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Potential of Lactic Streptococci to Produce Bacteriocin

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A survey was made on the bacteriocin-producing potential of lactic streptococci. Bacteriocin-like activities were isolated and partially purified from about 5% of the 280 strains investigated. The frequency of production varied from about 1% in *Streptococcus lactis* subsp. *diacetylactis* to 9 and 7.5% in *S. lactis* and *Streptococcus cremoris*, respectively. Eight strains of *S. cremoris* produced bacteriocins which, on the basis of heat stability at different pH values and inhibitory spectrum, could be divided into four types. From 54 *S. lactis* strains, 5 strains produced inhibitory substances, namely, three nisin-like antibiotics and two diffferent bacteriocins. Only 1 of 93 *S. lactis* subsp. *diacetylactis* strains produced a bacteriocin which was very similar to bacteriocins of type I in *S. cremoris*. All of the bacteriocins that were partially purified by ammonium sulfate precipitation showed very limited inhibitory spectra. Most of the lactic streptococci and a few members of the genera *Clostridium, Leuconostoc*, and *Pediococcus* were inhibited. None of the bacteriocins acted on gram-negative bacteria. The bacteriocinogenic strains were also characterized on the basis of plasmid content. All strains possessed between one and nine plasmids ranging from 1 to 50 megadaltons.

Bacteriocins are proteins with antibiotic activity that are excreted by bacteria. They are characterized by a narrow inhibitory spectrum against closely related bacteria. Many bacteriocin-like activities have been described in gram-positive bacteria, but only a few have been purified and characterized in detail (2, 12).

In lactic streptococci a bacteriocin-like activity was first detected by Whitehead and Riddet (14). Oxford (11) partially purified diplococcin, a bacteriocin produced by *Streptococcus cremoris*. Very recently, Kozak et al. (8) and Davey and Richardson (3) reported on bacteriocin production in lactic streptococci.

Lactic streptococci are used extensively as starter cultures in the dairy industry. The presence of a bacteriocin-producing strain can influence or alter the composition and stability of such a culture (6). In addition to phage infections, bacteriocin production could be a reason for fermentation problems.

The present work represents a survey of the production potential for bacteriocin-like substances of 280 strains of lactic streptococci; the latter are mostly isolates from commercially used starter cultures.

Although it was relatively easy to demonstrate antagonistic interaction by bacteriocin-like substances on solid agar medium, recovery and isolation of these substances were often difficult or unsuccessful. Therefore, inhibitors were only examined if they (i) were excreted into a liquid medium, (ii) could be precipitated by ammonium sulfate, (iii) were inactivated by proteolytic enzymes, and (iv) showed a narrow inhibitory spectrum. We will refer to these substances as bacteriocins.

MATERIALS AND METHODS

Organisms. A wide variety of lactic streptococci from our strain collection and isolates from commercially used starter cultures was used. The strains were normally grown in litmus milk and in lactic broth (5).

Clostridia and lactobacilli were grown anaerobically in RCM (1) and lactic broth, respectively.

Media. Lactic broth (5), M17 (13), RCM medium (1), a synthetic medium (15), and litmus milk were used as growth media.

Screening for antagonistic activities. Overnight cultures of lactic streptococci were spotted onto agar plates. The plates were incubated for 18 h at 30°C to allow colonies to develop. Soft agar (3.5 ml; 0.7%), inoculated with 10^8 cells of the indicator organisms in the late exponential phase of growth, was then poured onto the surface. The plates were checked for zones of inhibition surrounding the lactic streptococcal colonies after incubation at 30°C for 6, 12, and 24 h.

To exclude bacteriophage activity, material was picked out of the areas of inhibition and tested for the presence of bacteriophages by standard procedures.

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Inhibitory activity due to the action of H_2O_2 was excluded by the addition of catalase (5 mg/ml) to the overlayer agar.

In the deferred antagonism test, the bacteria were grown on lactic agar for 18 h. The bacteria were then killed by exposure to chloroform vapor for 30 min or by heat (80°C for 30 min).

