ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1976, p. 299-306
Vol. 10, No. 2
Printed in U.S.A.
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Genetic Basis of Streptococcin A-FF22 Production

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Received for publication 16 March 1976

Spontaneous, low-frequency loss of ability to produce streptococcin A-FF22 (SA) by group A streptococcus strain FF22 was observed. The proportion of non-SA-producing (SA^-) derivatives occurring in strain FF22 cultures grown in Todd Hewitt broth supplemented with 1% of yeast extract (THBY) was increased on treatment with ethidium bromide, acriflavin, or rifampin. The highest incidence of SA^- organisms, however, was found in untreated THBY cultures that had been aging by incubation at 37°C for several months. The possibility of selective effects in these experiments, operating to enhance the apparent frequency of SA^- bacteria, was discounted. The survival of SA^- derivatives in association with populations of SA^+ bacteria was dependent upon the use of culture conditions inimical to SA activity, since a consistent finding was that the loss of ability to produce SA was associated with loss of immunity to the killing action of this bacteriocin. Whereas selective killing of SA^- derivatives was evident in mixed cultures of SA^+ and SA^- strains in tryptic soy broth, no such effect was demonstrable in THBY. In these experiments, elimination of SA^- cells seemed directly related to the presence of active SA. Purified clones of SA^- substrains did not seem revertible to SA production, either spontaneously or on treatment with nitrosoguanidine. It is suggested that the property of production of SA by group A streptococcus strain FF22, together with that of host cell immunity to the homologous bacteriocin, may be mediated by plasmid-borne genetic determinants.

Streptococcin A-FF22 (SA; formerly called streptocin A) is a bacteriocin produced by group A streptococcus strain FF22 (20-22). In the course of studies of this bacteriocin, it was observed that non-SA-producing (SA^-) variants occasionally appeared during the routine transfer of stock cultures of the parent strain. An investigation of the genetic basis of this phenomenon is the subject of the present study. The findings suggest that the genes determining both the production of, and immunity to, SA may possibly be located on a single plasmid.

(This paper was presented in part at the 75th Annual Meeting of the American Society for Microbiology, New York, N.Y., 1975 [abstract no. H-94, p. 111].)

MATERIALS AND METHODS

Bacterial strains. The prototype SA-producing (SA^+) organism, group A streptococcus strain FF22, has been described before (21) and has been identified serologically as T type 3/13 and M type 52. Incorporation of the host strain designation (FF22) allows complete specification of the bacteriocin as streptococcin A-FF22.

SA^- derivatives of strain FF22 were numbered consecutively upon isolation, together with a prefix designating the specific chemical "curing" agent utilized; rifampin (RIF), ethidium bromide (EB), and acriflavine (AF). Spontaneous SA^- substrains were identified by the prefix SPON.

Indicator strains used for the detection of SA were *Micrococcus luteus* and *Staphylococcus aureus* CIT. These indicator organisms are extremely susceptible to SA and have also been utilized in an investigation of bacteriocin production by group B streptococci (23). SA-resistant strains used in adsorption experiments were *Escherichia coli* and *S. aureus* 502A. Group A streptococcus strains M28, M57, and M58 were specific prototype-M strains from our departmental culture collection. Group A streptococcus strain FF38, an M-type 13 strain susceptible to SA, has been described previously (21). Spontaneous streptomycin-resistant mutants were selected on blood agar containing 1 mg of streptomycin per ml. All strains were stored at −70°C or in the lyophilized state.

Media and chemicals. Bacteriological media was obtained from Difco. Sodium dodecyl sulfate (SDS) and AF were from Fisher Scientific Co., Fairlawn, N.J. RIF was from Sigma Chemical Co., St. Louis, Mo., and EB, B grade, was from Calbiochem, San Diego, Calif. Mitomycin C was from Sigma Chemical Co., and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine

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(NTG) from Aldrich Chemical Co. Inc., Milwaukee, Wis.

