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Streptococcin A-FF22: Nisin-Like Antibiotic Substance Produced by a Group A Streptococcus

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Streptococcin A-FF22 (SA) was shown to occur as both a cell-associated (SA-CA) and an extracellular (SA-EX) component of cultures of the producer bacterium, group A streptococcus strain FF22. SA-CA was solubilized by chemical, enzymatic, and mechanical procedures, similar to those used to release M protein. The independence of SA and M protein in strain FF22 was established by chromatographic separation of the two proteins on the basis of molecular weight and isoelectric point differences between the two substances. Media supporting optimal growth of strain FF22 did not necessarily favor SA production. SA was not produced either at elevated temperatures (39°C) or if the culture was maintained at pH 7 or higher. The release of SA from producer cells was enhanced at lower culture pH values. Much of the SA-CA activity seemed associated with the cell walls of the producer strain, and the nature of the binding appeared to be largely nonspecific in nature and attributable to electrostatic interaction.

Streptococcin A-FF22 (SA) is an antibiotic-like substance produced by group A streptococcus strain FF22 (40). In previous studies it has been established that SA is a proteinaceous substance of approximately 8,000 molecular weight (38) and that it is released extracellularly when the producer strain is grown in certain media, including Todd-Hewitt agar supplemented with 1% glucose (40) and tryptic soy broth (TSB) (38). SA has been found to be bactericidal for a susceptible group A streptococcus (38), and, although it is predominantly active against streptococci, the spectrum of its inhibitory action ranges over a variety of other gram-positive (but not gram-negative) bacteria (40). The genetic determinants of SA production and of producer cell immunity to SA appear to be plasmid-borne (42), and cotransduction of these determinants has been achieved by use of the virulent phage A25 (41).

All of these characteristics of SA seem consistent with the rather loosely defined requirements for a substance to be classified within the subgroup of the antibiotics known as bacteriocins (37). The problem of differentiating some bacteriocin-like substances from other types of inhibitory bacterial products was emphasized to us when it was observed that the "classical" antibiotic nisin, produced by *Streptococcus lactis* (32), is in many respects very similar to SA, a substance we had considered to be a bacteriocin.

In the present study, a cell-associated form of SA (SA-CA) was identified, and its relationship to the previously described extracellular SA (SA-EX) was examined.

MATERIALS AND METHODS

Bacterial strains. The SA-producing (SA^+) organism, group A streptococcus strain FF22 (M type 52, T type 3/13), the non-SA-producing derivatives of strain FF22, SPON 1 (M positive) and SPON 6 (M negative), and the sensitive indicator *Staphylococcus aureus* CIT have been used before (42).

Strains of other bacterial species tested for susceptibility to SA were obtained from culture collections of the Departments of Microbiology and Pediatrics at the University of Minnesota and were selected from those used in a previous study (36). Additional M type 52 group A streptococci identified as strains 71-571, 71-230, 70-1562, 70-1563, and CV-830B were obtained from the Department of Pediatrics culture collection. Organisms in regular use were subcultured weekly on blood agar and stored at 4°C. Stock cultures of all strains were stored at −70°C and lyophilized.

Media, chemicals, and enzymes. Basic media tested as substrates for the production of SA were Todd-Hewitt broth, tryptic soy broth (TSB), tryptose phosphate broth, and brain heart infusion, all obtained from Difco Laboratories, Detroit, Mich. Blood agar plates were made with Columbia blood agar base (Difco) plus 5% (vol/vol) sheep blood.

Medium supplements used in various experiments were neopeptone (Difco), yeast extract (Difco), glucose (Sigma Chemical Co., St. Louis, Mo.), and TES [*N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid; Sigma]; urea, bovine serum albumin (stan-

dard for protein assay), Pronase, and protease (type V) were all from Sigma.

Group C streptococcal phage-associated lysin was prepared and assayed by the methods of Fischetti et al. (11).

Detection and assay of SA. Inhibitory activity associated with preparations was demonstrated by surface spotting onto standardized lawn cultures of the susceptible indicator strain *S. aureus* CIT (38).

The inhibitory titer of SA preparations was determined by a modification of the punch-hole method (39). The highest twofold dilution (0.05 ml) to give definite inhibition of the indicator lawn was defined as containing 1 arbitrary unit (AU) of SA per ml (38). The titer of SA was the reciprocal of this dilution. Specific SA activity was expressed in terms of AU per milligram of protein, after quantitation of the amount of protein in the samples by the method of Lowry et al. (30).

Solubilization of SA-CA. The methods used to release cell-associated SA were based upon those used for the extraction of M proteins from group A streptococci (14) and included chemical, enzymatic, and mechanical processes.

