# Characterization of a *Staphylococcus aureus* Bacteriocin

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The bacteriocin produced by a strain of *Staphylococcus aureus* has been isolated and designated staphylococcin (414), and a study was made of its chemical, physical, and biological properties. The staphylococcin is released in appreciable quantities after breakage of the cells and can be purified through differential centrifugation and column chromatography. In the native state, it appears to be a lipoprotein-carbohydrate complex with a molecular weight in excess of 200,000. The complex can be dissociated by sodium dodecyl sulfate into smaller subunits which retain activity. The gross chemical and physical properties of the bacteriocin closely resemble those ascribed to certain preparations of cell membranes. Staphylococcin (414) is not a lytic enzyme like lysostaphin and does not have the same spectrum of activity. Like other bacteriocins from gram-positive microorganisms, it does not inhibit any gram-negative bacteria, but does inhibit several other genera.

The ability of various bacteria to inhibit the growth of other bacteria has long been recognized and sometimes attributed to such products as metabolic wastes, antibiotics, and bacteriophages (8, 39). To this list could be added the bacteriocins, inhibitory substances of a proteinaceous nature, the production of which is lethal and the action of which is limited to closely related species possessing the necessary specific receptor sites (20).

The colicins, bacteriocins elaborated by *Escherichia coli* and related species, were the first to be discovered (12), and, over the years, much knowledge has been accumulated concerning their chemistry, genetics, and mode of action. No such body of information exists for the bacteriocins produced by gram-positive organisms (10, 19, 35, 40). In 1946, Fredericq (9) noted the similarity between colicins and inhibitory substances produced by staphylococci and named the latter substances staphylococcins. Since then there seems to have been no concerted effort to further characterize these bacteriocins. This report deals with the isolation and preliminary characterization of a staphylococcin.

## MATERIALS AND METHODS

**Source of microorganisms.** The various isolates of *Staphylococcus aureus* used in this study were generously provided by J. B. Wilson of this department. Cultures used daily were maintained on BHI (Brain Heart Infusion, Difco) agar slants. Stock cultures were kept in a freeze-dried state or on porcelain beads (17).

**Screening of cultures.** Prospective bacteriocinogenic cultures were stabbed into BHI agar plates and incubated at 37 C for 24 hr. Surface growth was then killed by exposure to chloroform vapor and the plates were overlayered with soft agar (BHI broth + 0.8% agar) inoculated with a prospective indicator culture. The plates were reincubated at 37 C for 12 to 18 hr and then examined for a zone of inhibition in the bacterial lawn just above the stabs. In this way, both producer and indicator strains were identified. To determine whether the zones of inhibition were due to phage activity, blocks of agar were cut out of these zones, crushed in tubes of sterile broth, and incubated at 37 C overnight. Samples from the tubes showing no bacterial growth were then mixed with soft agar inoculated with the indicator culture and poured onto plates. After incubation, the plates were examined for the presence of phage plaques. If no phage plaques were found, this was considered presumptive evidence that the inhibition was due to a bacteriocin.

**Bacteriocin assays.** The specific activity of various staphylococcin preparations was determined by first making serial dilutions in BHI broth and then placing 0.01 ml of the dilutions on sections of a dry BHI plate. The plates were briefly exposed to the air to allow the drops to dry and were then overlayered with 3 ml of soft agar containing approximately $10^7$ indicator cells. The highest dilution resulting in a clear zone of inhibition was said to contain one unit of staphylococcin per ml and the titer of the bacteriocin preparation thus became the reciprocal of this dilution.

**Enzymatic studies.** The ability of three enzymes to inactivate solutions of staphylococcin was tested. The enzymes were used at a final concentration of 1 mg/ml in different buffers as follows: Ficin (Nutritional Biochemicals Corp., Cleveland, Ohio) was suspended in a buffer consisting of 0.02 M cysteine-hydrochloride,

