4^{\circ}\text{C}$  for 2 h for crude bacteriocin diffusion, then incubated at the appropriate temperature for each sensitive strain and examined for zones of inhibition. For the bacteriocin titer determination a modification of the critical dilution assay was used [6]. Serial two-fold

dilutions were made and  $50\text{ }\mu\text{L}$  from each dilution were placed in wells in plates seeded with the sensitive strain. The titer was defined as the reciprocal of the highest dilution exhibiting inhibition of the sensitive strain, and was expressed in arbitrary units (AU) per milliliter. Due to the extensive range between two serial dilutions of the critical dilution assay, we also tested the intermediate between the higher and following dilution in order to be more accurate in the bacteriocin titer estimation. *L. lactis* ssp. *cremoris* CNRZ 117 was routinely used as the target organism at  $30^{\circ}\text{C}$ . For the determination of the inhibition spectrum of crude bacteriocin, several sensitive strains were used with the agar well diffusion test, using the appropriate agar media and incubation conditions for their growth (Tab. I).

## 2.3. Kinetics of growth and bacteriocin production

Elliker broth medium was chosen for bacteriocin production studies, taking into consideration the data of Geis et al. [8] and Piard et al. [21] for higher bacteriocin production from lactic streptococci. Elliker broth medium (100 mL) was inoculated with 1 mL from a 16-h culture of *S. thermophilus* ACA-DC 0001 and incubated at  $37^{\circ}\text{C}$ , without agitation. At appropriate intervals, samples were removed for measurement of optical density (OD) at 600 nm and bacteriocin activity, as previously described.

## 2.4. Properties of crude bacteriocin

For a preliminary determination of the size of the bacteriocin produced by *S. thermophilus* ACA-DC 0001, 50 mL supernatant was subjected to ultrafiltration through a series of Diaflo membranes (Amicon Co., Lexington, MA, USA). The diaflo membranes employed included XM300, XM100, XM50, YM30 and YM10. The volumes of retentates and filtrates were recorded and the bacteriocin titers were determined.

**Table I.** Antimicrobial spectrum of crude bacteriocin from *S. thermophilus* ACA-DC 0001.

Sensitive strains	Inhibition zone (mm)
<i>L. lactis</i> ssp. <i>lactis</i> ACA-DC 127 *	11
<i>L. lactis</i> ssp. <i>cremoris</i> CNRZ 117 *	15
<i>Lb. delbrueckii</i> ssp. <i>bulgaricus</i> ATCC 11842 *	-
<i>Lb. delbrueckii</i> ssp. <i>bulgaricus</i> ACA-DC 0085	(8) ‡
<i>Lb. delbrueckii</i> ssp. <i>bulgaricus</i> ACA-DC 0086	(9) ‡
<i>Lb. delbrueckii</i> ssp. <i>bulgaricus</i> ACA-DC 0100 *	-
<i>Lb. delbrueckii</i> ssp. <i>bulgaricus</i> ACA-DC 0104 *	-
<i>Lb. fermentum</i> ATCC 9338 *	-
<i>S. thermophilus</i> ST 20 †	10
<i>S. thermophilus</i> ST 112 †	9
<i>C. sporogenes</i> C 22/10 †	-
<i>C. tyrobutyricum</i> NCDO 1754 †	-
<i>E. faecalis</i> ATCC 10741	11
<i>E. faecalis</i> EF1 †	11
<i>L. innocua</i> BL 86/26 †	11
<i>S. aureus</i> ATCC 29996	10
<i>S. carnosus</i> MC1 †	-
<i>Xanthomonas campestris</i> pv. <i>graminis</i> BPIC 1660 §	11
<i>Erwinia rubrifasciens</i> BPIC 1710 §	9
<i>Pseudomonas syringae</i> pv. <i>lisi</i> BPIC 1549 §	8

\* strains from ACA-DC Culture Collection; † strains from TNO Culture Collection; § strains from Benaki Phytopathological Institute of Athens; ‡ fade zone of partial inhibition; - no inhibition, diameter of well is 6 mm.

The data represent the diameter of the inhibition zone observed, using the agar diffusion test with several sensitive strains as bioassay strains.

