BACTERICIDAL SUBSTANCE FROM STAPHYLOCOCCUS AUREUS

Biological Properties*

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The majority of *Staphylococcus aureus* strains recovered in pure form from superficial skin lesions have been shown to belong to phage type 71 (1, 2). Certain unique features of this phage type have been described previously, among which are the common resistance to penicillin G (1, 2), inhibition of *Corynebacterium diphtheriae* on solid media (1, 3), and the production of opacity in horse serum agar, but not in egg yolk broth medium (3).

In a previous communication from our laboratory (4), a bactericidal substance has been described in supernatant fluids from broth cultures of phage type 71 *S. aureus*. This substance was active against streptococci belonging to Groups A, C, and D, pneumococci, and corynebacteria. No inhibition of Group B streptococci or Gram-negative rods was noted, and inhibition of Group G streptococci and staphylococci was variable. The bactericidal substance has been shown to be a protein or a polypeptide. The present investigations further describe the biological properties of this bactericidal substance and indicate that it is distinct from other known staphylococcal products.

Materials and Methods

Media.—Tryptic soy broth (TSB)[1] (Difco) was used to cultivate all the staphylococcal strains. A dialysate medium of TSB was prepared as previously described (4) by dissolving

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[1] *Abbreviations used in this paper:* DNase, deoxyribonuclease; RNase, ribonuclease; RTD, routine test dilution; TSB, tryptic soy broth.

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30 g of TSB in 250 ml of distilled water and dialyzing this against 750 ml of distilled water at 4°C for 18–24 hr. Blood agar plates were prepared by using tryptose blood agar base (Difco) and 5% sheep blood.

Organisms.—Staphylococcal strains were isolated from nose, throat, or skin cultures. Several of the strains were kindly supplied by Dr. Hugh Dillon. All strains were subcultured weekly on tryptic soy agar plates and stored at 4°C. Phage typing was done by standard techniques (5) at routine test dilution (RTD); if no reaction occurred, concentrated phage was used (RTD $\times$ 10^3). Phage typing was repeated periodically to assure purity of strains.

The strains of the other bacterial species were isolated from various sources in this laboratory or were obtained from the standard strain collections of the Department of Microbiology at the University of Minnesota College of Medical Sciences.

Preparation of Bactericidal Substance.—Staphylococcal strains tested for production of the bactericidal substance were grown in TSB or TSB dialysate medium. After incubation at 37°C with constant shaking for 36–48 hr, the supernatant fluids were harvested by centrifugation at 4000 *g* for 15 min in a Sorvall refrigerated centrifuge. The pH of the supernatant fluids was readjusted to 7.2 with 1 N NaOH, and the fluids were sterilized by filtration through a membrane filter with an average pore diameter of 0.45 mμ (Millipore Corp., Bedford, Mass.). The filtered supernatant fluids were stored at 4°C.

Effect of Ultraviolet Light on Production of Bactericidal Substance.—To study the effect of ultraviolet irradiation, 200 ml of TSB was inoculated with the C55 strain of *S. aureus*. After incubation at 37°C for 3 hr, 50 ml volumes of the logarithmic phase culture were dispensed into Petri dishes (15 cm diameter) so that the depth of the fluid was approximately 2 mm. One plate was not exposed to ultraviolet light and was used as a control. The other three plates were each exposed for 10, 30, and 60 sec, to ultraviolet light source (George W. Gates Co., Franklin Square, N. Y.) at a distance of 30 cm. The cultures were then transferred individually to sterile bottles and reincubated at 37°C. After 4 and 22 hr of incubation, 25 ml portions were removed from each bottle, centrifuged, and the filtered supernatant fluids assessed for their bactericidal activity on a strain of Type 49 streptococcus.

Effect of Mitomycin C on Production of Bactericidal Substance.—Two flasks each containing 250 ml of TSB were inoculated with the C55 strain of *S. aureus*. After 3 hr of incubation at 37°C, 25 μg of mitomycin C (0.1 μg per ml final concentration) were added to the logarithmic phase organisms in one flask, and the other flask was untreated. The same amount of mitomycin C was also added to 250 ml of fresh uninoculated TSB. All flasks were incubated at 37°C and at intervals, 50 ml portions were removed from each flask, and the supernatant fluids were harvested, filtered, and assessed for bactericidal activity.

