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Plasmid-Mediated Production of Staphylococcin in Bacteriophage Type 71 *Staphylococcus aureus*

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Staphylococcin production by *Staphylococcus aureus* strain C55 was eliminated when producing strains were exposed to various curing treatments. Acridine orange, ethidium bromide, and sodium dodecyl sulfate each effected less than 15% cure rates in treated cells. Rifampin eliminated the staphylococcin in 83% of treated isolates, and growth at 42 C resulted in a 97% cure rate. Curing of staphylococcin production and elimination of penicillinase plasmid were independent of one another. Several characteristics of the cured strains were examined and compared with the bacteriocin-producing strains. Whereas bacteriocin-producing cells were resistant to the bactericidal action of the staphylococcin and failed to adsorb it, cured cells adsorbed the staphylococcin and were susceptible to its lethal effect.

A large proportion of isolates of *Staphylococcus aureus* lysed by bacteriophage 71 produce a staphylococcin that can be detected on solid and in liquid media (4-8). This bacteriocin is bactericidal to a variety of gram-positive bacteria, including Group A beta-hemolytic streptococci (4, 6, 7).

In some gram-negative bacteria, bacteriocin production has been shown to be controlled by plasmids (14). Since several heritable characteristics in staphylococci have been shown to be extrachromosomal (2, 11, 12, 15, 16; R. P Novick, Fed. Proc., p. 29-38, 1967), the present investigations were designed to study the mechanism of bacteriocin production in phage type 71 *S. aureus*. The data to be presented strongly suggest a plasmid mediation for this production.

MATERIALS AND METHODS

Organisms. Strain C55 was used as a prototype of staphylococcin-producing phage type 71 *S. aureus*. It is a penicillinase-producing staphylococcus. Strain 502A was utilized as an example of a bacteriocin-nonproducing, penicillin-sensitive staphylococcus (6, 7). The staphylococcal strains were maintained in tryptic soy broth (Difco Laboratories, Detroit, Mich.) and stored at −20 C until needed. Subcultures were made on tryptic soy agar plates prior to the use of the strains.

Group A beta-hemolytic streptococcal strain PF 1643, an M-type 57, was utilized as a staphylococcin indicator strain, and its susceptibility to the phage type 71 staphylococcin has been documented previously (7, 8). This streptococcal strain was grown in Todd-Hewitt broth (Difco) for 6 h at 37 C, and 2-ml portions were then stored at −20 C until needed.

Demonstration of bacteriocin production. A modification of the deferred antagonism technique (8) was adapted to detect staphylococcin production. Single staphylococcal colonies were picked and spread in a circular manner to cover an area of approximately 2 cm in dimeter on the surface of a tryptic soy agar plate. The plates were incubated at 37 C for 36 to 48 h. The growth was scraped off the surface of the plates, which were then exposed to chloroform vapor for 30 min. An overlay of 6 ml of blood agar containing viable streptococci (M-type 57) was then placed on the surface of the agar, and the plates were reincubated at 37 C for 18 to 24 h. Streptococcal growth was inhibited over the area where bacteriocin-producing staphylococci had been grown previously (see Fig. 1 under Results).

Curing agents. Various agents were utilized to cure staphylococci of bacteriocin production. Sodium dodecyl sulfate (SDS) and acridine orange were obtained from Fisher Scientific Co., Fair Lawn, N.J. Rifampin (Sigma Chemical Co., St. Louis, Mo.) and ethidium bromide, B-grade, (Calbiochem, San Diego, Calif.) were also used. Staphylococcal strain C55 was grown in 10-ml portions of tryptic soy broth, each containing different concentrations of a curing agent. The cultures were incubated at 37 C for 18 h, after which time loopfuls were streaked on tryptic soy agar plates and the plates were incubated overnight at 37 C. Individual colonies were then picked and tested for staphylococcin production. In addition, growth at elevated temperature (42 C) was employed as a means to cure bacteriocin production (12).

