

## Characterization of a bacteriocin produced by *Streptococcus thermophilus* ST134

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**Abstract** A pH-dependent adsorption/desorption technique was used to screen *Streptococcus thermophilus* strains for the production of bacteriocins. Agar-diffusion tests with *S. thermophilus* strains as targets identified 13 out of 41 strains as producers of antibacterial activity. Thermophilin A, the bacteriocin-like substance present in the culture supernatant of *S. thermophilus* ST134 was purified to homogeneity by ammonium sulfate precipitation and ion-exchange chromatography, followed by ultrafiltration. Thermophilin A is a relatively heat-stable and apparently glycosylated bacteriocin with a bactericidal mode of action against sensitive cells.

### Introduction

Bacteriocins are a heterogeneous group of proteins of varying molecular mass and biochemical properties that exhibit bactericidal activity against strains and species closely related to the producer culture (Tagg et al. 1976). The synthesis of bacteriocins may confer a selective advantage by killing other bacteria that are in competition with the producing culture (Farkas-Himsley 1980). Most lactic acid bacteria used in the production of fermented dairy foods synthesize metabolites (hydrogen peroxide, diacetyl, and organic acids) with general antibacterial properties. In addition, many strains of lactic acid bacteria are known to synthesize bacteriocins (Nettles and Barefoot 1993), with antagonism toward related species and, in some cases, other bacteria associated with food spoilage and food-borne illnesses. In contrast to the mesophilic group of starter cultures, information is limited on bacteriocin production in *Streptococcus thermophilus*, an essential starter mi-

crobe in yogurt as well as Swiss and Italian-style cheese fermentations (Cilano et al. 1990).

Components of starter cultures are most often chosen on the basis of bacteriophage resistance and the production of lactic acid and other flavor and aroma compounds (Heap and Lawrence 1976). Inclusion of a bacteriocin-producing strain as a component of a multiple starter culture may interfere with the growth of other strains and negatively influence starter-culture performance. Thus, characterization of bacteriocin-producing strains is an important aspect of culture selection.

In this study, we surveyed the production of bacteriocin-like substances by 41 strains of *S. thermophilus* and report the isolation and characterization of the antibacterial properties of thermophilin A, a bacteriocin-like protein produced by *S. thermophilus* ST134.

### Materials and methods

#### Bacteria and growth conditions

All strains of *Streptococcus thermophilus* were from an in-house culture collection. For the production of antibacterial substances, cultures were grown overnight in tryptone/yeast extract/lactose (TYL) broth, pH 6.5 (Somkuti and Steinberg 1986), at 37°C. Strains of *S. thermophilus* used as indicators were grown in TYL broth for 4–5 h at 37°C.

#### Preparation of bacteriocins

For general screening, antagonistic activity was detected by the acid extraction method (Yang et al. 1992). For isolation of thermophilin A, 1 l TYL broth was inoculated with *S. thermophilus* ST134 and incubated at 37°C. After overnight growth the cell suspension was adjusted to pH 6.5 with 5 M NaOH, and stirred for 15 min. Cells were collected by centrifugation at 10000 g and washed twice with 10 mM sodium phosphate pH 6.5 (P<sub>i</sub> buffer). The cell pellet was resuspended in 10 ml of P<sub>i</sub> 100 mM NaCl buffer, adjusted to pH 2.0 and stirred for 1 h at 4°C, it was then centrifuged at 29000 g for 20 min. The supernatant was adjusted to pH 6.5, filter-sterilized, and tested for bacteriocin activity. The cell-free supernatant of an overnight culture of *S. thermophilus*

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ST134 was also tested following centrifugation and dialysis against  $P_i$  buffer.

### Bacteriocin assays

Bacteriocin activity was assayed by a modified agar diffusion method (Tagg and McGiven 1971). Briefly, agar plates were overlaid with 5 ml 0.75% TYL agar inoculated with 0.1 ml overnight culture *S. thermophilus* ST113 as the indicator strain. Wells of 5 mm diameter were cut into the agar plates and sealed with agar. After adding 100  $\mu$ l of the bacteriocin preparation to each well, diffusion was allowed for 2 h before incubation at 37°C. Plates were scored for zones of inhibition after 16 h.