The cells from a 1-liter TSB culture (18 h/35°C) of group A streptococcus strain FF22 were collected by centrifugation and washed twice in distilled water. The washed cells were divided into five aliquots and resuspended in 4 ml of: (i) distilled water; (ii) 1 M NaCl in 0.05 M phosphate buffer (pH 7); (iii) 7 M urea in 0.05 M phosphate buffer (pH 7); (iv) saline adjusted to pH 10 with NaOH; (v) saline adjusted to pH 2 with HCl. Each of these cell suspensions was divided into 2 × 2-ml volumes; one was boiled for 10 min, and the other was held at 4°C for 24 h. The cells were then removed by centrifugation and preparations (iv) and (v) were adjusted to neutrality. All supernatants were boiled for 5 min to kill residual bacteria and then assayed for activity. Controls included the extractant solutions and extracts prepared (as above) from washed strain SPON 1 (SA^-) cells.

Washed strain FF22 cells from a 100-ml TSB culture (18 h/35°C) were resuspended in 2 ml of 0.05 M phosphate buffer (pH 6) containing 200 U of group C streptococcal phage-associated lysin and 0.01 M 2-mercaptoethanol. After incubation at 35°C for 45 min, cell debris was sedimented by centrifugation, and the supernatant was boiled for 5 min to inactivate residual lysin before assaying the SA activity. The control extractant was a heat-inactivated lysin preparation.

A pellet of washed strain FF22 cells from a 100-ml TSB culture was resuspended in 2 ml of 0.05 M phosphate buffer (pH 6) and then shaken in a Mickle disintegrator for 15 min with an equal volume of Ballotini grade 12 beads. Cell walls and intact cells were removed by centrifugation at 10,000 × *g* for 30 min. After the mixture was boiled for 5 min, the supernatant was assayed for SA.

Mechanical fragmentation of strain FF22 cells. Strain FF22 cells from a 5-liter (18 h/35°C) TSB culture were washed twice in distilled water and resuspended in 150 ml of distilled water. The cells were disrupted by shaking with an equal volume of Ballotini grade 12 glass beads in a Vibrogen cell mill for 30 min. The broken cell debris was removed from the beads by washing with 50 ml of distilled water and pooled together with the fragmented cell suspension. Centrifugation at 3,000 × *g* for 10 min removed most of the remaining intact cells. A cell wall-enriched pellet was then obtained by centrifugation of the cell fragments at 10,000 × *g* for 30 min. The supernatant (containing cell membranes) was retained for further study. The pellet was washed twice in distilled water and resuspended in 200 ml of 0.05 M phosphate buffer (pH 7.3) containing 0.001 M $MgCl_2$. The cell wall fraction was then digested with DNase and RNase at 37°C for 45 min, and the cell walls were washed with distilled water before extraction by boiling in 5 ml of 1 M NaCl for 10 min. SA activity was assayed after removal of the cell wall debris by centrifugation. The cell membrane-enriched supernatant was centrifuged at 30,000 × *g* for 30 min to sediment the membranes. These were washed in distilled water, and SA was extracted (as above) by boiling in 1 M NaCl. The supernatant (after removal of the membranes) was tested for SA activity after precipitation with 80% (wt/vol) ammonium sulfate and resolubilization in 10 ml of distilled water.

Chromatographic separation of SA and M protein. Strain FF22 cells were collected from a 2-liter (35°C/18 h) culture in TSB supplemented with 1% neopeptone and washed twice in distilled water. M protein and SA were extracted by boiling the cells for 10 min in 4 ml of saline (adjusted to pH 2). The supernatant was neutralized after removal of the cells and then assayed for SA and M protein.

Separation of SA and M protein activity was achieved by chromatography on Sephadex G-25 and on carboxymethyl cellulose, using previously established procedures (40). M protein was detected by the Ouchterlony immunodiffusion assay, using M-52 antiserum (34).

RESULTS

Solubilization of SA-CA activity from strain FF22 cells. In previous studies, SA activity has been detected in and purified from the supernatant fluid of cultures of the producer strain (38). Attention was now turned to the determination of the extent of association of SA with the producer cells.

Washed cells from a TSB culture of group A streptococcus strain FF22 were treated with a variety of chemical, enzymatic, and mechanical agents in an attempt to solubilize any SA-CA (Table 1). The yield of SA-CA was greatest after extraction at 4°C in pH 2 saline and by boiling either at pH 2 or in 1 M NaCl. The highest specific activity of SA-CA was recovered after extraction at 4°C in pH 2 saline. No inhibitory activity was detected in extracts prepared from SPON 1 (SA^-) cells. For practical purposes, we found that the most convenient method for extraction of SA-CA was to boil the producer cells in 1 M NaCl for 10 min. Unless stated otherwise, this was the procedure used in all subsequent studies.

Characterization of SA-CA. Preparations of SA-CA and SA-EX were obtained from a 100-ml TSB culture (35°C/18 h) of strain FF22 and were adjusted to pH 7 and to 4 AU of SA per ml. SA-CA was obtained by boiling the washed cells in pH 2 saline for 10 min.