Association of Bactericidal Substance with Staphylococcal Cells.—Strain C55 staphylococcus was grown in 500 ml of TSB for 36 hr at 37°C. The supernatant was harvested as usual, and in addition, the sedimented cells were also saved. The cells were washed twice with phosphate buffer, resuspended in 5 ml of the same buffer, and disrupted on a mechanical shaker (H. Mickle, Gomshall, Surrey, England) for 20 min. Total disruption of cells occurred as assessed by Gram-staining of the preparation. After centrifugation at 10,000 *g* at 4°C for 30 min, the supernatant fluid was sterilized by Millipore filtration. 5 ml of the original TSB supernatant fluid was similarly treated, and the bactericidal activities of the two preparations were compared.

Relation of Bactericidal Substance to Other Staphylococcal Products.—Cultures of *S. aureus* strain C55 were grown in 10 liter volumes of a dialysate medium of tryptic soy broth (4). The supernatant fluids were harvested and filtered as described above and brought to 85% saturation with ammonium sulfate. The precipitate was collected on Filter Cel (Hy Flo Filter Cel, George T. Walker & Co., Inc., Minneapolis, Minn.) and eluted with 0.067 M phosphate buffer, pH 7.2. After dialysis against the same buffer, the dissolved products were

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fractionated by 10% saturation increments of ammonium sulfate. The precipitates were collected by centrifugation at 10,000 *g* in a refrigerated Sorvall centrifuge and dissolved in phosphate buffer. After dialysis and Millipore filtration, the specific activity of the bactericidal substance and of various known staphylococcal products was determined in each fraction.

Determination of Activity of Staphylococcal Products.—Bactericidal activity was determined by the lawn spotting method (4) using a strain of *Diplococcus pneumoniae* and of M Type 49 Group A β-hemolytic streptococcus as indicator lawns. Staphylokinase, ribonuclease (RNase), and deoxyribonuclease (DNase) activities were determined by methods previously used in this laboratory (6–8). All other staphylococcal products were assayed according to the methods described by Chesbro et al. (9).

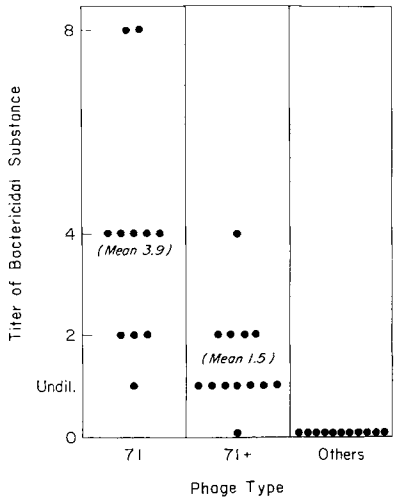

FIG. 1. Comparison of strains of *S. aureus* of various phage types in their capacity to produce the bactericidal substance. Type 71 are lysed only by phage 71; type 71+ are lysed by other phages in group II (3A, 3C, and 55) in addition to 71; the others represent various phage types in groups I, II, and III.

RESULTS

Association of Bactericidal Substance with Phage Type 71 S. aureus.—In a previous communication (4) staphylococcal skin isolates belonging to pure phage type 71 were shown to produce the bactericidal substance. Staphylococci lysed by other phages as well as a single strain lysed by phages 3A and 71 failed to produce the substance. 35 additional staphylococcal strains were assessed for their capacity to produce the bactericidal substance. These strains were isolated from skin lesions as well as from nose and throat cultures. The purpose of the present experiment was to examine a larger number of staphylococci lysed by phage 71, some that are lysed by other phages in addition to 71, and some other unrelated phages. The staphylococcal strains were grown separately in 50 ml of tryptic soy broth for 18–24 hr. The supernatant fluids were harvested

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as described under Materials and Methods. The bactericidal capacity of various dilutions of the filtered supernatant fluids against Type 49 streptococcus was determined by the lawn spotting method (4). The results are illustrated in Fig. 1. Strains of *S. aureus* lysed only by phage 71 produce the bactericidal substance in higher concentrations than ones lysed by other phages in addition to phage 71, whereas other staphylococcal phage types fail to produce the bactericidal substance. No difference regarding the source from which they were originally