For semi-quantitative analysis, bacteriocin activity was determined by the critical-dilution assay (Mayr-Harting et al. 1972). Serial twofold dilutions of crude and partially purified bacteriocins were made in 100- $\mu$ l samples of TYL broth in a 96-well microtiter plate. Each well was then inoculated with 50  $\mu$ l 100  $\times$  diluted stationary-phase culture of the indicator organism, *S. thermophilus* ST113. Growth at 37°C was monitored at 660 nm. One bacteriocin unit was defined as the highest dilution of the preparation that prevented the detection of turbidity after overnight incubation. The activity of the bacteriocin preparations was expressed as activity units (AU) per milliliter.

### Kinetics of production

The effect of various media on the production of bacteriocin by *S. thermophilus* ST134 was also evaluated. A 100-ml sample of TYL, micro inoculum broth (MI; Difco Laboratories, Detroit, Mich.), and M-17 broth (Difco) were each inoculated with a 1% inoculum from a stationary-phase culture of *S. thermophilus* ST134. The flasks were incubated at 37°C. Samples were taken at various intervals to determine  $A_{660}$ , culture pH, and bacteriocin activity.

### Effect of enzymes, pH and heat treatment

Samples of partially purified thermophilin A were incubated with several proteolytic enzymes (Sigma Chemical Co., St. Louis, Mo.). Incubation mixtures contained partially purified bacteriocin and 20  $\mu$ l of either trypsin (1 mg/ml), pronase (2 mg/ml), or papain (1 mg/ml). Amyloglucosidase,  $\alpha$ -amylase and  $\beta$ -amylase (Sigma Chemical Co., St. Louis, Mo.) were used at 100  $\mu$ g/ml final concentrations. Bacteriocin/enzyme mixtures were incubated for 1 h at 37°C, filter-sterilized and assayed for inhibitory activity.

To determine the effect of pH on bacteriocin activity, samples of the cell-free supernatant were adjusted between pH 3.0 and 10.0, and held for 2 h at 37°C. Indicator cells (*S. thermophilus* ST113) were suspended in  $P_i$  buffer to yield a concentration of about  $1.0 \times 10^7$  cfu/ml and added to bacteriocin preparations. Mixtures were incubated for 1 h at 37°C and 100- $\mu$ l samples were removed to determine viable counts. Controls consisted of indicator cells in TYL broth adjusted to the corresponding pH values.

To examine thermal stability, 1.0-ml samples of partially purified bacteriocin suspended in  $P_i$  buffer were incubated at various temperatures for 1 h, except for one sample which was autoclaved for 15 min at 121°C. The samples were rapidly cooled in a 4°C ice bath and assayed for activity.

### Mode of action

A 50-ml of TYL broth was inoculated with a 1% dilution (v/v) of an overnight culture of *S. thermophilus* ST113. After reaching early stationary phase of growth ( $A_{660} = 0.6$ ), cells were centrifuged, washed, and resuspended in  $P_i$  buffer. Cell suspensions were diluted to yield about  $10^7$  cfu/ml. Following the addition of bacteriocin, samples were periodically removed, plated in 1.5% TYL agar plates and scored for viable counts after 24 h at 37°C.

### Plasmid influence and bacteriocin production

Plasmids were isolated from *S. thermophilus* ST134 by a standard procedure (Somkuti and Steinberg 1986), and used for the electrotransformation of *S. thermophilus* ST128, a plasmidless, bacteriocin-negative strain (Somkuti and Steinberg 1988). Plates were incubated overnight and transformants were tested for bacteriocin activity as previously described.

### Partial purification of bacteriocin

Solid ammonium sulfate was added to the cell-free supernatant of an overnight (18-h) *S. thermophilus* ST134 culture to 60% saturation. After 16 h at 4°C, the precipitate was recovered by centrifugation and dialyzed against  $P_i$  buffer. The dialysate was concentrated either by lyophilization or ultrafiltration with 10-kDa cut-off membranes (Amicon, Beverly, Mass.). The retentate was resuspended in  $P_i$  buffer and passed through a carboxymethylcellulose column. Proteins were eluted with a linear NaCl gradient (0.0–0.8 M) in  $P_i$  buffer and 2-ml fractions were checked for absorption at 280 nm. Fractions showing activity against the ST113 indicator strain were combined and further purified by ultrafiltration using Amicon membranes (30-kDa cut-off). Protein concentration was determined by the Bio-Rad Bradford protein assay (Bio-Rad Laboratories, Hercules, Calif.) as directed by the manufacturer, with bovine serum albumin as the standard.

### Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Bacteriocin preparations were analyzed in 15% SDS-PAGE as described by Laemmli (1970). After electrophoresis at 10 mA for 5 h, the gel was stained with Coomassie blue R-250.

## Results

### Spectrum of activity

Cell free extracts of 41 strains of *S. thermophilus* were assayed for bacteriocin-like antagonism (Table 1). Us-

**Table 1** Bacteriocin production by *Streptococcus thermophilus*. + Inhibition of the indicator strain, – no inhibition. Cells were grown into the stationary phase. Bacteriocins were isolated by acid adsorption desorption as described in Materials and methods

Indicator Strains	Crude bacteriocin extracts of producing strains															
	101	110	111	122	123	125	126	129	130	131	134	136	137			
ST109	–	+	–	–	–	–	–	+	+	–	+	–	–			
ST110	–	–	–	–	–	–	–	+	+	–	–	–	–			
ST112	–	+	–	–	–	–	–	–	–	–	+	+	–			
ST113	–	+	+	+	+	–	–	+	+	–	+	–	+			
ST118	+	+	+	+	+	+	–	+	+	–	+	+	–			
ST120	+	+	–	+	+	+	–	+	+	–	+	+	–			
ST125	–	+	–	+	+	–	–	+	+	–	+	+	–			
ST127	+	+	+	+	+	+	–	+	+	+	+	+	–			
ST131	–	+	+	+	–	–	+	–	+	–	+	–	–			
ST134	+	+	+	+	+	+	+	+	+	+	–	–	+			
ST132	+	+	+	+	+	+	+	+	+	+	+	+	–			
ST135	–	+	+	+	+	+	+	+	+	+	+	+	–			
ST141	–	+	+	–	–	+	+	+	+	–	+	+	–			
ST142	–	+	–	–	–	–	–	–	–	–	+	–	–			

ing the well-diffusion assay, 13 of the 41 strains tested exhibited antagonistic activity against at least 2 indicator strains. Of these, *S. thermophilus* ST110, ST130 and ST134 exhibited the broadest activity spectrum against other *S. thermophilus* strains. Activity was not detectable against lactobacilli (*L. bulgaricus* and *L. casei*), *Staphylococcus aureus*, or *Listeria monocytogenes*. The putative bacteriocin produced by *S. thermophilus* ST134 (thermophilin A) was selected for further characterization.

#### Kinetics of production

The production of the bacteriocin-like substance was monitored in several media. Figure 1 shows that in TYL broth the maximum bacteriocin titer was reached after 8 h (culture pH of 5.2) and remained unchanged up to 28 h. In M-17 and MI broth, the maximum bacteriocin titer was reached after 24 h and 8 h respectively. The maximum inhibitory titer in all three media was 800 AU/ml and bacteriocin production, for the most part, paralleled growth.