Samples of crude bacteriocin (2560 AU·mL<sup>-1</sup>) were examined for susceptibility to proteolytic and other enzymes. The following enzymes (1 mg·mL<sup>-1</sup>) and respective buffers were employed: pronase E, trypsin,  $\alpha$ -chymotrypsin proteinase K, rennin and lysozyme in 0.01 mol·L<sup>-1</sup> sodium phosphate (pH 7.0); ficin in 0.02 mol·L<sup>-1</sup> cysteine hydrochloride, 0.01 disodium EDTA, 0.15 mol·L<sup>-1</sup> NaCl (pH 7.0); pepsin in 0.02 mol·L<sup>-1</sup> HCl; proteinase-K in 0.1 mol·L<sup>-1</sup> sodium acetate, 0.005 mol·L<sup>-1</sup>

calcium acetate (pH 7.5);  $\alpha$ -amylase in 0.01 mol·L<sup>-1</sup> potassium sulphate (pH 7) – all the above enzymes from Sigma Aldrich Co. (St Louis, MO, USA) – and lipase (Serva GmbH, Heidelberg, Germany) in 0.1 mol·L<sup>-1</sup> potassium sulphate (pH 6.0). Crude bacteriocin was mixed with equal volumes of the enzymatic solutions and incubated at 37 °C for 1 h, after which the remaining activity was determined by the agar diffusion method described previously. As a control, bacteriocin with added respective

buffer solution was treated in the same manner as the test preparations.

Samples of crude bacteriocin (2560 AU·mL<sup>-1</sup>, pH 5.5) were adjusted to pH values in the range of 1–12 using 1.0 mol·L<sup>-1</sup> HCl or 1.0 mol·L<sup>-1</sup> NaOH. After storage at 4 °C for 24 h, the samples were neutralized and the remaining antimicrobial activities were measured by the ADT method. An equal to the respective amount of the HCl and NaOH ratio of Ringer solution was added to the samples as a control, taking into consideration the dilution of the bacteriocin titer during the pH adjustment of the samples.

Aliquots of the crude bacteriocin were subjected to two different heat treatments, at 50 °C and 60 °C for 10 min, respectively. The remaining bacteriocin activity was determined by the ADT assay.

Finally, sterile supernatant fluid was stored at 25 °C, 4 °C and –30 °C. At different time intervals, samples were taken from the stored material for determination of the remaining activity.

## 2.5. Mode of action of bacteriocin

To study the effect on actively growing cells, the bioassay strain *L. lactis* ssp. *cremoris* CNRZ 117 was grown in M17 broth at 30 °C, and during the logarithmic phase various concentrations of crude bacteriocin were added. During this experiment, samples were taken and the optical density at 600 nm and number of colony forming units (cfu·mL<sup>-1</sup>) were determined. The cfu of *L. lactis* ssp. *cremoris* CNRZ 117 were determined by plating appropriate dilutions on M17 agar followed by overnight incubation at 30 °C.

To study the effect on stationary phase bacteria, cells of the bioassay strain from the stationary growth phase were used to test the viability against the bacteriocin. *L. lactis* ssp. *cremoris* CNRZ 117 cells (2.5 × 10<sup>7</sup> cfu·mL<sup>-1</sup>), taken from a 18-h culture, were prepared in sodium phosphate buffer 5 mmol·L<sup>-1</sup> (pH 6.2). Sterilized concen-

trated crude bacteriocin was added to the cell suspensions to give titers ranging from 320 to 10 240 AU·mL<sup>-1</sup>. The suspensions were incubated at 30 °C. At intervals of 5, 30, 60 and 90 min samples were taken and cells were removed by centrifugation at 12 000 × *g* for 5 min, washed twice with the phosphate buffer, and finally, the cell viability was determined by counting the colony forming units (cfu·mL<sup>-1</sup>). Surviving cells were enumerated on M17 agar as described previously.

## 2.6. Purification procedure

*S. thermophilus* strain ACA-DC 0001 was grown in 1 L of Elliker broth at 37 °C to stationary phase without agitation. The cells were removed by centrifugation at 10 000 × *g* for 15 min, and the cell-free supernatant was used as starting material for bacteriocin purification (fraction I). All the purification steps were performed at 4 °C.

### 2.6.1. Ammonium sulfate precipitation

Ammonium sulfate was added to a final concentration of 50% (wt/vol), and stirring continued for 3 h at 4 °C. The precipitate was collected by centrifugation at 10 000 × *g* for 30 min and redissolved in 20 mL of buffer A (20 mmol·L<sup>-1</sup> Bis-Tris buffer, pH 7.5) (fraction II).