TABLE I

Susceptibility of Various Organisms to the Bactericidal Substance

Sensitive organisms	Resistant organisms*
1:8 or >‡:	
Diplococcus pneumoniae (3)§	*Escherichia coli* (5)
Corynebacterium diphtheriae (1)	*Klebsiella* spp. (5)
Micrococcus lysodeikticus (1)	*Pseudomonas* spp. (10)
Bacillus megaterium (1)	*Mima* (1)
Bacillus subtilis (1)	*Herellea* (1)
	Enterobacteriaceae (1)
1:2–1:4:	
Corynebacterium diphtheriae (3)	*Staphylococcus aureus* (11)
Staphylococcus aureus (10)	*Staphylococcus epidermidis* (18)
Bacillus subtilis (1)	*Corynebacterium equi* (1)
Bacillus cereus (1)	*Candida albicans* (1)
Streptococcus pyogenes M49 (1)	*Candida tropicalis* (1)
Undiluted:	
Staphylococcus aureus (10)	
Listeria monocytogenes (1)	

* Resistant to all concentrations of bactericidal substance.

‡ Dilution of bactericidal substance.

§ Number in parenthesis indicates number of strains tested.

isolated was noted among the staphylococci that produce the bactericidal substance.

Spectrum of Activity of Bactericidal Substance.—The earlier demonstration (4) of the susceptibility of various streptococci to the bactericidal substance prompted the investigation of the effect of this substance on a variety of other microorganisms. Using the same preparation, the bactericidal effect on several Gram-positive and Gram-negative organisms was determined simultaneously by the lawn spotting technique. The results are shown in Table I. Among the sensitive organisms, a definite and reproducible variation in susceptibility was found, and some variation was evident not only among various species, but also among different strains of the same species. The Type 49 streptococcus strain has been shown to be the most sensitive among Group A streptococci

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(4) and occupies an intermediate position among the species listed here. All 18 strains of *Staphylococcus epidermidis* tested were resistant and so were all the Gram-negative rods tested. Among 31 different staphylococcal strains tested, 11 were resistant, and 20 were sensitive. The strains represented various phage types and no correlation of susceptibility to phage type was noted. Strains belonging to phage types 3A, 3C, 55, and 71 (group II) were similarly either sensitive or resistant. In no instance did the bactericidal substance exert any demonstrable effect when it was tested against the producing strain.

Factors Affecting Production of Bactericidal Substance.—It has been demonstrated previously (4) that certain environmental factors affect the degree of production of the bactericidal substance. Best yields were obtained when the producing staphylococcal strain was grown in TSB for 24–48 hr. Additional

TABLE II

Effect of Repeated Subculturing and Storage at 4°C on Production of Bactericidal Substance

No. of times subcultured	Duration of storage	Titer of bactericidal substance	
		Pneumococci	Streptococci
	wk		
13	0	1:8	1:4
12	1	1:8	1:4
11	3	1:8	1:4
9	5	1:8	1:2
7	7	1:4	1:4
4	10	1:8	1:4
1	13	1:4	1:2
0	25	1:4	1:4

experiments were carried out in an attempt to investigate further the effect of various other environmental factors on the production of the bactericidal substance.

The effect of repeated subculturing and storage of the staphylococci at 4°C on their capacity to produce the bactericidal substance was studied. *S. aureus* strain C55 was subcultured for 18 hr at 37°C on tryptic soy agar plates every week for a period of 25 wk. The agar plates were stored at 4°C for the remainder of the 25 wk period. At the end of this time one colony from each plate was inoculated into 50 ml of TSB and incubated for 24 hr at 37°C. The bactericidal capacity of two-fold dilutions of the supernatant fluids against *D. pneumoniae* and Type 49 Group A streptococcus was assessed by the lawn spotting method. The results are shown in Table II and indicate that there was no appreciable alteration in the capacity of the C55 strain to produce the bactericidal substance after multiple passage or prolonged storage at 4°C.

Since the bactericidal factor has a spectrum of activity similar to a staphylo-

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coccin described by Hamon and Peron (10), and some bacteriocins have been shown to be produced in large quantities after exposure of the culture to inducing agents (10), the effect of ultraviolet light and mitomycin C on the production of the inhibitor was investigated. Table III indicates that exposure to ultraviolet light for the indicated lengths of time totally inhibited the production of the bactericidal substance.