Demonstration of antibiotic activities in liquid media and milk. Strains positive in the antagonism assay were grown in different liquid media to the late exponential phase of growth. The cells were removed by centrifugation, and the growth medium was neutralized by the addition of NaOH. The bacteriocin titers were determined by a quantitative serial dilution test. In milk, the bacteriocin titer could not be readily determined by serial dilution. Therefore, 5 ml of sterile milk was inoculated by 10^8 colony-forming units of the test strain and a streptomycin-resistant indicator strain (6). After growth at 30°C for 16 h the number of colony-forming units was determined on lactic agar and on lactic agar supplemented with streptomycin (0.5 mg/ml) and compared with a control culture incubated with 2×10^8 colony-forming units of the indicator strain.

Quantitative serial dilution test. For bacteriocin activity 1 ml of indicator culture (about 10^7 cells per ml) was added to 1 ml of dilutions of the growth media or the partially purified bacteriocins and incubated at 30°C for 4 h. The bacterial growth was determined by measurement of the turbidity. The unit of bacteriocin activity was arbitrarily defined as the reciprocal of the dilution showing 50% growth inhibition compared with a control sample without bacteriocin. As the indicator strain we used a streptomycin-resistant mutant of *Streptococcus lactis* subsp. *diacetylactis*, strain C2-20 (6). This strain was inhibited by all of the bacteriocins so far detected by us.

The bactericidal mode of action of the bacteriocins was determined by counting the number of colony-forming units per milliliter at appropriate intervals after mixing indicator cells with bacteriocin solution under the above-described conditions.

Partial purification of bacteriocin. The neutralized growth medium was concentrated to 1/10 its original volume with a rotary evaporator at 40°C. Ammonium sulfate was added to saturating concentrations, and the sample was stirred overnight at 4°C. The ammonium sulfate precipitate was sedimented by centrifugation (50,000 × *g* for 1 h), resolved in 10 mM Tris-hydrochloride buffer (pH 8.0), and extensively dialyzed against the same buffer. The dialyzed material was freeze-dried and stored until use at −20°C.

Determination of the inhibitory spectrum. Samples (2 to 5 μl) of a solution of the partially purified bacteriocins were spotted onto lactic agar. After drying, 3 ml of 0.7% soft agar containing about 2×10^8 cells of the indicator strain was poured onto the surface. The plates were incubated at the appropriate temperature and checked for inhibition zones after 6 and 18 h.

Isolation of bacteriocin-negative mutants. About 10^8 cells of an overnight culture were inoculated into 5 ml of lactic broth. After incubation for 24 h at 40°C the culture was diluted, and samples were spread onto the surface of lactic agar plates. The plates were incubated for 18 h at 30°C. Colonies were screened for bacteriocin production. Bacteriocin-negative colonies were isolated from replica plates and tested for bacteriocin production and sensitivity to the bacteriocin of the wild type in the serial dilution test.

RESULTS

Screening of lactic streptococci for bacteriocin production. A total of 280 strains were tested for bacteriocin-like antagonism against 4 indicator strains. The indicators were selected as the most sensitive representatives of *S. cremoris*, *S. lactis*, and *S. lactis* subsp. *diacetylactis*. Fifty-six of the tested strains inhibited the growth of at least one indicator strain on lactic agar. In *S. lactis* subsp. *diacetylactis* about one-third of the strains showed antagonism. All of the activities but one, however, were resistant to proteolytic enzymes and sensitive to chloroform. Only one strain excreted a bacteriocin-like substance into liquid media. In *S. lactis* and especially in *S. cremoris*, antagonistic effects were much less frequent, but bacteriocin production occurred more often (Table 1).

Altogether, 16 strains excreted inhibitory substances in lactic broth which were sensitive to proteolytic enzymes and were precipitated by ammonium sulfate.