### 2.6.2. Ion-exchange chromatography

Fraction II was desalted by dialysis with a Spectra/Por-5 dialysis membrane (Spectrum Laboratories, Los Angeles, CA, USA) with a molecular mass cut-off of 12 kg·mol<sup>-1</sup> against 5 L of buffer A for 18 h at 4 °C. The final volume of dialysate was 50 mL. The bacteriocin preparation was subsequently applied to a sepharose QHP 16/10 anion-exchange column (Pharmacia, Uppsala, Sweden) equilibrated with buffer A using a Pharmacia FPLC system. The flowthrough fraction III containing bacteriocin activity

was pooled and concentrated by ultrafiltration through a Diaflo membrane YM30 to a volume of 5 mL. The ultrafiltrate was rediluted with 20 mL sodium acetate ( $20 \text{ mmol}\cdot\text{L}^{-1}$ , pH 5). This twice-repeated procedure resulted in a final 10 mL bacteriocin concentrate in sodium acetate solution ( $5 \text{ mol}\cdot\text{L}^{-1}$ , pH 5). The preparation was applied to a mono-S HR 16/10 cation-exchange column (Pharmacia) equilibrated with sodium acetate solution  $5 \text{ mol}\cdot\text{L}^{-1}$ , pH 5. The activity was eluted with a continuous gradient of  $2 \text{ mol}\cdot\text{L}^{-1}$  NaCl in the same acetate buffer ( $5 \text{ mol}\cdot\text{L}^{-1}$ , pH 5, buffer B) using a FPLC system.

### 2.6.3. Size-exclusion chromatography

The active fractions eluted from the cation-exchange chromatography were applied to a Superose 12 HR10/30 column (Pharmacia) equilibrated with sodium acetate solution ( $20 \text{ mmol}\cdot\text{L}^{-1}$ ) containing  $0.2 \text{ mol}\cdot\text{L}^{-1}$  NaCl, pH 5. Blue dextran 2000 was used to calibrate the column. The following molecular weight reference proteins were used: thyroglobulin (665 000), ferritin (440 000), catalase (232 000), aldolase (158 000), dimer-bovine serum albumin (134 000), bovine serum albumin (67 000), ovalbumin (45 000), chymotrypsinogen (25 000), ribonuclease (13 700) and cytochrome C (11 500). The flow rate was  $1 \text{ mL}\cdot\text{min}^{-1}$  and 1 mL fractions were collected and assayed for absorbance at 280 nm and inhibition activity. The titer of each active fraction was determined.

### 2.7. Protein determination

Protein concentrations of the fractions were determined by the Bicinchoninic acid (BCA) assay using the Tiertek Multiscan system at 540 nm [22].

### 2.8. SDS-PAGE Electrophoresis

SDS-Polyacrylamide gel electrophoresis (PAGE), in the presence of SDS and  $\beta$ -mercaptoethanol and a Tricine buffer system, was used to monitor the purification

steps of the bacteriocin and to determine the molecular mass of the purified thermophilin ST-1 [11, 27]. Polyacrylamide and N,N-methylenebisacrylamide concentrations were, respectively, 4 and 0.5% in the stacking gel, 10 and 0.5% in the spacer gel and 12 and 0.5% in the separating gel.

### 2.9. Plasmid analysis and curing experiments

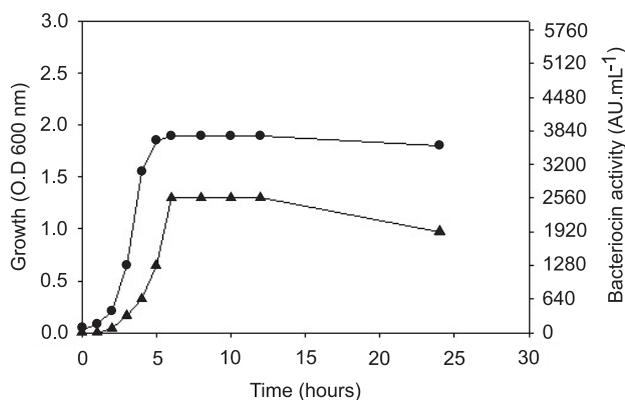
Plasmid DNA was isolated from the lactic cultures grown in Elliker broth with  $40 \text{ mmol}\cdot\text{L}^{-1}$  dl-threonine at  $37^\circ\text{C}$  for 18 h following the protocol described by Maniatis et al. [16]. Isolated plasmid DNA was visualized following electrophoresis in 0.8% (w/v) agarose gels in Tris-borate buffer at 30 V for 15 h, and staining with ethidium bromide solution ( $0.5 \mu\text{g}\cdot\text{mL}^{-1}$ ). *Escherichia coli* V517 containing marker plasmids employed as a single source of covalently closed circular (ccc) DNA molecules of different size [17].