Properties of the two preparations of SA were compared by application of previously described procedures (38, 40). The inhibitory activity associated with both preparations was dialyzable and was inactivated by treatment with 1-mg/ml preparations of Pronase or protease. Identical spectra of activity were demonstrated when the two preparations were spotted onto lawn cultures of a collection of known SA-susceptible and SA-resistant bacteria (36).

TABLE 1. *Solubilization of SA-CA activity associated with group A streptococcus strain FF22*

Treatment[a]	Yield of SA (AU)	Solubilized protein (mg)	Sp act of SA (AU/mg)
(A) Cells resuspended for 24 h at 4°C in:			
Distilled water	0	0.11	0
1 M NaCl	4	0.72	5.6
7 M urea	4	0.86	4.7
Saline (pH 10)	4	1.42	2.8
Saline (pH 2)	16	0.98	16.3
(B) Cells boiled for 10 min in:			
Distilled water	0	3.02	0
1 M NaCl	16	1.84	8.7
7 M urea	0	2.88	0
Saline (pH 10)	0	4.32	0
Saline (pH 2)	16	4.10	3.9
(C) Cells exposed to phage-associated lysin[b]	2	7.20	0.3
(D) Cells shaken for 15 min in a Mickle disintegrator	4	10.72	0.4

[a] For each treatment, the cells from 100 ml of a (18 h/35°C) TSB culture were collected, washed twice in distilled water, and resuspended in 2 ml of the extractant solution.

[b] 200 U for 45 min at 35°C.

Both SA-CA and SA-EX seemed to adsorb non-specifically to SA-susceptible and SA-resistant bacteria (42). When adsorption of SA-CA to M-positive (strain SPON 1) and M-negative (strain SPON 6) derivatives was compared, it was found that the M-negative cells bound approximately twice the SA activity that was bound to the M-positive cells. Similar results were obtained with preparations of SA-EX and were reported previously (42). It seems on the basis of these observations that SA-EX and SA-CA may represent the same substance in different physical states of association within the producer culture.

Effect of medium composition and growth conditions on SA production. In a previous study (38) it was determined that TSB (Difco) was the best of a number of tested basic media for the production of extracellular SA activity. Examination of a 100-ml TSB culture (35°C/18 h) of strain FF22 to determine the relative proportion of SA-EX and SA-CA indicated that the supernatant contained a total of 400 AU of SA (specific activity, 0.44 AU/mg of protein) and that 16 AU of SA (specific activity, 8.7 AU/mg of protein) was recoverable from the cell pellet by boiling in 1 M NaCl.

A selection of media were compared as substrates for the production of SA-CA and SA-EX (Table 2). The highest yield of both forms of SA was obtained in TSB supplemented with 1% (wt/vol) neopeptone. No detectable SA was recovered from brain heart infusion, tryptose phosphate broth, or Todd-Hewitt broth supple-

TABLE 2. *Effect of medium composition on the yield of SA in culture supernatants (SA-EX) and cell pellets (SA-CA)*

Medium	Culture supernatant				Cell pellet		
	Terminal pH of culture	SA activity (AU)	Protein (mg)	Sp act of SA-EX (AU/mg)	SA activity (AU)	Dry wt of cells (mg)	Relative yield of SA-CA (AU/mg of cells)
Brain heart infusion	6.4	0	160.0	0	0	4.74	0
Tryptose phosphate broth	6.0	0	78.4	0	0	2.65	0
THB	6.3	10	104.0	0.10	1	3.23	0.31
THB + 1% yeast extract	5.9	0	116.2	0	0	5.50	0
TSB	5.3	40	89.6	0.44	2	3.40	0.59
TSB + 1% yeast extract	5.7	40	102.4	0.39	2	7.53	0.27
TSB + 1% glucose	5.2	40	94.4	0.42	2	3.46	0.58
TSB + 1% neopeptone	6.1	80	117.6	0.68	4	4.56	0.88

[a] Ten milliliters of medium was inoculated with washed cells from 1 ml of an 18 h/35°C TSB culture of strain FF22 and then incubated at 35°C for 18 h. THB, Todd-Hewitt broth.

mented with 1% (wt/vol) yeast extract. Addition of 1% yeast extract to TSB and to Todd-Hewitt broth increased the cell mass but decreased the specific activity of SA-EX (AU of SA per milligram of protein) and the relative yield of SA-CA (AU of SA per milligram [dry weight] of cells).

The production of detectable SA-CA and SA-EX in relation to the time of incubation of strain FF22 in TSB was examined (Table 3). The time course of SA production was compared for cultures inoculated with cells either with (preincubated in TSB) or without (preincubated in brain heart infusion) detectable SA-CA activity. It seemed that SA-CA was formed first and that detectable SA-EX activity first appeared when the pH had fallen to around 6.6.