Assay for various markers. Penicillinase production was detected by using the starch-iodine technique as modified by Baldwin et al. (1). Assay for hemolysins was done according to the methods described by Chesbro et al. (3). The tube method for coagulase production was utilized, and mannitol fermentation was tested on mannitol salt agar plates. Phage typing was performed according to standard techniques.

RESULTS

Spontaneous loss of bacteriocin. Repeated subculturing of certain staphylococcal strains on agar slants has been shown to result in spontaneous elimination of staphylococcin production by these strains (10, 13). To assess the degree of spontaneous loss by strain C55, 474 single colonies were tested for staphylococcin production as outlined under Materials and Methods. Examples of bacteriocin-producing (Scn^+) and bacteriocin-nonproducing (Scn^-) strains are shown in Fig. 1. Of the 474 single isolates tested, five (1%) demonstrated spontaneous loss of staphylococcin production.

Effect of chemical agents. The effect of various curing agents on staphylococcin production was investigated next, and distinct differences among these agents were observed. The effects of varying concentrations of acridine orange are shown in Table 1. At a concentration of 10 μg/ml, 13.9% of the strains tested were cured, and the percentage of cured strains decreased with concentrations of the acridine orange above and below 10 μg/ml. In staphylococci, acridine orange was shown to be a poor curing agent for elimination of penicillinase plasmid, and other agents were shown to be superior.

Ethidium bromide is one agent that results in a high frequency of curing of staphylococcal penicillinase plasmid (2). When tested in the present system, however, it did not seem to be superior to acridine orange in eliminating staphylococcin production (Table 2). A concentration of 10 μg/ml seemed to be the most effective; however, only 14.4% of the strains were cured.

Another curing agent tested, SDS (16), was also only slightly effective (Table 3). Maximal curing was 7.4% with an SDS concentration of 0.001%. Higher concentrations were similar to the untreated controls.

The one agent that resulted in the highest cure rate was rifampin (11). In Table 4 the effects of five different concentrations of rifampin are shown. All concentrations above 0.01 μg/ml resulted in a substantial rate of cure; 0.075 μg/ml was the optimal concentration.

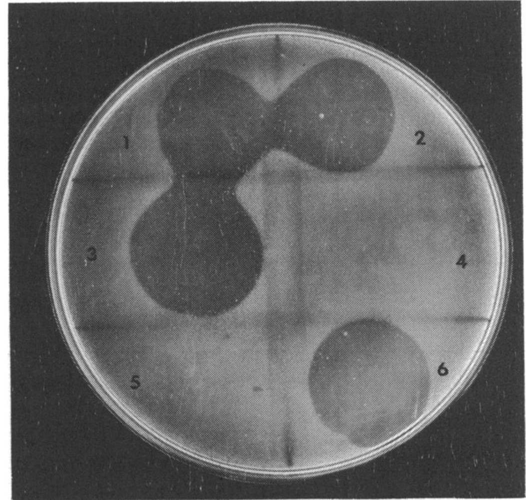

FIG. 1. *Examples of Scn^+ (sections 1, 2, 3, and 6) and Scn^- (sections 4 and 5) strains.*

TABLE 1. *Elimination of staphylococcin production by acridine orange*

Acridine orange concn (μg/ml)	No. cured/no. of colonies tested	Percent cured
5	5/72	6.9
10	5/36	13.9
20	0/24	0
50	2/108	2.0
100	1/48	2.1

TABLE 2. *Curing of staphylococcin production by varying concentrations of ethidium bromide*

Ethidium bromide concn (μg/ml)	No. cured/no. of colonies tested	Percent cured
1	20/174	11.5
2	5/66	7.5
5	18/222	8.1
10	32/222	14.4
20	2/100	2.0
50	0/24	0

Incubation at elevated temperature. A stock culture of strain C55 was streaked and grown at either 37 or 42 C on tryptic soy agar plates for 24 h. Individual colonies were picked and maintained for 48 h at 37 or 42 C and then tested for staphylococcin production. As shown in Table 5, almost all (96.9%) of the isolates grown and maintained at 42 C lost the capacity to produce staphylococcin. The determining factor in curing was the initial incubation temperature and selection of the cured colonies.