The process of plasmid curing followed the protocol described by Caro et al. [4]. Bacteria were incubated for 24 h at  $43^\circ\text{C}$  in Elliker broth without glycerophosphate in the presence of acriflavine ( $20 \mu\text{g}\cdot\text{mL}^{-1}$ ). The cultures were serially diluted and spread onto Elliker broth agar and then incubated until small colonies were formed (48 h). Plates were then overlaid with semi-solid agar seeded with *L. lactis* ssp. *cremoris* CNRZ 117 as the indicator strain and incubated for an additional 24 h at  $30^\circ\text{C}$ . Colonies were studied for the absence of a zone inhibition, indicating phenotypic loss of the (Bac<sup>+</sup>) trait.

## 3. RESULTS

### 3.1. Antimicrobial spectrum

The crude bacteriocin of *S. thermophilus* ACA-DC 0001 showed inhibition of a wide range of LAB strains of different species and other non-LAB strains. The susceptibility



**Figure 1.** Production of thermophilin ST-1 during growth of *S. thermophilus* ACA-DC 0001. (●) Optical density at 600 nm; and (▲) activity of thermophilin ST-1 in AU·mL<sup>-1</sup>.

of representative strains is shown in Table I. *Lb. delbrueckii* ssp. *bulgaricus* strains were not inhibited by the antimicrobial substance; however, three of them showed a zone of partial clearing. On the contrary, all the *L. lactis* and *S. thermophilus* strains were strongly inhibited. The most notable antimicrobial activity was found against food spoilage and pathogenic bacteria, such as *Enterococcus faecalis* EF1, *E. faecalis* ATCC 1074, *Listeria innocua* BL 86/26 and *Staphylococcus aureus* ATCC 29996 (Tab. I). However, the antimicrobial activity of bacteriocin was not restricted to Gram-positive bacteria, but also included some Gram-negative phytopathogenic bacteria such as *Xanthomonas campestris* pv. *graminis* BPIC 1660, *Erwinia rubrifasciens* BPIC 1710 and *Pseudomonas syringae* pv. *pisi* BPIC 1549.

### 3.2. Thermophilin ST-1 production

The excretion of thermophilin ST-1 into the growth medium started early in the exponential growth phase (after 2 h of incubation) and reached a maximum titer of 2560 AU·mL<sup>-1</sup> after 6 h of incubation. During the stationary phase, after 24 h of fermentation, the bacteriocin activity decreased to 1920 AU·mL<sup>-1</sup> (Fig. 1).

### 3.3. Properties of crude bacteriocin

During preliminary ultrafiltration studies of crude bacteriocin, 80% of the activity was retained by the YM30 Diaflo membrane (cut-off 30 kg·mol<sup>-1</sup>). Only 2.5% of the activity passed through the membrane to the filtrate and about 17.5% was lost on the membrane (data not shown).

We tested the crude bacteriocin sensitivity (loss of activity) to various enzymes, different pH levels, heat treatments and preservation. The antimicrobial activity was inactivated only with the proteolytic enzymes pronase and trypsin (Tab. II).

Crude bacteriocin was found to be stable at around pH 6 and completely inactivated at pH values between 1 and 3 and 10 and 12. The crude bacteriocin was very sensitive to heat treatment and completely lost its activity after exposure for 10 min at 60 °C (Tab. II).

Crude bacteriocin could be stored at -30 °C or 4 °C for at least 3 and 2 months, respectively, without loss of activity. However, during storage at 25 °C, 50% of activity was lost within 2 months (data not shown).

### 3.4. Mode of action

When the lower amount (40 AU·mL<sup>-1</sup> final inhibitory titer) of crude bacteriocin was added to exponentially growing *L. lactis*

**Table II.** Effect of various treatments on inhibitory activity of crude bacteriocin from *S. thermophilus* ACA-DC 0001.