The culture pH appeared to have a role in the stability of SA and in its occurrence as either SA-CA or SA-EX. Washed cells from a brain heart infusion culture (35°C/18 h) were used to inoculate TSB and TSB supplemented with either 0.25 or 0.5 M TES buffer at pH 7.0 (Table 4). Although TSB containing TES at 0.5 M gave excellent growth of strain FF22 and maintained the pH at 7, it did not appear to support the production of any SA. An active SA control preparation to which 0.5 M TES (pH 7) was added showed 50% loss of activity, indicating that although TES at pH 7 may possibly interfere with the production of SA, it can also bring about the inactivation of preformed SA. TES (0.5 M, pH 6) did not decrease the titer of SA preparations, showing that it was the elevated pH and not the TES that had contributed to the loss of activity of SA preparations in 0.5 M TES at pH 7. In a related experiment (data not shown) no detectable SA was recovered in TSB cultures of strain FF22 that had been maintained at a pH higher than 7 during growth by the periodic neutralization of the formed acid by addition of 3 M NaOH.

The influence of culture pH on the distribution of SA activity between cells and supernatant was examined using TSB cultures of strain FF22 (35°C/18 h) that had been adjusted to and left (for 4 h at 4°C) at different pH values before removal of the cells and assay of both SA-CA and SA-EX activity (Table 5). Cultures adjusted to pH 7 or higher showed a net loss of SA activity. Association of SA with cells was favored by more neutral pH values. At pH 4 or lower, SA-CA was reduced, presumably due to its extraction into the culture supernatant.

The temperature of incubation of the producer culture has previously (38) been shown to affect the yield of extracellular SA, with optimal production at 35°C and no activity recoverable at 39°C. A similar distribution of SA-CA activity with respect to incubation temperature was observed in the present study. The relative yields

TABLE 4. *Yield of SA-CA and SA-EX in cultures[a] of strain FF22 in TSB and in TSB supplemented with TES buffer at pH 7*

Medium	Terminal pH	Cell mass (mg, dry wt)	SA-CA (AU)	SA-EX (AU)
TSB	5.6	3.4	2	40
TSB plus 0.25 M TES	6.7	4.8	2	10
TSB plus 0.5 M TES	7.0	5.2	0	0

[a] Cells from 1-ml aliquots of a brain heart infusion culture (18 h/35°C) of strain FF22 were washed twice in distilled water and resuspended in 10 ml of the test inoculum at 35°C.

TABLE 3. *Time course of production of SA-CA and SA-EX in TSB cultures*

Sample[a] taken at time (h):	Origin of culture inoculum					
	Cells[b] from brain heart infusion			Cells[b] from TSB		
	Culture pH	SA-CA (AU)	SA-EX (AU)	Culture pH	SA-CA (AU)	SA-EX (AU)
0	7.2	0	0	7.2	0	0
1	7.2	0	0	7.2	0	0
2	7.2	0	0	7.1	0	0
3	7.1	0	0	7.0	0	0
4	6.8	1	0	6.6	1	10
5	6.5	2	10	6.0	2	20
6	5.9	2	20	5.5	2	40
7	5.7	2	40	5.3	2	40
10	5.3	2	40	5.3	2	40
24	5.3	2	40	5.3	2	40

[a] Ten-milliliter samples of the culture were taken, the cells were pelleted by centrifugation, and SA-CA was extracted by boiling in 1 ml of 1 M NaCl.

[b] Cells from a 10-ml (18 h/35°C) culture of strain FF22 were washed twice in distilled water and resuspended in 100 ml of fresh TSB (at 35°C).

of SA-CA (AU per milligram [dry weight] of cells) from cultures grown in TSB at 30, 35, and 39°C were 0.52, 0.59, and 0, respectively.

Subcellular localization of SA-CA activity in strain FF22. The distribution of SA-CA in the producer cells was examined after mechanical disintegration of the organisms and differential centrifugation of the cell fragments to obtain cell wall- and cell membrane-enriched fractions (Table 6). Although a considerable loss of activity apparently occurred in the course of the various processing steps, it seems that much of the SA-CA is firmly complexed within the cell wall-enriched material.

Washed strain FF22 cells from a 50-ml TSB culture (35°C/18 h) were resuspended in 5 ml of 0.05 M phosphate buffer (pH 7) containing 10 mg of protease and incubated at 37°C for 2 h. The cells were then collected by centrifugation and extracted by boiling in 1 ml of 1 M NaCl. No SA activity was detected in either this extract or the protease-containing supernatant recovered after centrifugation, indicating that a large proportion of the salt-extractable SA-CA activity in the cells had been accessible to protease digestion.