#### Effect of proteolytic enzymes, amylases, pH, and temperature

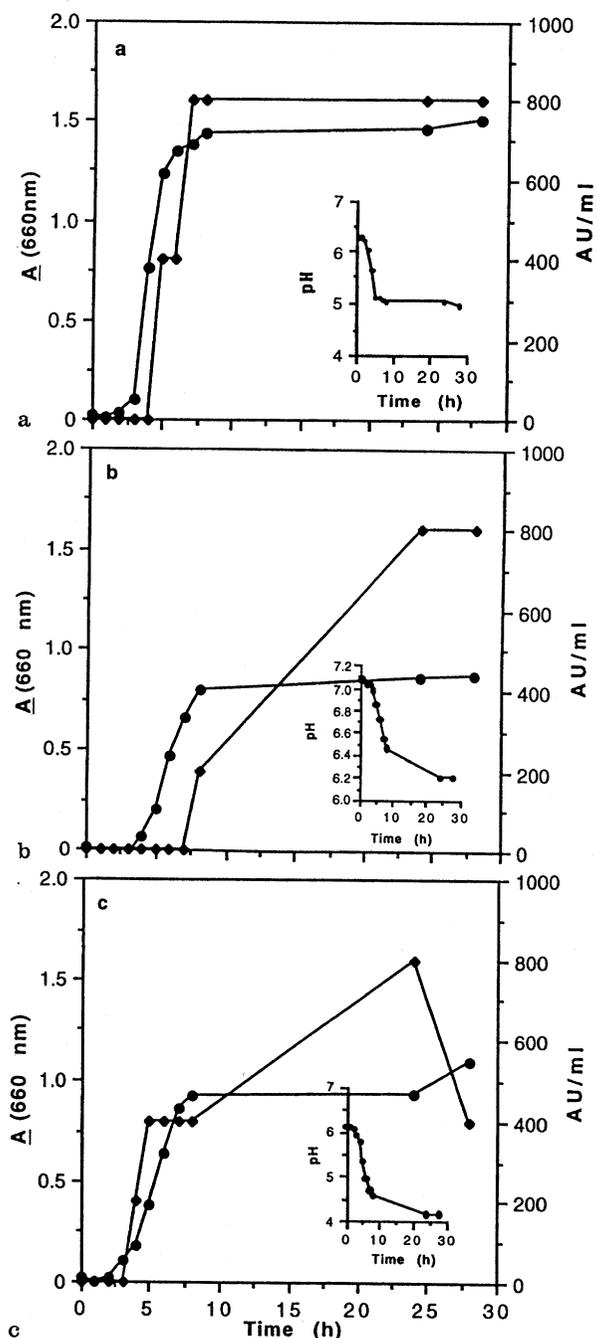
Treatment of the partially purified bacteriocin of ST134 with catalase for 1 h at 37°C did not result in loss of activity. However, thermophilin A was completely inactivated by trypsin, papain, and pronase but not by lysozyme. Treatment with amyloglucosidase and  $\beta$ -amylase had no effect on bacteriocin activity. On the other hand, thermophilin A was totally inactivated after exposure to  $\alpha$ -amylase.

Thermophilin A remained stable between pH 3 and 7 with maximum inhibitory activity detectable at pH 6.5. Bacteriocin activity was lost by exposure to pH 8.0.

There was no reduction in antibacterial titer after heat treatments from 60°C to 100°C for 45 min. However, autoclaving resulted in a nearly 50% loss of bacteriocin activity.

#### Mode of action

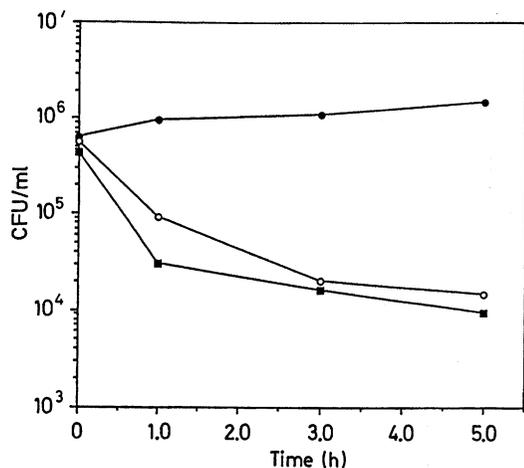
The effect of partially purified bacteriocin on the growth of indicator strain *S. thermophilus* ST113 was determined at two concentrations (Fig. 2). When stationary-phase cells were incubated for 1 h with partially purified bacteriocin at a titer of 25600 AU/ml, an almost tenfold reduction in viable count was observed, while exposure of ST113 for 5 h resulted in a 100-fold reduction. Absorbance values of treated cell suspensions remained constant throughout the experiment indicating the absence of cell lysis.