Treatment	Activity*
Pronase	-
Trypsin	-
$\alpha$ -Chymotrypsin	+
Protease	+
Proteinase	+
Pepsin	+
Ficin	+
Rennin	+
$\alpha$ -Amylase	+
Lysozyme	+
Lipase	+
24 h pH 1–3	-
24 h pH 4–9	+
24 h pH 10–12	-
10 min 50 °C	+
10 min 60 °C	-

\* the data represent the presence (+) and loss (-) of activity observed using the agar diffusion test with *L. lactis* ssp. *cremoris* CNRZ 117 used as bioassay strain.

ssp. *cremoris* CNRZ 117, growth stopped and the viability ( $\text{cfu}\cdot\text{mL}^{-1}$ ) dropped by 2 log units within 24 h (Figs. 2a and 2b). At inhibitory titers of 80 and 160  $\text{AU}\cdot\text{mL}^{-1}$ , a drop in  $\text{cfu}\cdot\text{mL}^{-1}$  of 4 log units was observed during the first 2 h of incubation and growth was not resumed after prolonged incubation (Fig. 2b). Although the viability dropped dramatically upon addition of crude bacteriocin, the optical density of the sensitive culture did not decrease, suggesting that lysis did not occur (Fig. 2a).

Exposure of washed resting cells of *L. lactis* ssp. *cremoris* to 10 240  $\text{AU}\cdot\text{mL}^{-1}$  crude bacteriocin resulted in the killing of over 92% (1 log reduction) of the cells within 5 min and 97% within 90 min (Fig. 3).

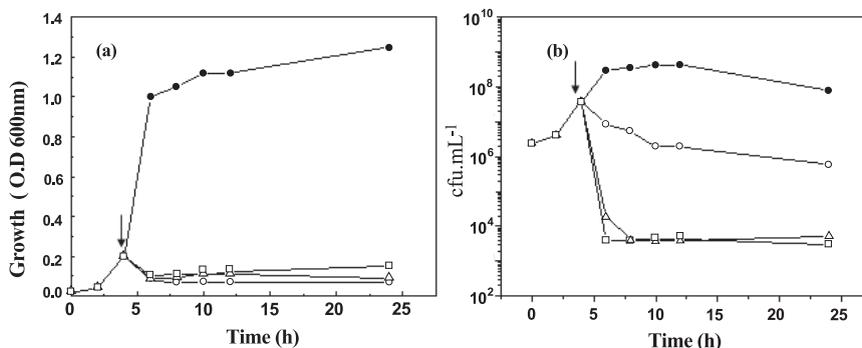
### 3.5. Purification of thermophilin ST-1

The results from the purification procedure are summarized in Table III. The mono-S cation-exchange and gel-filtration chromatographic elution profiles of the bacteriocin are shown in Figures 4a and 4b, respectively. The bacteriocin of *S. thermophilus* ACA-DC 0001 was recovered following 55% saturation of the culture broth with ammonium sulfate, with a simultaneous increase in its specific activity at 10 970 (fraction II). Upon anion-exchange chromatography on sepharose QHP, the bacteriocin was not bound and eluted during washing of the column with buffer A in a volume of 50 mL. The specific activity at this stage was increased almost two-fold and the recovery was only 10% (Tab. III, fraction III). Following the concentration and buffer exchange of bacteriocin with the ultrafiltration procedure, a high specific activity of 29 257 was achieved, but the yield was reduced to 4% (fraction IV). Then fraction IV was passed through a mono-S cation-exchange column, where a strong binding of activity was observed on the column (Fig. 4a). After elution of thermophilin ST-1, the specific activity was increased by four-hundred-fold followed by a low recovery of 0.8% (Tab. III, fraction V).

Upon size-exclusion chromatography, the majority of the bacteriocin activity was eluted in 2 fractions of 1 mL each, with a titer of 1280  $\text{AU}\cdot\text{mL}^{-1}$ . Finally, after this purification step, the specific activity increased five-hundred-fold with an overall recovery of bacteriocin at 0.1% (Tab. III, fraction IV). The elution volume ( $V_e$ ) of thermophilin ST-1 activity from the superose 12 column corresponded to a molecular mass of ca. 30  $\text{kg}\cdot\text{mol}^{-1}$  (Fig. 4b).

### 3.6. SDS-PAGE Electrophoresis

SDS-PAGE of samples of the active size-exclusion chromatography fraction IV showed a protein band migrating close to the 29  $\text{kg}\cdot\text{mol}^{-1}$  marker, confirming the



**Figure 2.** The effect of crude thermophilin ST-1 on (a) growth and (b) survival of sensitive strain *L. lactis* ssp. *cremoris* CNRZ 117 in Elliker broth at 37 °C. Thermophilin ST-1 concentrations of (●) 0; (○) 40; (△) 80; and (□) 160 AU·mL<sup>-1</sup> were added to the growing culture at the beginning of the log growth phase (↓). During the incubation, optical density at 600 nm (OD) and viable cell counts were measured (cfu·mL<sup>-1</sup>).