TABLE 5. *Influence of pH on the distribution of preformed SA as SA-CA and SA-EX in TSB cultures*[a]

pH adjusted to:	SA-EX (AU)	SA-CA (AU)
2	40	1
3	40	1
4	40	1
5	40	2
5.3[b]	40	2
6	40	4
7	20	8
8	10	8
9	10	4
10	10	4

[a] A 100-ml TSB culture (18 h/35°C) of strain FF22 was divided into 10 × 10-ml aliquots, and each was adjusted to the appropriate pH by use of 3 M NaOH or 3 M HCl. After 4 h at 4°C, the cells were pelleted by centrifugation and boiled in 1 ml of 1 M NaCl in 0.05 M phosphate buffer (pH 7.0) to extract SA-CA.
[b] This aliquot was left at the unadjusted pH of 5.3.

TABLE 6. *Distribution of SA-CA in various fractions of disrupted strain FF22 cells*[a]

Fraction	SA activity (AU)	Protein (mg)	Sp act (AU/mg)
Washed cells	480	85.20	5.6
Cell wall enriched	94	0.58	161.1
Cell membrane enriched	22	4.22	5.2
Supernatant fluid	30	68.12	0.4

[a] Cells from a 5-liter (18 h/35°C) culture of strain FF22 were washed twice in distilled water and disrupted in a Mickle disintegrator. Fractionation was as outlined in Materials and Methods.

Independence of SA and M protein in strain FF22. Certain observations had indicated a possible relationship between SA and the M-52 protein of strain FF22. Both proteins were extracted by boiling in saline at pH 2, and in an earlier study it had been remarked that SA production (like that of M protein) may be enhanced by passage of the producer strain through mice (38). However, a series of experiments was able to establish the independence of SA and M52 proteins.

Physical separation of the M-52 protein and SA activity in an extract (obtained by boiling the producer cells in pH 2 saline) was achieved by column chromatography. On Sephadex G-25, all of the M-52 protein activity was excluded at the column void volume, indicating a molecular weight in excess of 25,000, whereas SA was eluted later in a single peak of activity. Separation on the basis of charge differences between the two molecules was obtained by use of carboxymethyl cellulose (at pH 6.5). The M-52 protein (isoelectric point lower than 6.5) did not adsorb to the cellulose, whereas SA (isoelectric point higher than 7.0) was retained and could be recovered from the gel by elution with 0.5 M NaCl. Further evidence for the independence of M-52 protein and SA was obtained from the observation that none of five other tested strains of M type 52 group A streptococci appeared to produce either SA-CA or SA-EX in TSB cultures.

DISCUSSION

The present study has demonstrated that SA occurs in cultures of strain FF22 as both an extracellular product and a cell-associated component. The amount of SA detected after extraction of the cells was 5 to 10% of that recoverable from broth supernatants. The observation of a firm physical attachment of bacteriocins to their producer cells is not uncommon. Several other bacteriocins produced by gram-positive (4, 9, 25) and gram-negative (8, 19, 23) bacteria have also been observed to occur in cultures in both free and bound forms. Staphylococcin 1580 (25) was found to be 80% supernatant and 20% cell associated in Trypticase soy broth (BBL) cultures of the producer strain. Colicin K appears to be either part of or firmly associated with the somatic antigen of the producer strain (17). Similarly, colicin I seems to be adsorbed to specific colicin I receptors on the surface of the producer strain (23).

It appears that the optimal method of extraction of different cell-associated bacteriocins varies considerably according to the particular bac-

teriocin (37). Favored extractants have included 7 M urea for staphylococcin 462 (18), trichloroacetic acid for an inhibitor produced by phage type 71 staphylococci (2), 1 M NaCl for staphylococcin 1580 (25), colicins E2 and E3 (19), and bacteriocin JF246 of *Serratia marcescens* (13), and Triton X-100 in 1 M NaCl for butyricin 7423 (4). Other bacteriocins seem to be released from the cell mass only after physical breakage of the cells (7, 16) or the enzymatic digestion of other supporting cell wall components (9). The successful elution of SA-CA by 1 M NaCl in the present study suggests that much of this bacteriocin may be bound to the cell surface electrostatically. SA is a basic protein and seems to adsorb rather non-specifically to a wide range of both SA-susceptible and SA-resistant bacteria. Other bacteriocins that appear to lack adsorption specificity for susceptible strains include staphylococcins 414 (16) and 1580 (24) and streptococcin B-74628 (36). The greater adsorption of SA to M-negative group A streptococci is of interest in that it may indicate that sites for M protein attachment on the cell surface (35) are also suitable for attachment of SA. Mechanical fragmentation of the producer cells in 0.05 M buffer solubilized only about 25% of the SA-CA activity, indicating that much of it remained firmly bound to the cell wall fragments of the producer cells.

Evidence was obtained that SA-CA and SA-EX may be the same substance. Their identical spectra of inhibitory activity, protease susceptibility, and similar molecular size suggested that we may be dealing with the same substance in different degrees of association with the producer cells.