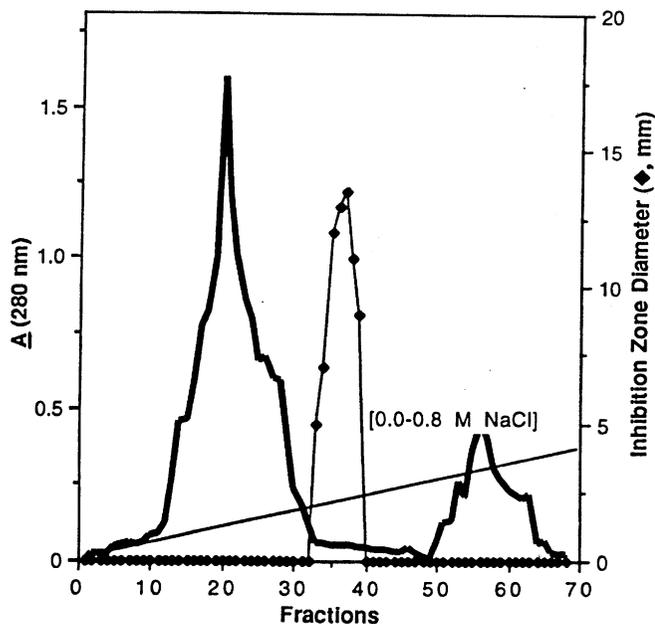
**Fig. 1a-c** Production of bacteriocin by *S. thermophilus* ST134 in (a) tryptone/yeast extract/lactose (TYL), (b) micro-inoculum, and (c) M-17 broths at 37°C. At 1-h intervals aliquots of cultures were removed and pH (●),  $A_{660}$  (●), and bacteriocin activity (◆) were determined

#### Purification of bacteriocin

The bacteriocin of *S. thermophilus* ST134 was recovered following the 60% saturation of the culture broth with ammonium sulfate. Further purification was achieved by ion-exchange chromatography on CM-52 carboxymethyl/cellulose (Table 2). Activity could be

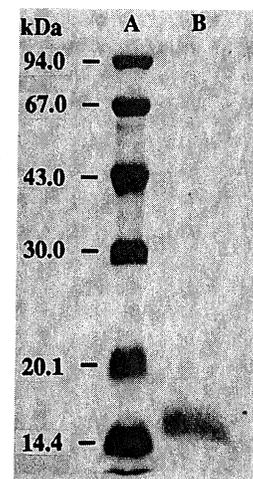


**Fig. 2** Effect of bacteriocin on the growth of *S. thermophilus* ST113. Partially purified thermophilin A was added at (●) zero, (○) 6400 AU/ml, and (■) 25600 AU/ml to cells from an early stationary-phase culture ( $1.0 \times 10^7$  cfu/ml). Survivors were determined by plating on TYL agar



**Fig. 3** Chromatography of bacteriocin on CM-52 cellulose. Ammonium sulfate precipitate (60%) was applied to a CM-52 column equilibrated with  $P_i$ -buffer. Activity was eluted with a linear NaCl gradient (0.0–0.8 M):  $A_{280}$  ◆ thermophilin A activity

**Fig. 4** Sodium dodecyl sulfate/polyacrylamide gel electrophoresis analysis of purified bacteriocin from *S. thermophilus* ST134. A: molecular mass markers, B: thermophilin A



eluted by a linear NaCl gradient (Fig. 3), with the major active peak detectable at 0.15–0.2 M NaCl. Final purification of the bacteriocin was achieved by ultrafiltration of the combined active fractions through an Amicon membrane (30-kDa cut off). Analysis by SDS-PAGE indicated that final bacteriocin preparation contained a single band with a molecular mass corresponding to about 17 kDa (Fig. 4).

## Discussion

Of the 41 strains of *Streptococcus thermophilus* screened, 13 showed antagonism against indicator cultures. the growth of lactobacilli, *S. aureus*, and *L. monocytogenes* was not affected and none of the producing strains demonstrated autoantagonism. The variability in the susceptibility of target strains suggested that *S. thermophilus* strains may produce a variety of antibacterial substances.

The antibacterial activity of the bacteriocin of *S. thermophilus* ST134 was retained under conditions that eliminated antagonistic activity attributable to hydrogen peroxide and organic acids. Inhibition was completely eliminated by treatment with proteolytic enzymes and  $\alpha$ -amylase, indicating the putative presence of a glycosidic moiety in thermophilin A, which may be required for activity. Similar observations were made with apparently glycosylated bacteriocins of other lactic acid bacteria (West and Warner 1988; Lewus et al. 1992; Jimenez-Diaz et al. 1993; Schved et al. 1993). In accordance with the guidelines outlined by Tagg et al.