TABLE III

Effect of Ultraviolet Irradiation on Production of Bactericidal Substance

Incubation time after exposure	Titer of bactericidal substance against M49 streptococcus			
	Duration of UV exposure			Untreated
	10 sec	30 sec	60 sec	
hr				
4	0	0	0	Undiluted
22	0	0	0	1:4

TABLE IV

Effect of Mitomycin C (0.1 μg/ml) on Production of Bactericidal Substance

Incubation time after exposure	Titer of bactericidal substance					
	Mitomycin-treated		Untreated		Mitomycin control	
	Pneumococci	Streptococci	Pneumococci	Streptococci	Pneumococci	Streptococci
hr						
0	Undiluted	0	Undiluted	0	0	0
1	"	0	"	0	0	0
2	"	0	1:2	0	0	0
4	1:2	0	1:4	Undiluted	0	0
22	1:8	Undiluted	1:16	1:4	0	0

The effect of mitomycin C, another bacteriocin-inducing agent, on the production of the bactericidal substance is shown in Table IV. In the concentrations used, mitomycin C alone had no inhibitory effect on the growth of pneumococci or Type 49 streptococcus. Comparison of optical densities of the mitomycin C-treated staphylococci and the untreated ones at the indicated times showed no evidence of lysis of cells at this concentration of mitomycin C. As can be noted from Table IV, however, the mitomycin C-treated staphylococci had lower titers of the bactericidal substance as compared to the untreated controls at the indicated times. When a similar experiment was performed using 0.5 μg/ml of mitomycin C, lysis of the staphylococci occurred, but a certain degree of inhibition of the pneumococci and M49 streptococcus was also observed with this concentration of mitomycin C. However, the same finding of

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inhibition of production of the bactericidal substance by mitomycin C was again noted.

Location of the Bactericidal Substance.—Recovery of the bactericidal substance has been achieved in all previous experiments from supernatant fluids of broth cultures. Experiments were designed to assess whether the bactericidal substance is exclusively an extracellular product of phage type 71 *S. aureus* or is also cell-associated. The C55 strain of *S. aureus* was grown in TSB, and after appropriate incubation, the supernatant fluid was harvested and the sedimented cells were also saved. The bactericidal activity of the original supernatant fluid and the supernatant fluid obtained after disrupting the staphylococcal cells

TABLE V

Per Cent Distribution of the Specific Activities of the Various Staphylococcal Products

Staphylococcal product	Per cent ammonium sulfate saturation					
	0–40	40–50	50–60	60–70	70–80	80–90
Bactericidal substance	13	4	6	27	50	0
Fibrinolysin	0	8	77	15	0	0
Hyaluronidase	8	0	0	0	36	56
Staphylokinase	0	100	0	0	0	0
Nuclease*	0	0	40	60	0	0
Muramidase	69	23	8	0	0	0
Leukocidin	12	44	44	0	0	0
α-Hemolysin	10	43	35	7	5	0
β-Hemolysin	7	48	40	5	0	0
Coagulase	—‡	—	—	—	—	—
Lipase	—	—	—	—	—	—

* Both RNase and DNase activities are comparable in the indicated fractions.

‡ —, not detectable.

was determined against *D. pneumoniae* and Type 49 streptococcus. No bactericidal effect was noted with the supernatant fluid of the disrupted cells, whereas the usual activity of the original TSB supernatant fluid was again noted. These data further indicate that the bactericidal substance is a diffusable extracellular product of phage type 71 staphylococci.

Purification of the Bactericidal Substance and Its Relationship to Other Staphylococcal Products.—The possible relationship of the bactericidal substance to other known staphylococcal products was examined. Table V shows a comparison of the relative distribution of the specific activity for each of the indicated staphylococcal products in the different ammonium sulfate fractions. Specific activities were determined as the concentration of each product per mg protein in each fraction.