It has already been established that the composition of the growth medium is a critical factor in the production of SA-EX (38) and of many other bacteriocins (37). TSB is the most satisfactory basic medium for production of SA, and when supplemented with 1% neopeptone the yield and specific activity of both SA-CA and SA-EX was significantly increased. This effect is probably attributable to the interference of neopeptone with the elaboration of streptococcal proteinase (6) and the resulting reduction in the inactivation of SA by this enzyme (38). Addition of 1% yeast extract to TSB or to Todd-Hewitt broth had the reverse effect on SA yield to that of neopeptone. Although the cell mass was increased, the relative yield of both forms of SA was decreased. Yeast extract has been demonstrated to stimulate proteinase production in cultures and, as a consequence, to reduce the amount of M protein (6). The failure to recover any SA activity in brain heart infusion or tryptose phosphate broths may be due to high levels of proteinase or to some ill-defined deficiency of the media.

The role of pH in the production, stability, and distribution of SA in cultures was of interest. It seemed that creation of an acidic pH in the course of carbohydrate fermentation by the growing culture was essential for the recovery of detectable SA. Producer cultures maintained during growth at pH 7 or higher failed to produce SA. Controls established that SA was inactivated at elevated pH values, but the necessity for an acidic medium to promote SA production also appeared likely. In earlier studies of SA production (in Todd-Hewitt agar), growth of the cultures at a pH lower than 6.5 had been found to be a critical requirement for SA production (40). The culture pH also seemed to have an effect on the distribution of SA activity. SA is a basic protein, and its solubility increases as the pH decreases.

The location of SA-CA activity within the producer cell was studied in several ways. Treatment of washed producer cells with protease removed all detectable SA activity from the cells, indicating that most of the SA-CA that is extractable by boiling in 1 M NaCl had been accessible to the enzyme. Lancefield (28) used a similar test to show that M protein was removed from viable cells on treatment with trypsin and interpreted this to mean that the M protein was located on the cell surface.

Mechanical fragmentation of the producer cells followed by differential centrifugation indicated that much of the SA-CA activity was firmly associated with the cell wall-enriched fraction of the cells. That the cell walls are not, however, necessary for SA production has been demonstrated by previous studies that showed that L-forms of the producer strain can also produce extracellular SA (21).

In the course of the current study certain characteristics of SA were observed to be similar to those of the M proteins associated with group A streptococci, so the possibility of a relationship between these two substances was examined. Differences in the isoelectric points and molecular weights of these two proteins in strain FF22 permitted their physical separation by both ion-exchange and Sephadex chromatography. Moreover, earlier studies of the genetic basis of SA production had indicated that the curing of SA and the loss of M52 protein in strain FF22 were separate events (42) and that the determinants of SA production and immunity were transduced independently of the determinants of M-52 protein (41).

On consideration of the information we now

had available regarding SA, we noticed, with particular interest, that many of the properties of SA were similar to those of the antibiotic nisin, produced by certain strains of *S. lactis*. Diplococcin (33) is yet another antibacterial substance produced by a "milk streptococcus" that has characteristics of both nisin and SA. We have tabulated some of the properties of SA, nisin, and M protein so that the common characteristics can readily be compared (Table 7). The remarkable likeness of SA and nisin, substances that have been categorized as a bacteri-

TABLE 7. *Some characteristics of SA, M protein, and nisin*

Characteristic	SA	M protein	Nisin
Chemical nature	Basic protein (40)	Acidic protein (14)	Basic protein (3)
Molecular weight	Approximately 8,000 (38)	Type-specific molecules have a minimum molecular weight of 6,000 (12), but occurs as multiple molecular structures of up to 300,000 depending on method of extraction (14)	Nisin A has molecular weight of 7,000 but occurs as multiple molecular forms (3)
Antigenicity	Poor (38)	Good to fair; occurs in more than 60 different antigenic types (14)	Data unavailable
Cellular location	Occurs both cell associated and extracellularly[a]	Associated with fimbriae on cell surface (35) but also released extracellularly (44)	Occurs both cell bound and extracellularly (20, 43)
Formation by L forms	Yes (21)	Yes (15)	Data unavailable
Location of genetic determinant	Possibly plasmid-borne (42)	Possibly plasmid-borne (5)	Possibly plasmid-borne (26)
Loss of character on storage or subculture	Yes (42)	Yes (14)	Yes (20, 26)
Extracted by boiling in saline at pH 2	Yes[a]	Yes (29)	Yes (1)
Extracted by cell breakage	Yes[a]	Yes (14)	Yes (1)
Enhancement of production by passage of strain through mice	Yes (38)	Yes (14)	Data unavailable
Incidence of production	1% of group A streptococci (40)	50% or more of group A streptococci and rare group A and G (14)	Data unavailable
Antibacterial activity	Predominantly against other streptococci and closely related genera; not active against gram-negative bacteria	Data unavailable	Active against various strains of gram-positive bacteria; not active on gram-negative bacteria (32)
	Autoinhibitory in high concentrations (40)		Autoinhibitory in high concentrations (22)
Nonspecific adsorption to *Escherichia coli*	Yes (42)	Yes (27)	Data unavailable
Inactivated by:			
Trypsin	Yes (40)	Yes (28)	Yes (31)
Alkali	Yes (40)	No (14)	Yes (31, 20)
Boiling	No (40)	No (29)	No (1)
Streptococcal proteinase	Yes (38)	Yes (10)	Data unavailable