**Table 2** Purification of thermophilin A

Purification step	Activity (AU/ml)	Protein (mg/ml)	Specific activity (AU/mg)	Yield (%)	Purification (×)
Supernatant	800	1.77	452	100	1
Ammonium sulfate	409600	0.64	640000	36	1415
Ion exchange	102400	0.02	5120000	9	11327

(1976), the antibacterial substance of *S. thermophilus* ST134 was classified as a true bacteriocin and was designated as thermophilin A.

Maximum bacteriocin production by ST134 was detected in the late exponential or early stationary phase of growth with bacteriocin levels remaining stable for at least 28 h. Similar results have been reported for mesenterocin 5 (Daba et al. 1991) and helveticin V.1829 (Vaughan et al. 1992). The exception in this study was MI broth in which the titer of thermophilin A was reduced.

Thermophilin A was stable when exposed to temperatures up to 100°C and even retained partial activity after autoclaving for 15 min. In this respect, thermophilin A exhibited properties similar to those of bacteriocins of several other lactic acid bacteria (Nettles and Barefoot 1993). Thermophilin A was active over a wide pH range and tolerated extreme acid conditions but, like the bacteriocins of other types of lactic acid bacteria, it was completely inactivated after exposure to alkaline pH. Unlike the lactostreptocins isolated from several lactococci (Kozak et al. 1978), thermophilin A apparently did not require an acidic environment to display inhibitory activity.

The antibacterial activity of thermophilin A appeared to be bactericidal to sensitive target cells. Cell death did not appear to be associated with lysis since there were no changes in the absorbances of cell suspensions. This finding was consistent with other reports on lactic acid bacteria (Joerger and Klaenhammer 1986; Schillinger and Lucke 1989), although bacteriocins causing lysis of sensitive cells have been reported (Pucci et al. 1988; Andersson et al. 1988). The bacteriocin of *S. thermophilus* appeared most active against stationary-phase cells.

Classical bacteriocins generally exhibit a narrow spectrum of activity (Tagg et al. 1976). In this respect, thermophilin A resembles true bacteriocins, inhibiting only other strains of *S. thermophilus*. Several strains of lactobacilli and food-borne pathogens such as *S. aureus* and *L. monocytogenes* were not affected by thermophilin A. Cilano et al. (1990) recently reported similar results with other *S. thermophilus* cultures that displayed antagonism only toward closely related strains.

There are several reports of plasmid-associated bacteriocin production in lactic acid bacteria (Davey 1984; Scherwitz et al. 1983; Ray et al. 1989; Mortvedt and Nes 1990), whereas in other cases, production is linked to chromosomal DNA (Joerger and Klaenhammer 1990). The results of this study suggested that the production of thermophilin A not associated with a plasmid in *S. thermophilus* ST134 that is known to harbor pER341 ( $2.7 \times 10^3$  base pairs, kbp) and pER342 (9.5 kbp) (Somkuti and Steinberg 1986). These plasmids failed to transform *S. thermophilus* ST128, a bacteriocin-negative strain, to a bacteriocin-positive phenotype.

In summary, we succeeded in identifying thermophilin A, a possibly glycosylated bacteriocin from *S. thermophilus* ST134. The 17-kDa bacteriocin was iso-

lated in a relatively pure form as indicated by SDS-PAGE analysis. The results also suggested that bacteriocin production in *S. thermophilus* strains is more widespread than recorded previously and should be carefully assessed to minimize strain incompatibility in dairy fermentations.