TABLE 3. *Effect of different concentrations of SDS on staphylococcin production*

SDS conc (%)	No. cured/no. of colonies tested	Percent cured
0.001	7/96	7.4
0.002	0/96	0
0.003	1/60	1.7
0.004	0/30	0

TABLE 4. *Effect of varying concentrations of rifampin on elimination of staphylococcin production*

Rifampin concn (μg/ml)	No. cured/no. of colonies tested	Percent cured
0.0075	0/30	0
0.010	27/96	28.1
0.025	8/60	13.3
0.075	25/30	83.3
0.100	43/96	44.8

TABLE 5. *Curing of staphylococcin production by incubation at elevated temperature*

Initial incubation temp	Maintenance temp	No. cured/ no. of colonies tested	Percent cured
37 C	37 C	3/96	3.1
37 C	42 C	2/55	3.6
42 C	37 C	42/60	70.0
42 C	42 C	93/96	96.9

Elimination of penicillinase and bacteriocin. The observations presented so far strongly suggest that the production of staphylococcin is controlled by an extrachromosomal mechanism. Since penicillinase production in staphylococci has been shown to be a function of plasmids (R. P. Novick, Fed. Proc., p. 29-38, 1967; 15), it was of interest to investigate whether staphylococcin production and penicillinase production are functions of the same or of different plasmids.

Ethidium bromide, SDS, and rifampin were tested for their capacity to eliminate penicillinase and staphylococcin productions in strain C55. The results are shown in Table 6. Different cure rates of staphylococcin and penicillinase productions were noted for the varying concentrations of each of the three agents. Furthermore, when representative strains cured for either function were tested for the simultaneous elimination of the other function, no positive correlation was observed. These findings indicate that the production of staphylococcin and penicillinase by strain C55 is controlled by two distinct and independent plasmids.

Alterations in the Scn$^-$ strains. Isolates cured of bacteriocin production remained in this state after repeated subculturing, with no spontaneous reversion to the producing state. All cured isolates were tested for several markers to assess whether certain other functions may be controlled by the same plasmid. In addition to loss of staphylococcin production, the cured isolates were uniformly susceptible to the bactericidal action of the bacteriocin (Fig. 2). Scn$^+$ and Scn$^-$ strains were grown in tryptic soy broth mixed with equal quantities of a staphylococcin preparation (4). Whereas producer strains were resistant to the action of the bacteriocin, cured isolates were killed by it. Using a previously described technique (7), Scn$^+$ strains failed to adsorb the bacteriocin even when incubated for 30 min at 37 C. However, Scn$^-$ strains very actively adsorbed the staphylococcin within 5 min after incubation at

TABLE 6. *Comparison of curing of staphylococcin production and elimination of penicillinase plasmid by various agents*

Curing agent	Concn	Staphylococcin cure rate	Penicillinase cure rate
Ethidium bromide	5 μg/ml	18/222	4/222
	20 μg/ml	2/110	14/110
SDS	0.001%	7/96	2/36
	0.002%	0/96	4/36
Rifampin	0.01 μg/ml	27/96	2/36
	0.10 μg/ml	43/96	0/36