Demonstration of SA production. It was determined that the most sensitive and convenient method for the testing of bacteriocin production was replicative stab inoculation. By use of tooth picks, individual colonies were inoculated in a regular grid pattern, first onto blood agar medium and then also on blood agar freshly seeded with a susceptible indicator lawn. These indicator lawns were prepared by spreading with a swab charged with logarithmic growth-phase cultures grown in Todd Hewitt broth (THB). From 36 to 64 colonies were tested for bacteriocin production on each lawn (Fig. 1). Bacteriocin production was evidenced by the development of inhibition zones within the lawn surrounding stabs of SA^+ organisms. Recovery of the test strains for additional studies was from the primary stab cultures on unseeded medium. Apparent SA^- clones were inoculated into 3-ml volumes of THB, incubated at 37°C for 6 h, and then streaked on blood agar to obtain single colonies. Repicking and further cycles of purification were repeated until pure, stable SA^- clones were obtained.

Loss of SA production. Preliminary experiments were conducted to establish optimal test conditions for the demonstration of the elimination of bacteriocin production by strain FF22.

An important consideration was the possible counter-selective effect of bacteriocin produced by SA^+ cells against any SA^- segregants that had concomitantly lost their host cell immunity to the killing action of SA. Our earlier studies (22) had shown that the composition of the growth medium and conditions of incubation of the SA producer strain have a great influence on the yield of SA. For the present purposes, it was desirable to select culture conditions that were not conducive to the production or persistence of biologically active SA. Preliminary experiments confirmed our previous finding that THB is relatively poor for SA production (22) and showed that when THB is supplemented with 1% yeast extract (THBY), no extracellular SA activity can be detected at any stage of the growth of SA^+ cultures under the conditions used in the present study. By comparison, it was found that when tryptic soy broth (TSB) was used as the growth medium, active SA accumulates in the supernatant, and no SA^- segregants can be detected in experiments involving either the aging of SA^+ cultures or their

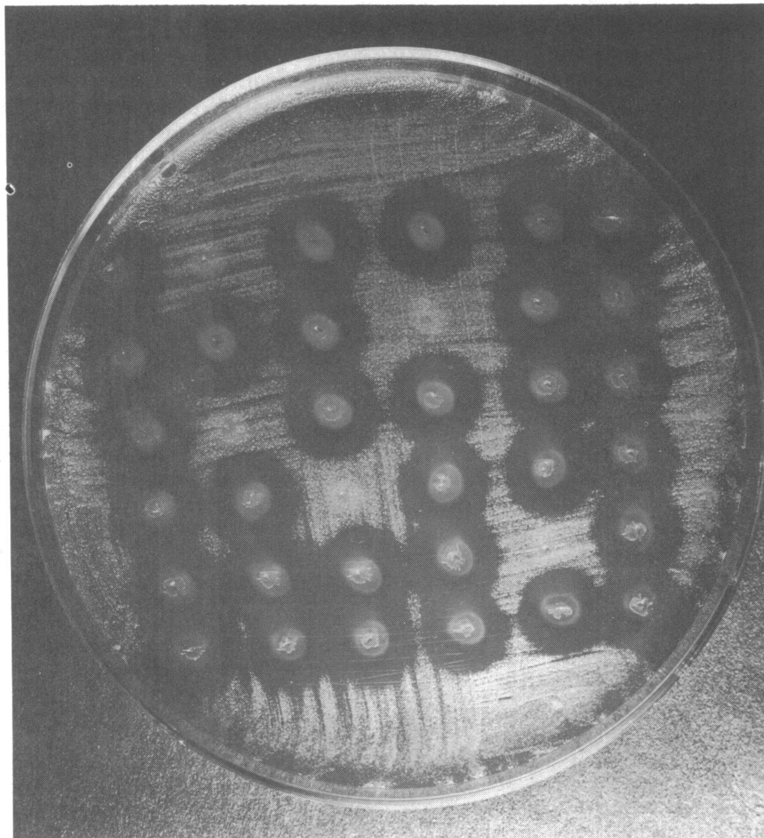

FIG. 1. *Detection of SA production after stab inoculation of individual clones into a lawn culture of M. luteus on blood agar medium. Failure to inhibit the growth of the indicator lawn by five of the test inocula indicates SA^- phenotype.*

treatment with curing agents. THBY was chosen as appropriate for use in the latter experiments. The general procedures adopted were as follows.