[a] Documented in the present paper.

ocin and a "classical" antibiotic, respectively, highlights some of the problems of classification of antibacterial substances that confront investigators in this area (37). Nisin appears to have some of the properties that have been presumed (37) to apply to the bacteriocins, such as protein nature, inhibitory spectrum centered about the homologous species, and plasmid-borne genetic determinants. However, the situation is certainly unclear, and this seems yet another good example of one investigator's "bacteriocin" being another's "classical antibiotic."

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LITERATURE CITED

1. **Bailey, F. J., and A. Hurst.** 1971. Preparation of a highly active form of nisin from *Streptococcus lactis*. Can. J. Microbiol. **17**:61-67.
2. **Barrow, G. I.** 1963. The nature of inhibitory activity by *Staphylococcus aureus* type 71. J. Gen. Microbiol. **32**:255-261.
3. **Cheeseman, G. C., and N. J. Berridge.** 1959. Observations on the molecular weight and chemical composition of nisin A. Biochem. J. **71**:185-194.
4. **Clarke, D. J., R. M. Robson, and J. G. Morris.** 1975. Purification of two *Clostridium* bacteriocins by procedures appropriate to hydrophobic proteins. Antimicrob. Agents Chemother. **7**:256-264.
5. **Cleary, P. P., Z. Johnson, and L. W. Wannamaker.** 1975. Genetic instability of M protein and serum opacity factor of group A streptococci: evidence suggesting extrachromosomal control. Infect. Immun. **12**:109-118.
6. **Cohen, J. O.** 1969. Effect of culture medium composition and pH on the production of M protein and proteinase by group A streptococci. J. Bacteriol. **99**:737-744.
7. **Dajani, A. S., M. C. Tom, and D. J. Law.** 1976. Viridins, bacteriocins of alpha-hemolytic streptococci: isolation, characterization, and partial purification. Antimicrob. Agents Chemother. **9**:81-88.
8. **Dandeu, J. P.** 1971. Chemical and immunological study of colicins E_1, K, A, and Q. Infect. Immun. **3**:1-9.
9. **Donoghue, H. D.** 1972. Properties and comparative starch-gel electrophoresis of megacins from several *Bacillus megaterium* strains. J. Gen. Microbiol. **72**:473-483.
10. **Elliott, S. D.** 1945. A proteolytic enzyme produced by group A streptococci with special reference to its effect on the type-specific M antigen. J. Exp. Med. **81**:573-592.
11. **Fischetti, V. A., E. C. Gotschlich, and A. W. Bernheimer.** 1971. Purification and physical properties of group C streptococcal phage-associated lysin. J. Exp. Med. **133**:1105-1117.
12. **Fischetti, V. A., E. C. Gotschlich, G. Siviglia, and J. B. Zabriskie.** 1976. Streptococcal M protein extracted by nonionic detergent. 1. Properties of the antiphagocytic and type-specific molecules. J. Exp. Med. **144**:32-53.
13. **Foulds, J.** 1972. Purification and partial characterization of a bacteriocin from *Serratia marcescens*. J. Bacteriol. **110**:1001-1009.
14. **Fox, E. N.** 1974. M proteins of group A streptococci. Bacteriol. Rev. **38**:57-86.
15. **Freimer, E. H., R. M. Krause, and M. McCarty.** 1959. Studies of L forms and protoplasts of group A streptococci. 1. Isolation, growth, and bacteriologic characteristics. J. Exp. Med. **110**:853-873.
16. **Gagliano, V. J., and R. D. Hinsdill.** 1970. Characterization of a *Staphylococcus aureus* bacteriocin. J. Bacteriol. **104**:117-125.
17. **Goebel, W. F.** 1973. The nature of the colicin K of *Escherichia coli* K235. Proc. Natl. Acad. Sci. U.S.A. **70**:854-858.
18. **Hale, E. M., and R. D. Hinsdill.** 1973. Characterization of a bacteriocin from *Staphylococcus aureus* strain 462. Antimicrob. Agents Chemother. **4**:634-640.
19. **Herschman, H. R., and D. R. Helinski.** 1967. Purification and characterization of colicin E2 and colicin E3. J. Biol. Chem. **242**:5360-5368.
20. **Hirsch, A.** 1951. Growth and nisin production of a strain of *Streptococcus lactis*. J. Gen. Microbiol. **5**:208-221.
21. **Hryniewicz, W., and J. R. Tagg.** 1976. Bacteriocin production by group A streptococcal L-forms. Antimicrob. Agents Chemother. **10**:912-914.
22. **Hurst, A., and H. Kruse.** 1972. Effect of secondary metabolites on the organisms producing them: effect of nisin on *Streptococcus lactis* and enterotoxin B on *Staphylococcus aureus*. Antimicrob. Agents Chemother. **1**:277-279.
23. **Isaacson, R. E., and J. Konisky.** 1973. Electron microscopy of colicin I-producing cells. J. Bacteriol. **113**: 452-460.
24. **Jetten, A. M., and G. D. Vogels.** 1972. Nature and properties of a *Staphylococcus epidermidis* bacteriocin. J. Bacteriol. **112**:243-250.
25. **Jetten, A. M., G. D. Vogels, and F. De Windt.** 1972. Production and purification of a *Staphylococcus epidermidis* bacteriocin. J. Bacteriol. **112**:235-242.
26. **Kozak, W., M. Rajchert-Trzpil, and W. T. Dobrzanski.** 1974. The effect of proflavin, ethidium bromide and an elevated temperature on the appearance of nisin-negative clones in nisin-producing strains of *Streptococcus lactis*. J. Gen. Microbiol. **83**:295-302.
27. **Krasner, R. I., and R. Heitmann.** 1968. The presence of a virulence factor in group A streptococcal acid extracts. II. The enhancement of virulence of heterologous streptococci and other organisms by these extracts. J. Infect. Dis. **118**:39-46.
28. **Lancefield, R. C.** 1943. Studies on the antigenic composition of group A hemolytic streptococci. 1. Effects of proteolytic enzymes on streptococcal cells. J. Exp. Med. **78**:465-476.
29. **Lancefield, R. C., and G. E. Perlmann.** 1952. Preparation and properties of type-specific M antigen isolated from a group A type 1 hemolytic streptococcus. J. Exp. Med. **96**:71-82.
30. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
31. **Mahmoud, S. A. Z., G. M. El-Sadek, and A. H. M. Dawood.** 1976. Mode of action of nisin on some representative bacterial spores. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 2 **131**:212-221.
32. **Mattick, A. T. R., and A. Hirsch.** 1947. Further observations on an inhibitory substance (nisin) from lactic streptococci. Lancet **ii**:5-8.
33. **Oxford, A. E.** 1944. Diplococcin, an anti-bacterial protein elaborated by certain milk streptococci. Biochem. J. **38**:178-182.
34. **Rotta, J., R. M. Krause, R. Lancefield, W. Everly, and H. Lackland.** 1971. New approaches for the laboratory recognition of M types of group A streptococci.