Bacteriocin production. When grown in lactic broth, bacteriocin production of all strains continued during the exponential phase of growth and stopped when the cells entered the stationary phase. Then, probably due to proteolytic exoenzyme(s) of the producing strains, the bacteriocin activities decreased slowly. Various media, including lactic broth, M17 broth, brain heart infusion, a synthetic medium, and milk were compared for bacteriocin production. The highest titers were obtained with unbuffered

TABLE 1. Screening of lactic streptococci for antagonism and bacteriocin production[a]

Species tested	No. of strains tested	No. of strain exhibiting antagonistic effects on agar[b]	No. of strains producing bacteriocin in liquid medium[c]
S. lactis	54	14	5
S. lactis subsp. *diacetylactis*	93	36	1
S. cremoris	133	15	10

[a] *S. lactis* subsp. *diacetylactis* strain C2-20 and Bu 2, *S. lactis* KC2, and *S. cremoris* AC1 were used as indicator strains.

[b] Strains which showed antagonistic activity grew as colonies which were surrounded by a clear inhibition zone. To exclude the inhibitory activity of H_2O_2, catalase (5 mg/ml) was added to the overlayer agar.

[c] Production of antibiotic substances which showed a narrow inhibitory spectrum, which were sensitive to proteolysis, and which could be precipitated by ammonium sulfate.

TABLE 2. Production of bacteriocins in various media and in milk

Producing strain[a]	Code	Bacteriocin concn[b] (U/ml)				
		Lactic broth	Brain-heart infusion	M17 medium	Synthetic medium	Milk[c]
S. cremoris	AC1	150	116	3	20	2
S. cremoris	1A1	53	25	17	2	4
S. cremoris	3A6	212	67	143	0	0.1
S. cremoris	3C6	67	6	38	0	0.6
S. cremoris	3E9	30	5	0	0	14
S. cremoris	4E9	28	5	0	0	7
S. cremoris	4G6	40	25	26	0	0.1
S. cremoris	9B4	80	22	3	0	0.4
S. cremoris	KC3	83	0	3	0	2
S. cremoris	W3	200	0	3	1	2
S. lactis	2F6	120	0	0	0	30
S. lactis	5D8	600	33	625	38	8
S. lactis	6F3	1,000	6	400	59	17
S. lactis	6F5	250	120	200	83	0.1
S. lactis	7C1	59	7	33	5	7
S. lactis subsp. *diacetylactis*	6F7	138	120	53	0	2

[a] Cells were grown into the late exponential phase. After neutralization of the growth medium, the activity was determined by the serial dilution test.

[b] The actual titers fluctuated from one experiment to the next. The data shown are the highest titers obtained in several experiments.

[c] Colony-forming units as the percentage of the survivors of the indicator strain in mixed milk culture (see text).

lactic broth. In the buffered M17 medium most of the strains showed markedly reduced titers or no activity. Even more reduced bacteriocin titers were found in the synthetic medium. All strains produced antibiotic activities in milk (Table 2).

Properties of the partially purified bacteriocins. All of the bacteriocins were non-dialyzable and could not be sedimented by centrifugation at 200,000 × *g* for 2 h. The bacteriocins were sensitive to proteolysis and resistant to heat (100°C for 30 min) at pH 4.5 and 7.0. At an alkaline pH of 9.4 some of the bacteriocins were inactivated by heat treatment (Table 3). Incubation of the indicator bacteria with the different bacteriocins at 30°C for 1 h resulted in a reduction of viable counts to less than 0.5% of the original values.

Action of bacteriocins on other bacteria (inhibitory spectrum). A variety of gram-positive and some gram-negative bacteria were tested for their susceptibility to the partially purified bacteriocins. On the basis of these results and the properties described in Table 3 the bacteriocins were divided into several types. Tables 3 to 5 allow some general conclusions. (i) All bacteriocins of the same type showed the same response to heat inactivation and to proteolytic digestion (Table 3). (ii) The inhibitory spectra of the bacteriocins of one type were very similar, but not identical (Table 4). (iii) A strain was always resistant to its own bacteriocin and to the other bacteriocins of its own type (compare Table 3 with Table 5). However, a bacteriocin producer may be sensitive to bacteriocins of other types (Table 5). (iv) Mutants, which lost the ability to produce bacteriocin also lost the resistance or immunity to its own bacteriocin and the bacteriocins of the same type (Table 5, compare AC1 with AC1-1 and AC1-2). (v) Only gram-positive bacteria were affected. The tested gram-negative strains (*Escherichia coli, Pseudomonas* sp., *Enterobacter* sp.) were not inhibited.