A single SA^+ colony was inoculated into 5 ml of THBY and incubated at 37°C for 18 h. Then: (i) to demonstrate spontaneous loss of SA, 0.1-ml portions were seeded into 25-ml volumes of fresh prewarmed THBY and incubated at the appropriate temperatures. Samples, at 24 h and various intervals thereafter, were plated on blood agar to obtain single colonies, and these were tested individually for the production of SA. (ii) To determine the effect of exposure to chemical curing agents, 0.1-ml portions were inoculated into 25-ml volumes of THBY containing concentrations (per milliliter) in the range of 0.01 to 0.05 μg of RIF, 0.02 to 0.4 μg of EB, 0.1 to 1.0 μg of AF, and 1.0 to 4.0 μg of SDS. The appropriate ranges of test doses of these chemicals were predetermined to be those inhibitory to the growth of strain FF22, such that viable cell counts (after incubation at 37°C for 24 h) were less by a factor of 10^2 to 10^3 colony-forming units (CFU) per ml compared with control cultures grown in the absence of the curing agents. Samples of the treated and control cultures were plated on blood agar, and individual colonies were treated as above. Variations of this procedure that were evaluated but later abandoned because of failure to increase the apparent rate of curing included: pretreatment of the 18-h seed culture with mitomycin C (1 μg/ml) or ultraviolet irradiation (99% kill dose); incubation of the test cultures at elevated (40°C) temperature; variation in sampling time within the range 4 to 48 h; treatment of the cultures with sonic oscillation before plating to shorten chains containing mixtures of SA^+ and SA^- units; and incorporation of the test chemical within solid medium.

Characterization of SA^- strains. Representative SA^- strains were serotyped to identify M and T antigens, and the content of M protein was determined by the classical method (13) of assessing the ability of the organisms to resist phagocytosis when rotated in fresh human blood.

Possible "back mutation" of SA^- strains to SA^+ phenotype was tested by exposing selected strains to 100 μg of NTG per ml, using previously described procedures (1).

A crude preparation of SA having a titer of 32 when assayed using *M. luteus* as indicator was obtained by established techniques (18), and this was used to test the susceptibility and also the adsorptive capacity of the parent FF22 strain and its SA^- derivatives. Susceptibility of the organisms to SA was determined by titration of the bacteriocin on blood agar lawn cultures of the test strains prepared from 1:20 dilutions of overnight THB cultures.

For demonstration of adsorption, the organisms from 25-ml THB cultures grown 18 h at 37°C were washed twice in phosphate buffer (pH 7.2) and then resuspended in 1.5 ml of the buffer. Then, to 0.4 ml of cell suspension was added 0.1 ml of a SA preparation of titer 20 (assayed on *M. luteus*). Incubation was at 37°C for 60 min, after which the organisms were pelleted by centrifugation. The supernatant fluid was titrated for bacteriocin activity after heating at 80°C for 10 min. Crude SA is stable to heating under these conditions (18). Organisms used in some adsorption experiments were first killed by heating at 80°C for 30 min. Controls consisted of the bacteriocin and the cell suspension, appropriately diluted in THB.

The relative susceptibility of parent and SA^- derivatives to growth inhibition by the curing agents was tested in two ways: (i) by drop testing with serial (THBY) dilutions of the different chemicals onto lawn cultures and estimating susceptibility as the end point of interference with growth of the lawn; (ii) by comparing the growth characteristics of the organisms in THBY supplemented with the curing agents in concentrations originally used to produce those SA^- organisms.

Growth of mixed cultures of SA^+ and SA^- strains. To aid differentiation between the two cell populations in mixed cultures, streptomycin-resistant mutants of the SA^- cultures were utilized. Separate cultures of the SA^+ and SA^- strains were established in either TSB or THBY at 37°C for 18 h. Portions, 0.5 ml, containing approximately 5×10^7 CFU of each strain were seeded into 25 ml of the appropriate prewarmed broths both independently (controls) and together (test). Incubation was at 37°C for 24 h, and 0.1-ml duplicate samples were withdrawn at intervals for viable counts on blood agar and on streptomycin blood agar. Counts for SA^- organisms were estimated on streptomycin medium, and the SA^+ counts were determined by difference.