0.01 M ethylenediaminetetraacetic acid (EDTA), and 0.15 M NaCl, and the pH was adjusted to 7.0; Pronase (B grade, Calbiochem, Los Angeles, Calif.) was dissolved in a sodium barbital-hydrochloride buffer (pH 7.8) containing 0.01 M CaCl₂ and 0.001 M CoCl₂; Trypsin (A grade, Calbiochem) was dissolved in tris-(hydroxymethyl)aminomethane(Tris) - hydrochloride buffer (pH 8.0), containing 0.01 M CaCl₂. A solution of staphylococcin (414) containing one of the enzymes listed above was incubated at 37 C for periods up to 4 hr, and samples were removed at intervals and assayed. Control experiments were also performed by using staphylococcin in the same buffer solutions, but without enzymes. In several instances, a quick check for enzyme inactivation was made by placing a drop of the enzyme in buffer directly on the growth of a stab plate which had previously been exposed to chloroform vapor. Failure of a zone of inhibition to appear where one had been noted on a previous test indicated enzyme inactivation of that particular bacteriocin.

**Induction and elimination techniques.** In certain instances, bacteriocinogenic factors have been induced (18, 21) and eliminated (16, 23) by methods used in phage work. Two procedures were used in an attempt to induce *S. aureus* strain 414 to produce greater quantities of staphylococcin. A BHI broth culture with an optical density (OD) of 0.50 measured at 650 nm was diluted 10-fold in fresh BHI broth containing mitomycin C (Nutritional Biochemicals Corp.) at concentrations of 0.5, 1.0, 2.0, 4.0, and 8.0 µg/ml. The cultures were reincubated at 37 C. At various intervals, OD measurements were made, and two 1-ml samples were removed from each flask. The first sample was heated at 65 C for 30 min; the second sample was sterilized with chloroform and then centrifuged. The supernatant fluid was removed and the pellet was resuspended to original volume. The heat-killed culture, supernatant fluid, and resuspended cells were then assayed for activity.

In a second procedure, the culture was grown up as before, and the cells were harvested by centrifugation. One-half of the cells was suspended in 0.15 M NaCl and the other half was suspended in BHI broth. The suspensions were placed in petri dishes (20 by 150 mm) and irradiated with an ultraviolet lamp (Westinghouse G-8T5, Arthur H. Thomas Co., Philadelphia, Pa.) placed 20 cm above the dish. At intervals between 30 and 300 sec, samples were taken and handled as in the first procedure.

Two dyes, acridine orange (Basic Orange 14; Matheson, Coleman and Bell, East Rutherford, N. J.) and neutral acriflavine (Sigma Chemical Co., St. Louis, Mo.) were tested for their ability to eliminate staphylococcin production in three strains of *S. aureus*. The dyes were dissolved in 5 ml of BHI broth in concentrations ranging from 1 to 100 µg/ml, and the tubes were then inoculated with 0.1 ml of an 18-hr broth culture of the producing strains. After 24 hr of incubation at 37 C, serial dilutions were made of the tubes with the highest dye concentration in which there was growth. The various dilutions were spread on dry BHI plates, and the plates were incubated at 37 C for 24 hr. Those plates which had well-isolated colonies on them

were then overlayed with the indicator culture as in the stab plate screening procedure.

**Column chromatography.** Sephadex G-200 (Pharmacia, Uppsala, Sweden) was prepared and packed to give a bed size of 50 by 3.4 cm (LKB, Stockholm, Sweden) by the usual procedure, using 0.05 M Tris-hydrochloride buffer (pH 8.0) for the packing and final equilibration.

**Acrylamide gel electrophoresis.** The procedures, reagents, and apparatus used were essentially those described by Maizel (31) and Dunkert and Rueckert (7). A 0.05 M phosphate buffer (pH 7.0) containing 0.1% sodium dodecylsulfate (SDS) was used to dissolve the samples and prepare the gels. An 8% gel (acrylamide-bis, 29:1, w/w) was prepared by using 0.025% ammonium persulfate as a polymerizer and 60 µliters of tetramethylenediamine per 100 ml of acrylamide solution as an accelerator. Sucrose was added to the samples to give a concentration of 10% (w/v), and 100 µliters of the sample was carefully layered on each gel. The gels measured 6 by 90 mm, and electrophoresis was carried out at 3 v/cm of gel (actual voltage across gel) or roughly 5 ma per tube for 4 hr. After electrophoresis, the gels were stained for protein by using Coomassie brilliant blue (7), carbohydrate by using Alcian Blue, or lipid by using oil red O (6). Non-Enzymic Protein Molecular Weight Markers (Mann Research Laboratories, New York, N.Y.) were used at the same time in adjacent gels at a concentration of 0.5 mg/ml. Samples of staphylococcin contained 0.5 mg/ml. The inhibitory activity of the staphylococcin could be located in the gel after electrophoresis by first soaking unstained gels for 12 hr in 0.05 M phosphate buffer (pH 7.0) to remove most of the SDS and then by embedding the gels in soft agar containing the indicator culture. The relative position of the zone of inhibition could then be compared with the bands which developed in stained gels run at the same time.