Coagulase and lipase activities could not be detected in any of the ammonium sulfate fractions. The experimental conditions are probably not optimal for

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coagulase production. Lipase is known to be primarily cell associated. Although a certain degree of overlap was found among the various extracellular staphylococcal products (Table V), ammonium sulfate fractionation is an effective method of separating the bulk of bactericidal substance from most of the other products. The specific activity of the bactericidal substance is highest in the

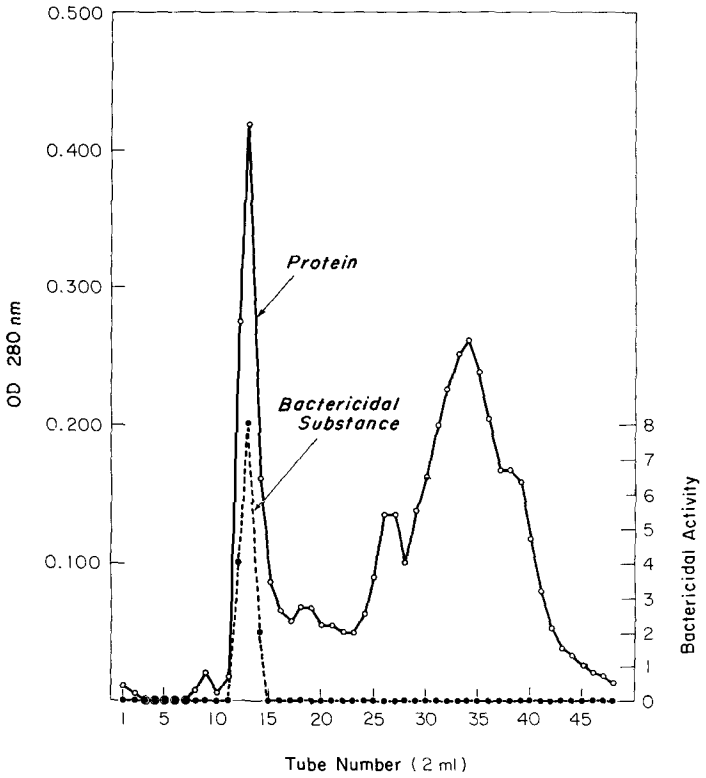

FIG. 2. Gel filtration of 60–80% ammonium sulfate fractions on Sephadex G-100 column (void volume 30 ml).

60–80% ammonium sulfate fractions. Its distribution appears to be different from that of all the other products.

Attempts have been made to further purify the bactericidal substance by applying the 60–80% ammonium sulfate fractions on various cellulose columns. Strong binding at pH 7.0 to diethylaminoethyl (DEAE)-cellulose occurs with no successful elution of the bactericidal substance in a 2 M sodium chloride solution. At the same pH, no binding to phosphocellulose or carboxymethylcellulose columns occurs, thus indicating that the bactericidal substance has a net negative charge at neutral pH.

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Considerable purification of the bactericidal substance was achieved by gel filtration on a Sephadex G-100 column. When the 60–80% ammonium sulfate fractions were put over such a column, the pattern illustrated in Fig. 2 was observed. A single peak of protein contained all the bactericidal activity, and was separate from the main protein peak. Activities of all the staphylococcal products were again determined in the fractions containing the bactericidal activity. In addition to the bactericidal substance, α-hemolysin was the only other product detected. The specific activities of the bactericidal substance and α-hemolysin were compared in the 60–80% ammonium sulfate fractions before and after fractionation on the Sephadex column. A 250-fold increase in the specific activity for the bactericidal substance was noted as compared to a reduction in α-hemolysin specific activity to one-half.

DISCUSSION

Enhancement or inhibition of growth of bacteria by certain other related or unrelated microorganisms is an observation of long standing (14). In vitro bacterial interference could be due to exhaustion of or competition for an essential nutrient, the production of toxic metabolites, or the liberation of a variety of antibiotic substances. The experimental conditions under which bactericidal activity was assessed in the present studies and previously (4) rule out the possibility of nutrient exhaustion or competition. A unique group of antibiotic substances produced by various microorganisms are the bacteriocins (11–13)

Two groups of bacteriocins have been recognized (11): (*a*) high molecular weight group, identified as phage or phage-like particles, which are trypsin-resistant, thermolabile, and sedimentable; and (*b*) low molecular weight bacteriocins that cannot be resolved by electron microscopy, and which are trypsin-sensitive and thermostable. Most bacteriocins isolated from Gram-negative organisms belong to the high molecular weight group and a great deal is available in the literature regarding their nature, production, and action (11). In contrast, very little is known about bacteriocins of Gram-positive organisms, although such substances have been described (13).