RESULTS

Spontaneous elimination of SA. Occasional, irreversible loss of ability to produce SA was observed on repeated subculture of group A streptococcus strain FF22. The extent of spontaneous elimination of SA upon long-term incubation of the producer strain in THBY cultures was tested at 30, 35, and 40°C. Table 1 summarizes the results of one such experiment. Repeat experiments of this type have given essentially similar results, although the maximum percentages of SA^- cells sometimes showed considerable variation in different cultures incubated under similar conditions. For example, the greatest proportion of SA^- cells in cultures incubated at 35°C has varied from a low of 20% to a high of 90%. Nevertheless, a consistent finding was the observation of very little increase in the proportion of SA^- segregants until after about 3 weeks of incubation, followed by a sharp increase between 4 and 5 weeks. The reason for the relatively low rate of loss of SA at 30°C is not apparent. Incubation at the elevated temperature of 40°C did not seem to enhance either the time course or the frequency of appearance of SA^- derivatives when compared with cultures incubated at 35°C. In the experiment documented in Table 1, a subculture was

TABLE 1. *Loss of SA synthesis by cells in aging broth cultures of group A streptococcus strain FF22*

Sample time (week)	% SA⁻ organisms[a] at temp					
	30°C		35°C		40°C	
	Original	Subculture[b]	Original	Subculture	Original	Subculture
0	0		0		0	
1	0		0		0	
2	0	0	1	0	0	0
3	0	0	3	3	0	0
4	1	0	4	1	0	0
5	ND[c]	ND	29	3	28	2
6	ND	1	54	33	ND	ND
7	1	1	60	80	33	3
8	2	0	68	62	44	20
9	1	2	82	88	69	28

[a] A total of 100 to 140 colonies was tested in each sample.
[b] Made from original culture at 2 weeks.
[c] ND, Not determined.

taken at 2 weeks from each of the established broths into fresh THBY medium at each of the temperatures. It was found that the pattern of appearance of SA^- derivatives was similar in each subculture to that observed with the corresponding primary culture (Table 1). There was little loss of ability to produce SA in the subcultures incubated at 30°C; however, after subculture at 35°C, the incidence of SA^- strains increased appreciably from 4 to 5 weeks.

Induced elimination of SA. Several of the chemical curing agents tested were found to give increased yields of SA^- derivatives by comparison with untreated control (THBY) cultures of strain FF22. Table 2 lists the aggregated results from numerous experiments. The curing rates obtained were low. RIF appeared to give the greatest yield of cured derivatives. SDS did not give rise to any SA^- organisms. It was significant that none of these chemicals appeared to eliminate bacteriocin production at a level comparable with that obtained by long-term incubation of the producer strain.

Origin of SA^- derivatives. A number of tests were conducted to establish whether the SA^- strains may have had some selective survival advantage under the test conditions.

Portions from cell-free membrane (Millipore Corp.) filtrates of 48-h- and 2-month-aged THBY cultures of strain FF22 were tested for capacity to support the growth of SA^+ strains and spontaneous SA^- derivatives (SPON1 and SPON6). Daily viable counts indicated that the growth and survival characteristics of the SA^- strains were similar to those of the SA^+ bacteria in each of the filtrates.

Possible selective effects of the chemical curing agents were tested by titrating preparations of each of the chemicals on lawn cultures of the parent and the corresponding SA^- substrains. By this procedure, it was found that the parent and the derivatives invariably had similar susceptibility to killing by the particular test chemicals. In other tests, growth curves of the parent and of SA^- substrains in the presence of test doses of the various curing agents were compared. These tests, e.g., the one illustrated in Fig. 2, indicated that the cured derivatives did not appear to have any selective growth advantage under the conditions of the test.

Stability of SA^- strains. All of the SA^- strains that had been obtained by exposure of actively multiplying SA^+ cultures to chemical

TABLE 2. *Elimination of SA in group A streptococcus strain FF22 by use of curing agents*

Treatment	Total colonies tested	No. of SA⁻ clones	Elimination (%)
Untreated	3,100	3	0.09
EB	620	3	0.48
AF	980	8	0.82
RIF	1,450	16	1.10
SDS	408	0	0