**Analytical methods.** Nitrogen determinations were performed by using the digestion technique of Koch and McMeekin (24) and a modified Nesslerization (45). Protein was determined by the method of Lowry et al. (28), by using Bovine Serum Albumin fraction V (Nutritional Biochemicals Corp.) as the standard. Other determinations included phosphorus by the method of Chen et al. (5), carbohydrate by the method of Loewus (27) and lipid by the gravimetric procedure used by Rude and Goebel (41).

**Electron microscopy.** A solution of staphylococcin (1 mg/ml) in Tris-hydrochloride buffer (pH 8.0) was dropped on Formvar-coated grids and negatively stained with a 1:1 mixture of 1.0% osmium tetroxide and 1.5% phosphotungstic acid in phosphate buffer (pH 7.5). The grids were washed in distilled water, dried, and then examined in an electron microscope (model HU-11E-1, Hitachi, Ltd., Tokyo, Japan).

**Immunological procedures.** Rabbits were immunized by two different methods. Animals in the first group received 50 mg of staphylococcin in 1 ml of Complete Freunds Adjuvant (Difco) administered subcutaneously in five equal parts. The animals were bled 8 weeks later by cardiac puncture. Rabbits in the second group received three intravenous injections of 5 mg of staphylococcin per week for three weeks. One

week after the last injection the animals were bled by cardiac puncture. Tests for neutralizing antibody were performed as previously described (11). Ring tests were carried out as described by Campbell et al. (4).

## RESULTS

**Screening of cultures.** For the initial screening, approximately 200 cultures of *S. aureus* were arbitrarily selected from the 600 available and were tested for their ability to inhibit 21 similarly selected cultures. Although many cultures seemed capable of producing very small zones of inhibition, very few were found which repeatedly produced zones of inhibition greater than 5 mm in diameter by using the stab plate procedure. Eventually, three producer strains (263, 414, and 462) and three indicator strains (19, 140, and 698) were selected for further study. Some of the characteristics of these cultures are shown in Table 1. It is possible to differentiate the three producing strains by their spectra of activity (Table 2). The biological activity of the producing cultures cannot be serially transferred in the sensitive culture as would be expected if activity were due to bacteriophage. Bacteriophage could not be harvested from the soft agar in the zone of inhibition on stab plates by the method described under screening procedures. Other evidence to be presented makes it unlikely that activity is associated with a defective phage or structural parts of phage such as tails.

Attention was drawn to strain 414 early in the study when zones of inhibition approaching 30 mm were observed after overlaying stabs with strain 698. As a result, the major emphasis in this investigation was placed on the bacteriocin produced by strain 414. Where the data are available, comparisons with strains 263 and 462 have been made.

**Isolation and purification.** An attempt was made to isolate the bacteriocin produced by strain 414 from the supernatant fluid of various broth cultures incubated at 30 or 37 C and under either aerobic or anaerobic conditions. The various broths used were BHI, Nutrient Broth (Difco), Trypticase Soy Broth (Difco), and Thioglycollate Broth (Difco). Although strain 414 grew reasonably well under all these conditions, little or no inhibitory activity could be demonstrated in the broth supernatant fluids. However, if the heat-killed cells were resuspended in broth to the original culture volume, they generally had an activity in the range of 100 to 200 units/ml. Under the conditions employed, neither irradiation with ultraviolet light nor the use of mitomycin C brought about an increase in levels of bacteriocin in either the cells themselves or the broth supernatant fluid.