On the basis of their chemical properties (Table 3) and their inhibitory spectra (Table 4 and 5) the bacteriocins were divided into eight types (I through VIII).

The inhibitory activities of *S. cremoris* bacteriocins (types I through IV) were restricted to other lactic streptococci and to a few members of the genera *Clostridium, Leuconostoc*, and *Pediococcus*. The bacteriocins of type I were resistant to heat inactivation at pH 9.4. They inhibited up to 80% of the tested *S. cremoris* and up to 100% of the tested *S. lactis* strains. The bacteriocins of type II were sensitive to heat treatment at pH 9.4 and much less active against lactic streptococci. Two bacteriocins (types III and IV), which exhibited significantly different inhibitory spectra did not fit into types I or II.

TABLE 3. Responses of the partially purified bacteriocins to digestion with trypsin and heat treatment

Bacteriocin source	Type	Response[a] of bacteriocin to:	
		Trypsin digestion[b]	Heat treatment[c]
S. cremoris			
AC1	I	S	R
3A6	I	S	R
4G6	I	S	R
9B4	I	S	R
1A1	II	S	S
3E9	II	S	S
KC3	II	S	S
W3	II	S	S
4E9	III	S	S
3C6	IV	S	R
S. lactis			
2F6	V	S	R
5D8	VI	R	S
6F3	VI	R	S
6F5	VI	R	S
7C1	VII	S	S
S lactis subsp. *diacetylactis* 6F7	VIII	S	R
Nisin		R	S

[a] S, Bacteriocin activity was sensitive to indicated treatment; R, bacteriocin activity was resistant to treatment.

[b] Digested with 0.5 mg of trypsin for 1 h at 25°C. All bacteriocins listed in this table were sensitive to digestion with chymotrypsin, pronase P, and proteinase K.

[c] Samples were boiled for 30 min in 0.5 M phosphate buffer (pH 9.4). All bacteriocins were resistant to heat treatment in 0.5 M phosphate buffer at pH 4.5 and 7.0.

S. lactis strains produced three types of inhibitors. *S. lactis* 2F6 produced a bacteriocin (type V) that was very similar to the *S. cremoris* type I bacteriocin.

Type VI was separated from all of the other bacteriocins by a broad inhibitory spectrum, including a wide variety of gram-positive bacteria (Table 4). The activity spectrum was similar but not identical to the spectrum of nisin, a polypeptide antibiotic known to be produced by some *S. lactis* strains. The resistance of this inhibitory substance against trypsin and the resistance of the producer strains to nisin suggest that these strains produce an antibiotic peptide of the nisin type.

S. lactis type VII bacteriocin was also very active against various gram-positive bacteria. In contrast to type VI, it did not act on enterococci and *Bacillus subtilis*, but inhibited the producer strains of the nisin-like compounds. The producer strain (*S. lactis* 7C1) was sensitive to nisin.

Only one strain of *S. lactis* subsp. *diacetylactis* could be found that produced a bacteriocin-like substance (type VII). This bacteriocin was very similar to those of type I from *S. cremoris*. It was resistant to heat inactivation at pH 9.4, very sensitive to proteolytic digestion, and acted mainly on lactic streptococci (Tables 4 and 5).

Plasmid profiles of the bacteriocinogenic lactic streptococci. The plasmid profiles of the bacteriocin-producing strains are shown in Fig. 1. The strains contained from one up to nine plasmids of 1 to 50 megadaltons. *S. cremoris* strains 4G6 and 9B4 showed very similar or even identical profiles. The profiles of the other strains were quite distinct.