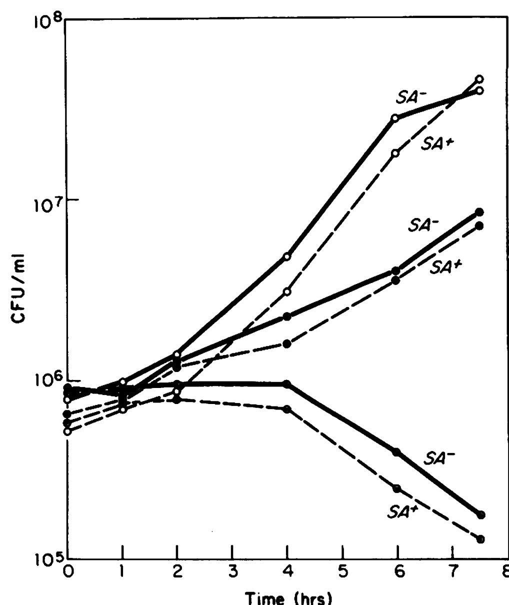

FIG. 2. *Growth curves of group A streptococcus strain FF22 (SA^+) and its cured derivative, strain EB1 (SA^-), in THBY containing 0.4 μg of EB per ml (⊙), 0.1 μg of EB per ml (●), and with no supplement (○).*

curing agents appeared to be stable on primary isolation, with no evidence of reversion of bacteriocin production upon repeated subculture. By comparison, almost 50% of the colonies appearing to be SA^- on preliminary isolation from prolonged incubation experiments were found to give rise to mixtures of SA^+ and SA^- colonies on continued testing. Occasionally, it was necessary to repeat three or four cycles of purification from the original SA^- clones to obtain pure cultures of SA^- derivatives that failed to revert on continued subculture.

Four SA^- derivatives (EB1, AF1, SPON1, SPON6) were subcultured weekly for 4 months on blood agar and showed no evidence of reversion to bacteriocin production. In addition, strains EB1, SPON1, and SPON6 were treated with (per ml) 100 μg of the potent mutagen NTG, in an attempt to demonstrate reversion by back mutation. None of 2,000 tested colonies of EB1, or 1,000 each of SPON1 and SPON6, showed evidence of reversion to SA^+ phenotype.

Characteristics of SA^- derivatives. No relationship between the loss of SA and colony appearance or production of hemolysis on blood agar was apparent. All 20 SA^- derivatives that were examined by serotyping had the characteristic 3/13 T pattern of the parental culture. Determination of M protein production indicated that all of 15 tested SA^- strains that had been obtained by exposure to curing agents were still M positive. Two of 20 examined SA^- cultures that had been obtained from aged cultures were M negative.

Little specificity was found in the adsorption of SA (Table 3). The extent of adsorption was similar by use of the parent SA^+ strain, two M-positive SA^- derivatives (EB1, SPON1), one M-negative SA^- substrain (SPON6), and two unrelated organisms that are not susceptible to SA (*E. coli* and *S. aureus* 502A). Similar results were obtained with use of either viable or heat-killed organisms.

In spite of the similar adsorptive capacity of the parent and SA^- substrains, considerable difference was found in the susceptibility of these strains to the inhibitory action of SA. All of 20 spontaneous and 15 induced SA^- cultures tested were found to be 16 to 32 times more susceptible to SA than the parent strain when examined by the drop dilution method of titration.

Thus, both after exposure to curing agents and in aged cultures, it appeared that producer cell immunity to the homologous bacteriocin had consistently been lost together with ability to produce the bacteriocin. Concomitant loss of both functions gave support to the contention

TABLE 3. *Adsorption of SA to susceptible and resistant bacteria*[a]

Test organism	Susceptibility to SA killing action	SA titer in supernatant
Group A streptococcus		
Strain M57	Susceptible	2
Strain EB1	Susceptible	2
Strain SPON1	Susceptible	2
Strain SPON6	Susceptible	1
Strain FF22	Resistant	2
Escherichia coli	Resistant	1
Staphylococcus aureus		
Strain 502A	Resistant	2
Strain CIT	Susceptible	1
None (control)		4

[a] Viable, washed cells incubated with SA for 30 min at 37°C.

that both determinants may be located on a single plasmid and did not seem consistent with a point mutation origin of SA^- strains in these experiments.