TABLE 1. *Characteristics of strains of Staphylococcus aureus selected for their staphylococcin sensitivity or production*

| Strain | Origin | Inorganic nitrogen utilization | Growth on mannitol-salt | Growth on tellurite-glycine | Coagulase production |
|--------|--------|-------------------------------|-------------------------|-----------------------------|----------------------|
| 19[a] | Sheep | − | + | − | + |
| 140[a] | Raccoon | − | + | + | + |
| 263[b] | Mink | − | + | + | + |
| 414[b] | Turkey | − | + | + | + |
| 462[b] | Mink | − | − | + | + |
| 698[a] | Cow | − | + | + | + |

[a] Sensitive to one or more staphylococcins.
[b] Staphylococcin producer.

TABLE 2. *Diameter (mm) of zones of inhibition on indicator cultures*

| Bacteriocin producer | Indicator cultures | | |
|----------------------|------|------|------|
| | 19 | 140 | 698 |
| 263 | 0 | 6 | 0 |
| 414 | 0 | 15 | 30 |
| 462 | 14 | 15 | 0 |

Attempts to elute the staphylococcin from the cells also met with failure. The eluants tried were 1.0 M NaCl, 0.05 M EDTA, 7 M urea, and 0.5% SDS. Extraction with water at 65 C was also attempted, by using a procedure which proved successful for obtaining colicin K from *Shigella sonnei* (15), but with negative results.

Disruption of the cells, however, released substantial quantities of the bacteriocin. *S. aureus* strain 414 was grown on a rotary shaker at 37 C for 18 hr in 1 liter of BHI broth, and the culture was centrifuged 7,600 × g for 30 min. The cells were collected, resuspended to 10% (w/v) in 0.15 M NaCl, and killed by heating at 65 C for 30 min. The cells were then spun down and washed twice by resuspension in 1.0 M NaCl followed by centrifugation. This step removed much colored material which was not characterized, but which had little inhibitory activity. The washed cells were suspended in 0.15 M NaCl and were subjected to breakage at 40,000 psi in a Ribi cell fractionator (model RF-1, Ivan Sorvall, Inc., Norwalk, Conn.) with the orifice temperature held at 15 to 20 C. Large cell fragments were removed by two centrifugations at 7,000 × g for 20 min. The supernatant fluid was then centrifuged at 38,000 × g for 3 hr, resulting in a pale yellow supernatant fluid and a pellet with four discernible layers. The bulk of the inhibitory

activity resided in the brown transparent jelly-like layer at the top of the pellet, and this material could be removed by careful scraping with a spatula. After resuspension in 0.05 M Tris-hydrochloride buffer (pH 8.0), the material was further purified by chromatography on G-200 Sephadex gel. The bulk of the activity was observed to come off in a peak eluted at the void volume of the column, and a moderate amount of colored material with little activity came off later. This step also removed uncharacterized inactivating substances.