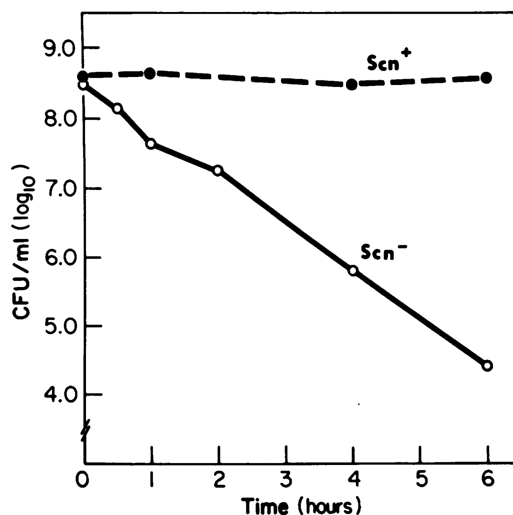

FIG. 2. *Effect of staphylococcin on the viability of Scn$^+$ and Scn$^-$ strains.*

37 C. When tested for susceptibility to lytic phages, the Scn$^+$ and Scn$^-$ strains were identical, being lysed only by bacteriophage 71. The strains were similarly identical in coagulase production, mannitol fermentation, and production of various hemolysins.

DISCUSSION

Bacteriocin production by staphylococcal strain C55 has been shown in the present report to be very likely mediated by an extrachromosomal mechanism. Elimination of staphylococcin production has been achieved through the use of various curing agents with distinct differences among such agents in their cure rates.

In a recent paper (10), Jetten and Vogels have also described an extrachromosomal control of bacteriocin production in *S. aureus*. The authors have been able to eliminate staphylococcin production by using different curing agents. Some similarities and some differences between their findings and ours as to the relative efficacy of the various curing agents are shown in Table 7 and deserve a brief mention. Both their strain 89 and our strain C55 are lysed by phage 71. Acridine orange is a poor agent to cure staphylococcin production in both studies, and growth at elevated temperature is exceedingly effective. Whereas SDS and ethidium bromide were found to be excellent curing agents by Jetten and Vogels, both agents were relatively poor in curing the staphylococcin of strain C55. This is very likely a reflection of the existence of two or more different plasmids that control bacteriocin production in different staphylococcal strains.

That more than one plasmid may control a specific staphylococcal function is not unusual. Several plasmids have been described in staphylococci to control penicillinase production (15). Furthermore, several distinct staphylococcins have been described (4-8, 9, 10, 13), and their controls may well be a function of distinct plasmids. The results of the present investigations also indicate that, whereas the control of both penicillinase production and staphylococcin production is extrachromosomal, such control is the function of at least two distinct plasmids.

The characteristics of the Scn$^-$ strains as compared with the parent Scn$^+$ C55 strain deserve some discussion. Of the several functions tested, no changes were noted in coagulase production, mannitol fermentation, or hemolysin production. Both Scn$^+$ and Scn$^-$ strains were lysed only by bacteriophage 71, a finding similar to that of Jetten and Vogels with their strain 89 (10). Certain other staphylococcal strains, however, have been described where either spontaneous (10, 13) or induced (10) elimination of staphylococcin production resulted in a broader susceptibility to lytic phages. The exact relationship of lytic phages to bacteriocin production in staphylococci remains obscure and is worthy of some exploration.

Perhaps the most significant alteration in the Scn$^-$ strains is their uniform susceptibility to the bactericidal effect of the staphylococcin. Scn$^+$ strains have been shown in the present report to be unable to adsorb the bacteriocin and to be resistant to its lethal effect. In marked contrast, Scn$^-$ strains adsorb the staphylococcin and are killed by it. The presence of the bacteriocin plasmid, therefore, renders the cell resistant to the action of the staphylococcin by a mechanism that is not apparent from the design of the experiments presented in this report.

TABLE 7. *Comparison of efficacy of various curing treatments on elimination of staphylococcin in two staphylococcal strains*

Treatment	Optimal elimination (%)	
	Strain 89[a]	Strain C55[b]
Controls	<0.1	1.0
Ethidium bromide	94.0	14.4
Acridine orange	12.1	13.9
Acriflavine	25.0	NT[c]
SDS	100.0	7.4
Rifampin	NT	83.3
Growth at 42 C	98.6	96.9

[a] Jetten and Vogels (10).
[b] Present report.
[c] NT, not tested.

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