Treatment of the producer strain with NTG was found to give rise to SA^- derivatives, the majority of which differed significantly from those described above. Of 20 such SA^- substrains, 18 were found to retain parental-strain levels of resistance to the bacteriocin, whereas the other two were 16 times more susceptible and appeared to correspond phenotypically to the SA^- strains obtained by aging or use of curing agents. Reexposure of representative SA^- SA-resistant mutants to NTG induced reversion to the SA^+ SA-resistant phenotype, indicating the occurrence of back mutation. Aging of cultures of the SA^- SA-resistant mutants gave rise to SA^- SA-susceptible derivatives, and these were not revertible to SA^+ phenotype on reexposure to NTG. Although it was not the subject of a special study, the frequency of appearance of SA^- SA-susceptible derivatives in aging SA^- SA-resistant cultures seemed similar to the observed frequency of appearance of SA^- SA-susceptible derivatives arising on aging of SA^+ SA-resistant cultures.

Growth of SA^+ and SA^- organisms in mixed cultures. Figure 3 shows growth curves representing the pure and the mixed growth of strain FF22 (SA^+) and its SA^- derivative EB1 in TSB medium at 37°C. The parent strain exhibited similar growth in both pure and mixed cultures, achieving a maximum population of 2×10^8 CFU per ml at 10 to 12 h after inoculation. By comparison, the SA^- strain was markedly inhibited by growth in association, with a strong decrease in viability after 6 h of incubation. A complete kill was effected within 10 h of

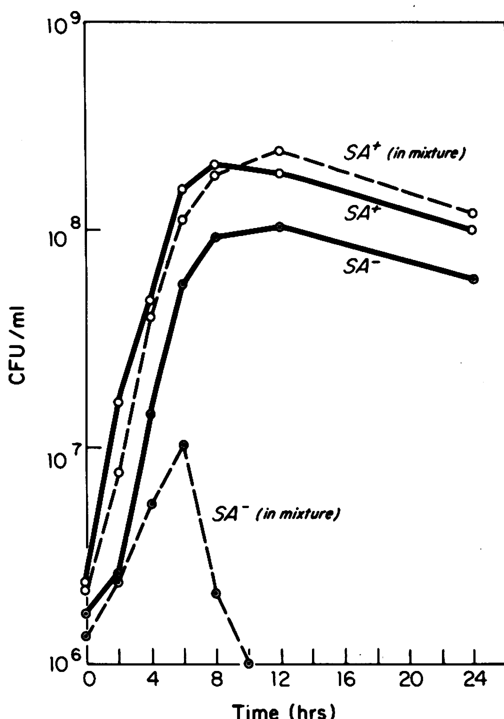

FIG. 3. *Growth characteristics of group A streptococcus strain FF22 (SA+) and its cured derivative, strain EB1 (SA−), in pure cultures (——) and in mixed culture (- - -).*

seeding the cultures. TSB was suitable for use in this experiment, since it is excellent for the production of SA (22). No inhibition of SA⁻ strains occurred in mixed cultures in either THB or THBY medium.

DISCUSSION

The reported data seem consistent with the interpretation that the genetic determinants of SA production and immunity are located on a plasmid in group A streptococcus strain FF22. Many, but not all (15), studies of the genetic basis of bacteriocin production have indicated the involvement of plasmids (18). Although most studies have been of bacteriocins of gram-negative species, exceptions include those of some of the staphylococcal bacteriocins (5, 10). Information of this kind about the streptococcal bacteriocins is lacking, although production of the bacteriocin-like antibiotic "nisin" by *Streptococcus lactis* was recently shown possibly to be plasmid determined (11).

In general, the replication and inheritance of plasmids is precisely controlled and the occurrence of plasmid-negative progeny is a rare event. However, under particular growth conditions or upon exposure to chemical curing agents, the rate of elimination of some plasmids may be substantially increased. In the present study, it was found that aging of a SA+ culture in THBY at 35°C for a minimum of 4 to 5 weeks promoted the widespread loss of SA. Other reports have also documented the spontaneous loss of ability to produce bacteriocin on prolonged storage of staphylococci (10, 12, 17), *Lactobacillus helveticus* (25) and group B streptococci (24). In other studies, the elimination of plasmid deoxyribonucleic acid having determinants of multiple antibiotic resistance was demonstrated in group D streptococci (3, 8) and in a strain of *Staphylococcus epidermidis* (19) in the course of lengthy incubation of cultures. In these studies, as we have found for the loss of SA, the rate of elimination by aging of cultures was far greater than that obtained by exposure to curing agents. Malke has noted that in some strains of group A streptococci, chemical curing agents have little effect on the stability of resistance to erythromycin and lincomycin (14). It was presumed, from other indirect evidence, that the genetic determinants of these properties were plasmid borne.