**Table III.** Purification of thermophilin ST-1.

Purification steps	Fraction No.	Volume (mL)	Activity (AU·mL <sup>-1</sup> )	Total act. (AU)*	Protein (mg·mL <sup>-1</sup> )*	Specific activity (AU·mg <sup>-1</sup> )*	Recovery (%)*	Purification (fold)*
Culture supernatant	I	1000	2560	2 560 000	10	256	100	1
Ammonium sulphate precipitate	II	20	15 360	307 200	1.4	10 970	12	43
Sepharose QHP	III	50	5120	256 000	0.4	12 800	10	50
Ultrafiltrate concentrated	IV	10	10 240	102 400	0.35	29 257	4	114
Mono-S	V	2	10 240	20 480	0.1	102 400	0.8	400
Superose-12	VI	2	1280	2560	0.01	128 000	0.1	500

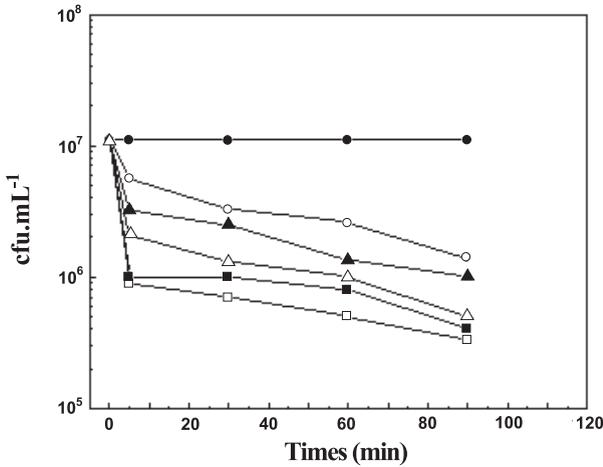
\* Total activity is determined by the multiplication of Volume with Activity. Protein concentration was determined by BCA chromatometric method. Specific activity is activity units divided by the protein concentration (AU·mg<sup>-1</sup>). Recovery is the remaining total activity as a percentage of the initial total activity. Purification fold is the increase of the initial specific activity.

molecular mass of thermophilin ST-1 obtained by the size-exclusion chromatography (Fig. 5).

### 3.7. Plasmid analysis

Plasmid analysis revealed that the original strain *S. thermophilus* ACA-DC 0001, wild-type, harbored one small plasmid of

approximately 2.7 kbp (Fig. 6, lane 2). Additional efforts were made to determine whether this plasmid carried genetic determinants responsible for thermophilin ST-1 production by incubating producer cells at 43 °C in the presence of acriflavine (20 µg·mL<sup>-1</sup>). No mutants deficient in thermophilin ST-1 production (Ther<sup>-</sup>) were detected during examination of single colony



**Figure 3.** The effect of different concentrations of crude thermophilin ST-1 on *L. lactis* ssp. *cremoris* CNRZ 117 suspended in 5 mmol·L<sup>-1</sup> phosphate buffer, (pH 6.2) at 30 °C. (●) 0; (○) 320; (▲) 1280; (△) 2560; (■) 5120; and (□) 10 240 AU·mL<sup>-1</sup>.