DISCUSSION

The present study was undertaken to estimate the frequency of bacteriocin production and related inhibitory compounds by lactic streptococci. It was shown that lactic streptococci produced a variety of antimicrobial substances which exhibited restricted inhibitory spectra, a bacteriocidal mode of action, and which were inactivated by proteolytic enzymes. These properties justify the name bacteriocin for these inhibitors (12). In addition, three strains of *S. lactis* synthesized nisin-like activities. With the exception of a more extended inhibitory spectrum against gram-positive bacteria, these antibiotics shared common properties with the bacteriocins. Therefore, they were included in our study.

Antibacterial activities were isolated and partially purified from about 5% of the 280 lactic streptococci strains investigated. The frequency of production varied from less than 1% in *S. lactis* subsp. *diacetylactis* to 9 and 7.5% in *S. lactis* and *S. cremoris*, respectively (nisin producers included).

Eight strains of *S. cremoris* were found to produce a bacteriocin. These bacteriocins were divided into four types on the basis of their heat stability at different pH values and their inhibitory spectra.

Diplococcin, a bacteriocin produced by some *S. cremoris* strains, was recently purified. Of 150 strains, 11 synthesized this inhibitor (3). Since no comparable detailed inhibitory spectrum has been reported and since bacteriocins have not been purified to homogeneity, we do not know whether diplococcin belongs to the bacteriocins described in this study. Diplococcin is heat sensitive to alkaline pH (3). In this respect, it resembles types II and III of the *S. cremoris* bacteriocins described here.

From 54 tested *S. lactis* strains, 5 strains produced inhibitory substances, namely, three nisin-like antibiotics (type VI) and two bacteriocins with quite different properties (types V and

TABLE 4. Activity spectrum of the partially purified bacteriocins from lactic streptococci against gram-positive bacteria

Indicator species	No. of strains tested	No. of strains susceptible to bacteriocins from:																
		S. cremoris											*S. lactis*					Nisin
		I				II				III	IV	V	VI			VII	VIII	
		AC1	3A6	4G6	9B4	1A1	KC-3	W3	3E9	4E9	3C6	2F6	5D8	6F3	6F5	7C1	6F7	
S. cremoris	86	53	68	68	60	39	24	17	23	43	60	65	74	80	78	81	74	81
S. lactis	21	19	21	21	18	10	5	0	2	9	21	19	10	10	12	20	19	15
S. lactis subsp. *diacetylactis*	20	13	14	14	12	7	2	4	0	9	13	13	15	15	17	19	14	20
Lactobacillus sp.	4	0	0	0	0	0	1	1	0	0	0	2	0	4	4	4	0	4
Streptococcus faecalis	10	0	0	0	0	0	0	0	0	0	0	0	9	10	10	0	0	10
Streptococcus sanguis	3	0	0	0	0	0	0	0	0	0	0	0	0	3	3	3	0	3
Pediococcus sp.	2	0	0	1	0	0	0	0	0	2	0	0	1	1	1	1	1	1
Leuconostoc sp.	3	0	0	1	0	1	0	0	0	3	0	0	3	3	3	3	1	3
Staphylococcus aureus	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bacillus subtilis	2	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	2
Clostridium sp.	42	2	5	6	6	1	4	6	1	6	3	3	31	40	38	31	4	36

TABLE 5. Cross-reaction of the partially purified bacteriocins with the bacteriocin producer strains