Staphylococcins have been isolated by Fredericq (15) and by Hamon and Peron (10). The term staphylococcin was used by these investigators to describe a proteinaceous substance from a variety of staphylococci that inhibits the growth of several Gram-positive organisms. Inhibition was demonstrated on agar plates only and no further characterization of these staphylococcins was described.

The classification of the bactericidal substance described in this report and in a previous communication (4) as a bacteriocin is probably justifiable. If the definition of bacteriocins as "bactericidal particles which are unable to multiply in a sensitive indicator" (11) is accepted, then the bactericidal substance described here from phage type 71 staphylococci may be so classified. Further-

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more, all previously described bacteriocins have been found to be macromolecular and to include, if not to consist of, polypeptide or protein (13). The previously described (4) thermostability and trypsin-sensitivity of the substance would categorize it under the low molecular weight bacteriocins (11). It should be obvious, however, that the above criteria for classification are not very well defined and that the bactericidal substance may indeed be unrelated to the classical bacteriocins.

Bacteriocins of Gram-positive bacteria are believed to differ from those of Gram-negative bacteria in their mode of action (13) and in their activity spectra (10). While most Gram-negative bacteriocins act on susceptible cells by inhibiting DNA, RNA, or protein synthesis (11), certain Gram-positive bacteriocins (megacins) seem to act on the cell membrane of sensitive bacteria. Moreover, in contrast to the effect of Gram-negative bacteriocins which is limited to closely related species, bacteriocins of Gram-positive organisms have a wider spectrum of activity. In this regard they resemble the bactericidal substance described here.

In some respects, a distinct difference exists between the bactericidal substance described in the present studies and bacteriocins. The production of almost all reported bacteriocins is increased by inducing agents such as mitomycin C or ultraviolet irradiation (11). Both these agents inhibit or totally abolish the production of the bactericidal substance. The proteinaceous nature of the bactericidal substance has been reported previously (4) and further confirmed in the present studies. Since both mitomycin C and ultraviolet irradiation are known inhibitors of protein synthesis, it is not surprising to observe their inhibition of production of the bactericidal substance. Furthermore, the substance has been shown to be a diffusable extracellular product of phage type 71 staphylococci distinct from all other known staphylococcal products. The extracellular location of the substance would be unusual for a bacteriocin.

A considerable degree of purification of the bactericidal substance was achieved by a combination of ammonium sulfate fractionation and gel filtration on Sephadex G-100 column. Only small amounts of α-hemolysin activities remain with the bactericidal substance after such treatment. This clearly suggests that the bactericidal substance is unrelated to any of the other known biologically or chemically active substances of *Staphylococcus aureus*. Thus, this substance seems to be unique both in regard to its distinction from other staphylococcal products and also with respect to its production by a limited group of strains of this species. The production of this substance only by staphylococci lysed by phage 71 may have certain significant genetic and epidemiologic implications. The ability to produce the bactericidal substance is a stable property of the staphylococci and it seems likely that it is genetically determined. The possibility of transferring this ability from phage type 71 staphylococci to other phage types certainly warrants investigation. The epidemiologic

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implications have been alluded to previously (4). Both the *S. aureus* and Group A streptococcus are known to produce skin infections; however, the interaction between these two groups of organisms is not well understood. If phage type 71 staphylococci are capable of producing the bactericidal substance in vivo, colonization with such a strain may prevent skin infection with a β-hemolytic streptococcus. Furthermore, such staphylococcal strains may secondarily invade an existing streptococcal skin lesion and eradicate the streptococci from it. Studies are currently in progress to assess the role of such interaction in natural and experimental skin infections.

Studies of the mechanism of action of this bactericidal substance and of its effects on the ultrastructural appearance of susceptible organisms will be published elsewhere (16, 17).

SUMMARY

A bactericidal substance previously isolated from phage type 71 *Staphylococcus aureus* has been further identified and characterized. Staphylococci belonging to phage type 71 produce the substance in higher titers than staphylococci lysed by other phages in group II in addition to phage 71. Other staphylococci do not produce the bactericidal substance. The bactericidal substance shares several of the properties of bacteriocins but differs from this group of antibiotic substances in some respects. A combination of ammonium sulfate fractionation and gel filtration on a Sephadex G-100 column resulted in considerable degree of purification of the bactericidal substance. The substance is a previously unrecognized product of *S. aureus* and is distinct from other extracellular products of this organism.

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