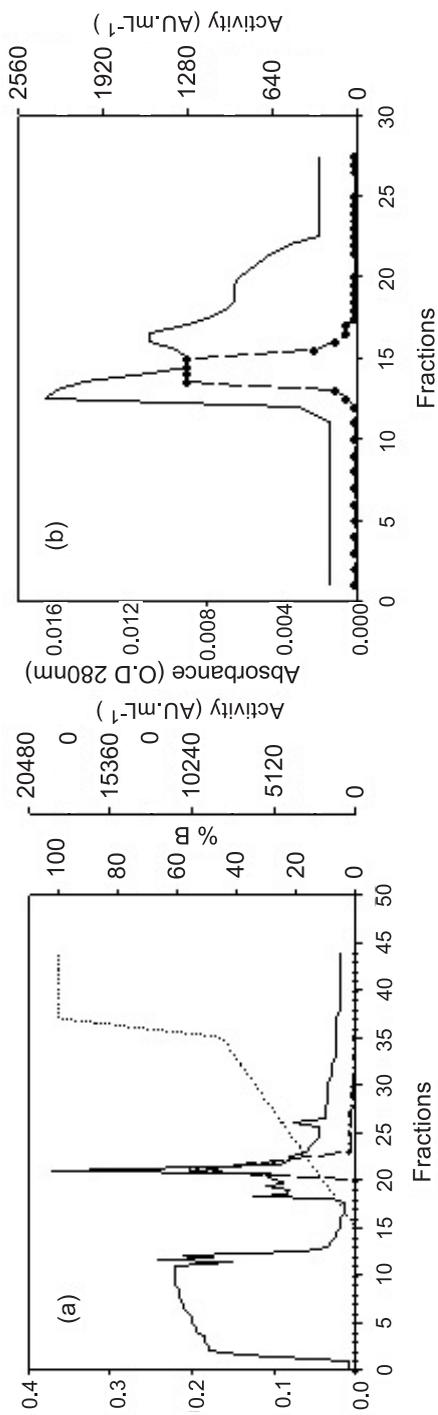
isolates for loss of the phenotype. Plasmid DNA isolations were performed on 8 Ther<sup>+</sup> colonies isolated after growth of *S. thermophilus* ACA-DC 0001 in Elliker broth containing acriflavin (20 µg·mL<sup>-1</sup>). Two of the Ther<sup>+</sup> isolates were found to be cured of the 2.7 kbp plasmid, as is shown in Figure 6 (lanes 5 and 8).

#### 4. DISCUSSION

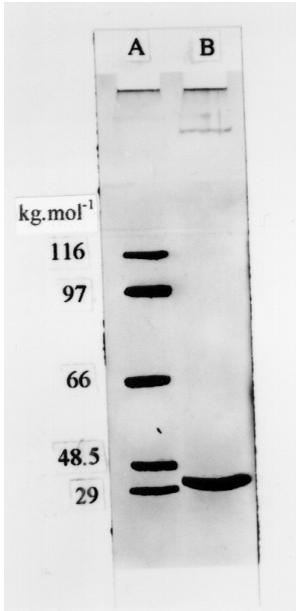
The data derived from this study demonstrate that thermophilin ST-1, the bacteriocin isolated from the strain *S. thermophilus* ACA-DC 0001, has a wide antimicrobial spectrum including lactic acid bacteria and also a range of various Gram-positive and Gram-negative microorganisms. In general, among the LAB tested, lactobacilli were resistant to thermophilin ST-1, whereas lactococci and other *S. thermophilus* strains were the most sensitive. The resistance of lactobacilli to thermophilin ST-1 should exert a favorable effect on the synergistic interference of both these thermophilic microorganisms used for the production of yogurt and other fermented dairy products. The most notable inhibition was that against some potentially food-borne pathogenic bacteria such as *E. faecalis*, *L.*

*innocua* and *S. aureus* strains; however, *Clostridium* sp. was not inhibited. Previous studies have shown that thermophilin 347 and thermophilin ST-13 exhibit an antilisterial activity [18, 36], whereas thermophilin T is inhibitory against *Clostridium* sp. [1]. However, the inhibitory spectrum of several thermophilins appears to be limited only to some closely related lactic acid bacteria [5, 9, 38]. In addition, this inhibitory ability of thermophilin ST-1 against Gram-negative phytopathogenic bacteria suggests that the producer strain could be used for the biological control of bacterial plant diseases, or for the control of post-harvest plant diseases of fruit and vegetables under storage conditions. Similarly, a variety of lactic acid bacteria has also been reported to be antagonistic to test strains of the phytopathogens *Xanthomonas campestris*, *Erwinia carotovora* and *Pseudomonas syringae* [37].

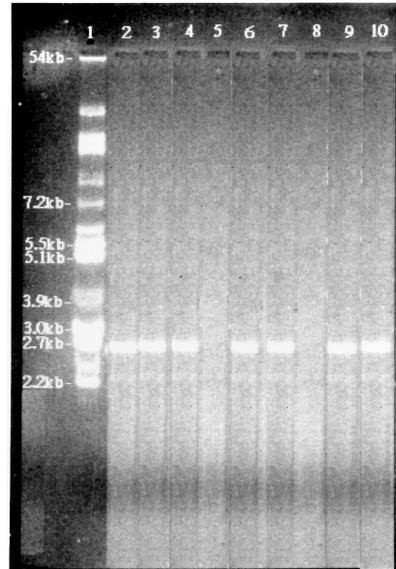
Thermophilin ST-1 production was related to the growth phase of the culture and the highest titers were obtained during the early stationary phase, while further incubation caused a decay in the titer of activity. Similar results have been reported for thermophilin ST10 [5], thermophilin T [1] and thermophilin A [38]. On the contrary,



**Figure 4.** Elution profile of thermophilin 1 on (a) mono-S cation-exchange column and (b) Superose-12 gel filtration column. (—●—) Absorbance; (—●—) activity (AU·mL<sup>-1</sup>); and (···) NaCl gradient (% buffer B).