Indicator strains	Cross-reaction[a] of bacteriocin producer strains with bacteriocin from:																	
	S. cremoris											*S. lactis*						
	I				II				III	IV	V	VI			VII	VIII	Nisin	
	AC1	3A6	4G9	9B4	1A1	KC-3	W3	3E9	4E9	3C6	2F6	5D8	6F3	6F5	7C1	6F7		
S. cremoris																		
AC1	−	−	−	−	−	−	−	−	+	−	−	+	+	+	+	−	+	
3A6	−	−	−	−	−	−	−	−	+	+	−	+	+	+	+	+	+	
4G6	−	−	−	−	−	−	−	−	+	+	−	+	+	+	+	−	+	
9B4	−	−	−	−	−	−	−	−	+	−	−	+	+	+	+	−	+	
1A1	+	+	+	+	−	−	−	−	+	+	+	+	+	+	+	+	+	
KC-3	−	−	−	−	−	−	−	−	+	+	−	+	+	+	+	−	+	
W3	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	+	−	
3E9	−	+	−	−	−	−	−	−	+	+	−	+	+	+	+	+	+	
4E9	−	−	−	+	−	−	−	−	−	+	−	+	+	+	+	+	+	
3C6	+	+	+	−	−	−	−	−	+	−	+	+	+	+	+		+	
S. lactis																		
2F6	+	+	+	+	+	+	−	+	+	+	−	+	+	+	+	−	+	
5D8	+	+	+	+	+	+	−	+	+	+	+	−	−	−	+	+	−	
6F3	+	+	+	+	+	+	−	+	+	+	+	−	−	−	+	+	−	
6F5	+	+	+	+	+	+	−	+	+	+	+	−	−	−	+	+	−	
7C1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	
S. lactis subsp. *diacetylactis*																		
6F7	−	−	−	−	−	−	+	−	+	−	−	+	+	+	+	−	+	
Mutant strains[b]																		
AC1-1	+	+	+	+	−	−	−	−	+	−	+	+	+	+	+	+	+	
AC1-2	+	+	+	+	−	−	−	−	+	−	+	+	+	+	+	+	+	

[a] +, Inhibition of the indicator strain; −, no inhibition. The rectangles mark the actions of the bacteriocins of one type against their producer strains.

[b] AC1-1 and AC1-2 are *S. cremoris* mutants unable to produce an active bacteriocin.