J. Exp. Med. **134**:1298–1315.

35. **Swanson, J., C. Konrad, and E. C. Gotschlich.** 1969. Electron microscopic studies on streptococci. 1. M antigen. J. Exp. Med. **130**:1063–1091.
36. **Tagg, J. R., A. S. Dajani, and L. W. Wannamaker.** 1975. Bacteriocin of a group B streptococcus: partial purification and characterization. Antimicrob. Agents Chemother. **7**:764–772.
37. **Tagg, J. R., A. S. Dajani, and L. W. Wannamaker.** 1976. Bacteriocins of gram-positive bacteria. Bacteriol. Rev. **40**:722–756.
38. **Tagg, J. R., A. S. Dajani, L. W. Wannamaker, and E. D. Gray.** 1973. Group A streptococcal bacteriocin. Production, purification and mode of action. J. Exp. Med. **138**:1168–1183.
39. **Tagg, J. R., and A. R. McGiven.** 1971. Assay system for bacteriocins. Appl. Microbiol. **21**:943.
40. **Tagg, J. R., R. S. D. Read, and A. R. McGiven.** 1973. Bacteriocin of a group A streptococcus: partial purification and properties. Antimicrob. Agents Chemother. **4**:214–221.
41. **Tagg, J. R., S. Skjold, and L. W. Wannamaker.** 1976. Transduction of bacteriocin determinants in group A streptococci. J. Exp. Med. **143**:1540–1544.
42. **Tagg, J. R., and L. W. Wannamaker.** 1976. Genetic basis of streptococcin A-FF22 production. Antimicrob. Agents Chemother. **10**:299–306.
43. **White, R. J., and A. Hurst.** 1968. The location of nisin in the producer organism *Streptococcus lactis*. J. Gen. Microbiol. **53**:171–179.
44. **Widdowson, J. P., W. R. Maxted, D. L. Grant, and A. M. Pinney.** 1971. The relationship between M-antigen and opacity factor in group A streptococci. J. Gen. Microbiol. **65**:69–80.