**Figure 5.** Tricine SDS-polyacrylamide gel electrophoresis containing the molecular size marker (lane A); and the active fraction from Superose-12 gel-chromatography (lane B).



**Figure 6.** Agarose gel electrophoresis of plasmid DNA isolated from *E. coli* V517 (54, 7.2, 5.5, 5.1, 3.9, 3.0, 2.7 and 2.2 kb)(lane 1); *S. thermophilus* ACA-DC 0001 (lane 2); and eight (Ther<sup>+</sup>) *S. thermophilus* ACA-DC 0001 after acriflavin curing (lanes 3-8).

the maximum activity of thermophilin 347 was detected in the mid-log phase of growth and rapidly declined after further incubation [36].

The antibacterial activity of thermophilin ST-1 was completely eliminated by treatment with the pancreatic-originating proteolytic enzymes pronase and trypsin, suggesting its proteinaceous nature. In addition, its sensitivity to these enzymes suggests that it may be used as a biological preservative in foods and feeds, as it will not affect the microbial flora of the gastrointestinal tract. Similarly to thermophilin 13 and thermophilin 347 [18, 36], thermophilin ST-1 was not affected by  $\alpha$ -amylase and lipase. Conversely, thermophilin A [38], bacteriocin ST10 [5] and thermophilin T [1], also isolated from *S. thermophilus* strains, were sensitive to  $\alpha$ -amylase. The ability of thermophilin ST-1 to withstand inactiva-

tion from rennin, makes it a more favorable agent for technological applications in cheese manufacturing.

The sensitivity of thermophilin ST-1 to heat (60 °C for 10 min), acid and alkaline treatments suggests its proteinaceous structure of a large molecular mass. The molecular mass of 30 kg.mol<sup>-1</sup>, determined by size-exclusion chromatography and SDS-PAGE electrophoresis, is consistent with the above biochemical properties of thermophilin ST-1 and classifies it as a large heat-labile protein, similar to helveticin J of *Lb. helveticus*, acidophilucin A of *Lb. acidophilus* and lactinins A and B of *Lb. delbrueckii* ssp. *bulgaricus* [13, 15, 34, 35]. On the contrary, other bacteriocins from different *S. thermophilus* strains such as thermophilin A [38], thermophilin 347 [36], thermophilin ST-13 [18] and thermophilin T [1] seem to be included in the non-lantibiotic heat-stable

group of bacteriocins with small molecular mass. The above properties differentiate thermophilin ST-1 and make it unique.

Thermophilin ST-1 showed marked bactericidal action against the sensitive strain *L. lactis* ssp. *cremoris* CNRZ 117, which was concentration-dependent. Cell death was not associated with lysis or leakage of the cell membrane, since no changes in optical density were observed. This finding is consistent with other reports, suggesting that thermophilin T, thermophilin A and several bacteriocins from different lactic acid bacteria can exert a similar effect [1, 13, 28, 38]. However, several bacteriocins causing lysis of sensitive cells have also been reported for several species of lactic acid bacteria [2, 23].

There are several reports of plasmid-associated bacteriocin production from lactic acid bacteria [7, 19, 25], whereas in other cases, bacteriocin production has been linked to chromosomal DNA [14]. The results of this study suggest that the production of thermophilin ST-1 was not associated with a 2.7 kb plasmid, which is harbored in the *S. thermophilus* ACA-DC strain. In addition, the genetic stability of thermophilin ST-1 production, under growth conditions that induce plasmid curing, provides further evidence for chromosomally-encoded thermophilin ST-1 production. Similar observations have also been reported for thermophilin A [38].

Further work will be necessary to test the efficiency of thermophilin ST-1 against more undesirable bacteria in food and in the biological control of phytopathogenic bacteria in vivo. However, the broad inhibitory spectrum of thermophilin ST-1, its genetic stability and sensitivity to pancreatic enzymes may be considered as desirable properties for potential applications as a biopreservative agent.

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