## References

- Andersson RE, Daeschel MA, Hassan HM (1988) Antibacterial activity of plantarian SIK-83, a bacteriocin produced by *Lactobacillus plantarum*. *Biochimie* 70:381-390
- Cilano L, Bossi MG, Carini S (1990) Bacteriocin production by *Streptococcus thermophilus*. *Microbiol Aliment Nutr* 8:21-30
- Daba H, Pandian S, Gosselin JF, Simard RE, Huang J, Lacroix C (1991) Detection and activity of a bacteriocin produced by *Leuconostoc mesenteroides*. *Appl Environ Microbiol* 57:3450-3455
- Davey GP (1984) Plasmid associated with diplococcin production in *Streptococcus cremoris* 346. *Appl Environ Microbiol* 48:895-896
- Farkas-Himsley H (1980) Bacteriocins, are they broad spectrum antibiotics? *J Antimicrob Chemother* 6:224-227
- Heap HA, Lawrence RC (1976) The selection of starter strains for cheese making. *J Dairy Sci Technol* 11:16-20
- Jimenez-Diaz R, Rios-Sanchez RM, Desmazeaud M, Ruiz-Barbra JL, Piard JC (1993) Plantaricins S and T, two new bacteriocins produced by *Lactobacillus plantarum* LPC010 isolated from a green olive fermentation. *Appl Environ Microbiol* 59:1416-1424
- Joerger MC, Klaenhammer TR (1986) Characterization and purification of helveticin J and evidence for a chromosomally determined bacteriocin produced by *Lactobacillus helveticus* 481. *J Bacteriol* 167:439-446
- Joerger MC, Klaenhammer TR (1990) Cloning, expression, and nucleotide sequence of the *Lactobacillus helveticus* 481 gene encoding the bacteriocin helveticin J. *J Bacteriol* 177:6339-6347
- Kozak W, Bardowski J, Dobrzanski WT (1978) Lactostreptocins, acid bacteriocins produced by lactic streptococci. *J Dairy Res* 45:247-257
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature* 227:680-685
- Lewis CB, Sun S, Montville TJ (1992) Production of an amylase-sensitive bacteriocin by an atypical *Leuconostoc paramesenteroides* strain. *Appl Environ Microbiol* 58:143-149
- Mayr-Harting A, Hedges AJ, Berkeley RCW (1972) Methods for studying bacteriocins. *Methods Microbiol* 7:315-422
- Mortvedt CI, Nes IF (1990) Plasmid associated bacteriocin production by a *Lactobacillus sake* strain. *J Gen Microbiol* 136:1601-1607
- Nettles CE, Barefoot SF (1993) Biochemical and genetic characteristics of bacteriocins of food-associated lactic acid bacteria. *J Food Prot* 56:338-356
- Pucci MJ, Vedamuthu ER, Kinka BS, Vanderbergh PA (1988) Inhibition of *Listeria monocytogenes* by using bacteriocin PA-1 produced by *Pediococcus acidilactici*. *Appl Environ Microbiol* 54:2349-2353
- Ray SS, Johnson MC, Ray B (1989) Bacteriocin plasmids of *Pediococcus acidilactici*. *J Ind Microbiol* 4:163-171
- Scherwitz KM, Baldwin KA, McKay LL (1983) Plasmid linkage of a bacteriocin-like substance in *Streptococcus lactis* subsp. *diacetylactis* strain WM<sub>4</sub>: transferability to *Streptococcus lactis*. *Appl Environ Microbiol* 45:1506-1512
- Schillinger U, Lucke FK (1989) Antibacterial activity of *Lactobacillus sake* isolated from meat. *Appl Environ Microbiol* 55:1901-1906

- Schved F, Lalazar A, Henis Y, Juven BJ (1993) Purification, partial characterization and plasmid-linkage of pediocin SJ-1, a bacteriocin produced by *Pediococcus acidilactici*. J Appl Bacteriol 74:67-77
- Somkuti GA, Steinberg DH (1986) Distribution and analysis of plasmids in *Streptococcus thermophilus*. J Ind Microbiol 1:157-163
- Somkuti GA, Steinberg DH (1988) Genetic transformation of *Streptococcus thermophilus* by electroporation. Biochimie 70:579-585
- Tagg JR, McGiven A R (1971) Assay system for bacteriocins. Appl Microbiol 21:943
- Tagg JR, Dajani AS, Wannamaker LW (1976) Bacteriocins of gram positive bacteria. Bacteriol Rev 40:722-756
- West CA, Warner PJ (1988) Plantacin B, a bacteriocin produced by *Lactobacillus plantarum* NCDO 1193. FEMS Microbiol Lett 49:163-165
- Vaughan EE, Daly C, Fitzgerald GF (1992) Identification and characterization of helveticin V-1829, a bacteriocin produced by *Lactobacillus helveticus* 1829. J Appl Bacteriol 73:299-308
- Yang R, Johnson MC, Ray B (1992) Novel method to extract large amounts of bacteriocins from lactic acid bacteria. Appl Environ Microbiol 58:3355-3359