The contents of the tubes making up the first peak were pooled, dialyzed, and freeze-dried. The powder was then extracted on a fine-sintered glass filter with chloroform and ether to remove unbound lipids. The resulting off-white granular substance was called staphylococcin (414) and had a specific activity on strain 698 of one unit per 0.5 μg. The isolation procedure is shown in Fig. 1, and the yields and activities are summarized in Table 3.
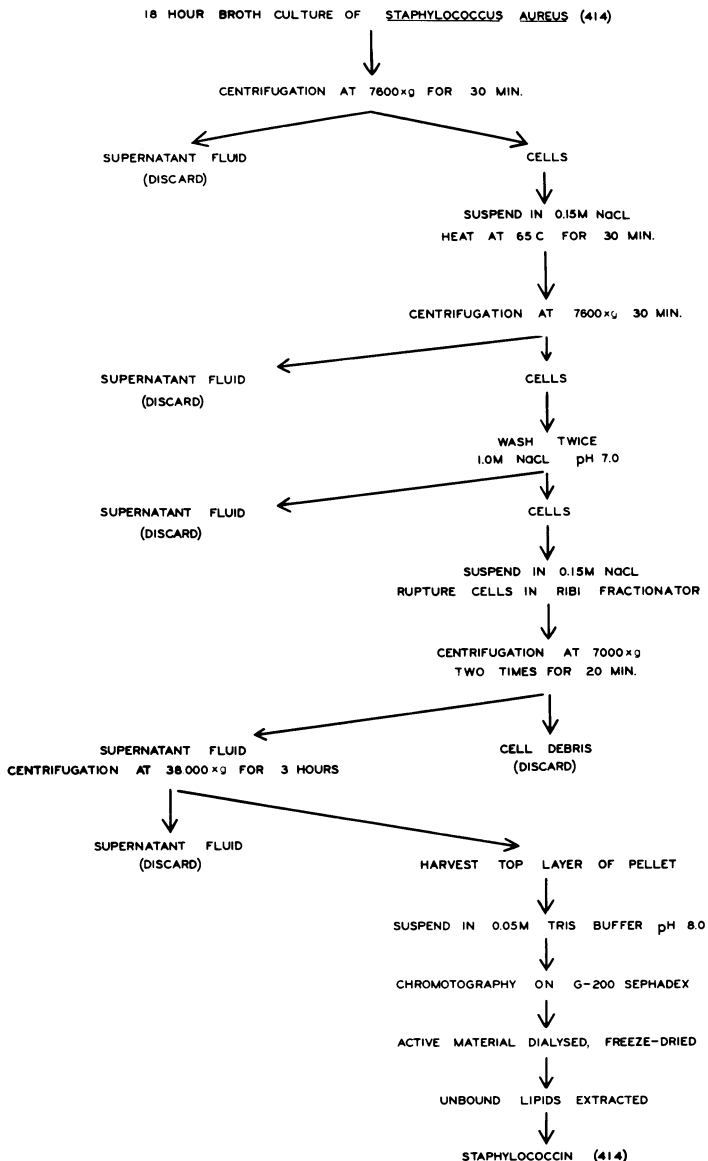
Attempts to extract an active material from

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        18 HOUR BROTH CULTURE OF   STAPHYLOCOCCUS  AUREUS  (414)
                                │
                                ▼
              CENTRIFUGATION  AT  7600×g  FOR  30 MIN.
                    ╱                           ╲
          SUPERNATANT  FLUID                   CELLS
             (DISCARD)                           │
                                                 ▼
                                    SUSPEND  IN  0.15M  NaCL
                                    HEAT  AT  65 C  FOR  30 MIN.
                                                 │
                                                 ▼
                               CENTRIFUGATION  AT  7600×g  30 MIN.
                    ╱                            │
          SUPERNATANT  FLUID                   CELLS
             (DISCARD)                           │
                                                 ▼
                                         WASH    TWICE
                                    1.0M  NaCL     pH 7.0
                    ╱                            │
          SUPERNATANT  FLUID                   CELLS
             (DISCARD)                           │
                                                 ▼
                                 SUSPEND  IN  0.15M  NaCL
                          RUPTURE  CELLS  IN  RIBI  FRACTIONATOR
                                                 │
                                                 ▼
                                  CENTRIFUGATION  AT  7000×g
                                  TWO  TIMES  FOR  20 MIN.
                    ╱                            │
          SUPERNATANT  FLUID                  CELL  DEBRIS
   CENTRIFUGATION  AT  38,000×g  FOR  3 HOURS   (DISCARD)
           ╱              ╲
  SUPERNATANT  FLUID       ╲
     (DISCARD)              ►   HARVEST  TOP  LAYER  OF  PELLET
                                                 │
                                                 ▼
                            SUSPEND  IN  0.05M  TRIS  BUFFER  pH 8.0
                                                 │
                                                 ▼
                            CHROMOTOGRAPHY  ON  G-200 SEPHADEX
                                                 │
                                                 ▼
                            ACTIVE  MATERIAL  DIALYSED,  FREEZE-DRIED
                                                 │
                                                 ▼
                                 UNBOUND  LIPIDS  EXTRACTED
                                                 │
                                                 ▼
                               STAPHYLOCOCCIN  (414)
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FIG. 1. *Isolation and purification of staphylococcin (strain 414).*

strains 263 and 462 by this same procedure were unsuccessful.