The reason why SA elimination is promoted in aged cultures has not been determined. The importance of using culture conditions unfavorable to the production of active SA is clear. No SA⁻ segregants could be detected in media such as TSB which are suitable for SA production. Our previous studies (22) of the requirements for SA formation indicated an inverse correlation between the production of active SA and that of streptococcal proteinase. In the present investigation, the yield of active SA was shown to be particularly poor in THBY medium. The enhancing effect of yeast extract on proteinase elaboration has been documented in earlier investigations (4).

Although we were unable to detect the presence of levels of SA that are inhibitory to SA⁻ strains in filtrates of young THBY cultures of SA+ bacteria, it is possible that under the test conditions sufficient SA may be present to select against the SA⁻ segregants. The role of some producer cell-bound form of SA in this effect cannot yet be dismissed. Alternatively, it may be speculated that a metabolite formed during aging of the culture facilitates plasmid elimination.

The failure of incubation at elevated temperature (40°C) to promote greater loss of SA is in marked contrast to studies of the staphylococcins, in which growth at elevated temperatures has been shown to promote elimination of bacteriocinogenic factors (5, 10). The observation of occasional concomitant loss of M protein in SA⁻

derivatives obtained from aged broths is of interest in view of the recent report (1) that M protein and serum opacity factor of group A streptococci may also be plasmid determined. Apparently, however, in group A streptococcus strain FF22, the determinants for M and for SA are not co-eliminated, and it is unlikely that they are located on the same plasmid.

Control tests indicated that the appearance of SA^- clones is not due to selection of SA^- organisms. Extensive tests failed to demonstrate the spontaneous or NTG-induced reversion of SA^- clones obtained from cultures either aged or exposed to curing agents. Since it is a characteristic of plasmids that cured cells are unable to regain functions linked to that plasmid by means of mutation, this can be taken as good indirect evidence against the occurrence of point mutation or phase variations in the present investigations. It seems that loss of a plasmid is the most likely interpretation of the observed facts. Strong supportive evidence is accrued from the concomitant loss of immunity to SA in SA^- substrains. In this regard, a difference between the antibiotic "nisin" and SA is apparent, since the loss of nisin production was not associated with loss of immunity to nisin (11). On the other hand, investigations of several colicins (18) and of a staphylococcin (5) have indicated that the genes determining immunity to a particular bacteriocin are located on the same plasmid that determines production of the bacteriocin. This immunity, presumably operating via the synthesis of specific immunity substances, is a necessary property to ensure the survival of bacteriocinogenic organisms and is different from bacteriocin resistance, which is due to loss of the specific bacteriocin receptor.

The lack of adsorption specificity of SA is of interest. Although most of the bacteriocins produced by strains of gram-negative species appear to have considerable specificity of attachment to susceptible bacteria (18), studies of some bacteriocins produced by staphylococci (7, 9) and lactobacilli (25) have clearly shown that adsorption may be quite nonspecific, being unrelated to the susceptibility of different bacteria to the killing action of bacteriocins.

The demonstration in the present study that the adsorption of bacteriocin activity from solution is comparable with use of either viable or heat-killed bacteria may be taken as indirect evidence that the removal of SA is attributable to adsorption of the bacteriocin and not to its enzymic inactivation. Streptococcal proteinase (6) and other known streptococcal enzymes are inactivated under the conditions of heating at 80°C for 30 min.

To the best of our knowledge, there have only been four other studies reported that relate to the presence of plasmids in group A streptococci, three of which show association with antibiotic resistance markers (2, 14, 16) and the other relating to M protein and serum opacity factor production (1).

Our recent studies (unpublished data) have demonstrated that the determinants of SA production and immunity are co-transducible. Presently we are attempting to isolate and characterize the bacteriocinogenic factor.

ACKNOWLEDGMENTS

We thank Christine Windler for excellent technical assistance and Paul Cleary for advice on curing techniques and for critical review of the manuscript.

This work was supported by Public Health Service grant AI 08724 from the National Institute of Allergy and Infectious Diseases. J.R.T. was supported by a Career Investigator Fellowship from the American Heart Association. L.W.W. is a Career Investigator of the American Heart Association.

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