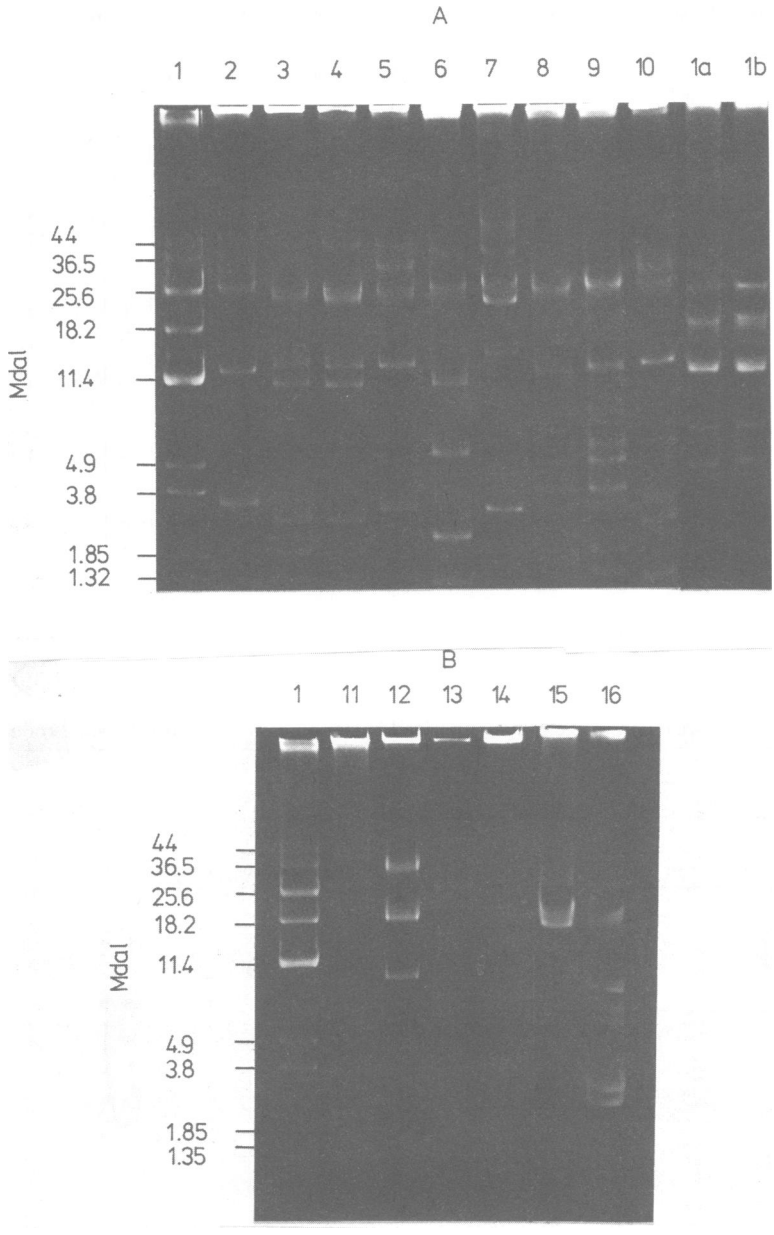

FIG. 1. Plasmid profiles of bacteriocinogenic lactic streptococci. *S. cremoris* strains: 1, AC1; 2, 3A6; 3, 4G6; 4, 9B4; 5, 1A1; 6, KC3; 7, W3; 8, 3E9; 9, 4E9; 10, 3C6; 1a, AC1-1; 1b, AC1-2. *S. lactis* strains: 11, 2F6; 12, 5D8; 13, 6F3; 14, 6F5; 15, 7C1. *S. lactis* subsp. *diacetylactis* strain: 16, 6F7. Plasmid DNA was extracted from cells grown in lysis broth (7) to the late exponential phase of growth by the method of LeBlanc and Lee (10). Plasmids were separated by electrophoresis on 0.6% agarose gels in TEA buffer. (Tris, 40 mM; sodium acetate, 20 mM; disodium EDTA, 1 mM) at 0.8 V/cm. The molecular masses (megadaltons [Mdal]) of *S. cremoris* AC1 plasmids were determined by electronmicroscopy and analysis of restriction fragments by agarose gel electrophoresis.

VII) (Tables 4 and 5). Although more than one-third of the tested *S. lactis* subsp. *diacetylactis* strains showed antagonistic effects on solid agar medium, only one strain produced an inhibitory substance in liquid medium. This bacteriocin was very similar to those of type I from *S. cremoris*. These results are in contrast to those of Kozak et al. (8), who observed bacteriocin (lactostrepcin) production in all of 47 non-nisin-producing *S. lactis* and in 13 of 14 *S. lactis*

subsp. *diacetylactis* strains. In contrast to these lactostrepcins, none of the bacteriocins in this report was inactivated at pH 7 to 8 or by digestion with phospholipase D. We cannot explain the discrepancy in the frequency of bacteriocin production in these two investigations. The use of different media and strains may be the reason. In our study, however, brain heart infusion medium, used by Kozak et al. (8), did not lead to significantly different results (Table 2). We therefore speculate that many very similar or even identical strains may have been used for the detection of lactostrepcins.

When the partially purified bacteriocins were tested against a large number of gram-positive bacteria, the bacteriocins of one chemical type showed differences in their inhibitory spectra (Table 4 and 5). This suggests that even bacteriocins of one type may be a heterogeneous group.

For a clear characterization, purification to homogeneity is necessary, but is difficult to achieve for several reasons. Bacteriocins in reasonable amounts were only produced in rich medium. Removal of high-molecular-weight components from these media by dialysis (data not shown) or growth in a synthetic medium led to unsufficient yields. Interference of peptide components from the medium, increasing instability during purification, and a strong tendency to aggregate complicate each purification procedure, which also has to be optimized for each individual bacteriocin.

All bacteriocin producers possessed up to nine plasmids ranging from 1 to 50 Mdal. There is increasing evidence for the involvement of plasmids in streptococcal bacteriocin production. In enterococci, the plasmid nature of the genetic determinant for two bacteriocin activities has been shown (4). Also, nisin synthesis by some strains of *S. lactis* was believed to be plasmid controlled (9). After growth at 40°C some bacteriocin-negative derivatives of *S. cremoris* AC1 were isolated that had lost two large plasmids (36.5 and 44 Mdal) (Fig. 1) and the resistance or immunity against the bacteriocin of the wild type (Table 5). Further experiments concerning purification and the genetic determination of bacteriocins are in progress.

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