**Chemical and physical properties.** The following values were obtained in the chemical analysis of staphylococcin: nitrogen, 8.7%; protein, 46%; carbohydrate, 1.2%; lipid, 24.3%; phosphorus, 0.2%; and ash, 3.2%. Staphylococcin appears to be remarkably stable, retaining its activity for more than 9 months when stored in the freeze-dried state or in Tris buffer at pH 8.0. Prolonged heating of staphylococcin in Tris buffer at 70 C or less does not decrease activity, but 50% of the activity is lost in 30 min at 100 C. Staphylococcin (414) is not inactivated by ficin under the conditions employed. Both trypsin and Pronase bring about a slow inactivation leading to an 80% loss in activity at the end of 2 hr. Since the inhibitory substances could not be isolated from strains 263 and 462, direct comparisons of enzymatic inactivations cannot be made with staphylococcin (strain 414). We found, however, that if the enzymes were used directly on the stab plates, as previously described, that we obtained the following results. The inhibitory activity of strain 414 was inactivated by trypsin and Pronase, that of strain 263 was inactivated by trypsin, Pronase and ficin, and only Pronase inactivated the inhibitor produced by strain 462.

Staphylococcin is generally difficult to dissolve without using such agents as 7 M urea, SDS, or sodium deoxycholate. Removal of these substances by dialysis causes the staphylococcin solution to become cloudy, but does not seem to lower activity. The fact that staphylococcin suspended in Tris buffer can be pelleted by low-speed centrifugation and that it is excluded from G-200 Sephadex gel suggests that it is a substance of high molecular weight. However, staphylococcin in Tris-hydrochloride buffer (pH 8.0) containing 0.1% SDS yields a clear solution and cannot be pelleted at 35,000 × g for 1 hr. When the staphylococcin was dissolved in a phosphate buffer containing SDS and was subjected to electrophoresis in polyacrylamide gel, the material migrated as single substance (Fig. 2). Only one band in the same location was observed whether the stain used was for protein, carbohydrate, or lipid. Inhibitory activity was also shown to be localized at this position in the gel. By comparison with substances of known molecular weight, the bacteriocin appears to have a molecular weight of 12,500 under these conditions.

Electron photomicrographs taken of negatively stained staphylococcin reveal an amorphous background containing numerous circular bodies with diameters of 16 to 64 nm. No other organized structures were observed (Fig. 3).

**Biological properties.** The inhibitory effects of

TABLE 3. *Yield and specific activity of staphylococcin (strain 414) at various stages in the purification procedure*

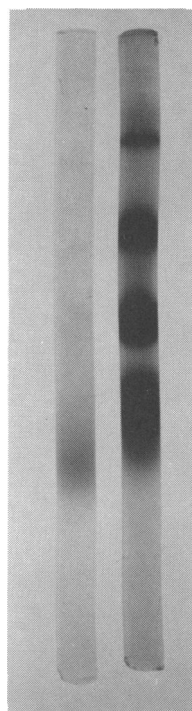| Preparation | Dry weight | Activity | Specific activity | Yield | Fold purification |
|---|---|---|---|---|---|
| | *mg* | *total units* | *units/mg* | *%* | |
| Cell lysate........... | 10,000 | 200,000 | 20 | 100 | 1 |
| Supernatant fluid from cell lysate .... | 193 | 94,184 | 488 | 47 | 24 |
| Top layer of pellet ... | 186 | 88,328 | 488 | 44 | 24 |
| Active peak from column........... | 95 | 142,500 | 1500 | 71 | 75 |
| Lipid extracted staphylococcin...... | 55 | 112,640 | 2048 | 56 | 102 |



FIG. 2. *Polyacrylamide gel electrophoretic patterns after staining for protein. Gel on left, staphylococcin 414; gel on right, protein markers used to estimate the molecular weight of the staphylococcin. From top to bottom: ovalbumin (2 bands), chymotrypsinogen A, cytochrome C.*

the staphylococcins are not restricted only to the *S. aureus* strains used in this investigation. All three producing strains inhibit a variety of other gram-positive species, but not gram-negative microorganisms (Table 4). By using the stab plate technique, strain 414 significantly inhibited 54% of the *S. aureus* strains tested, including
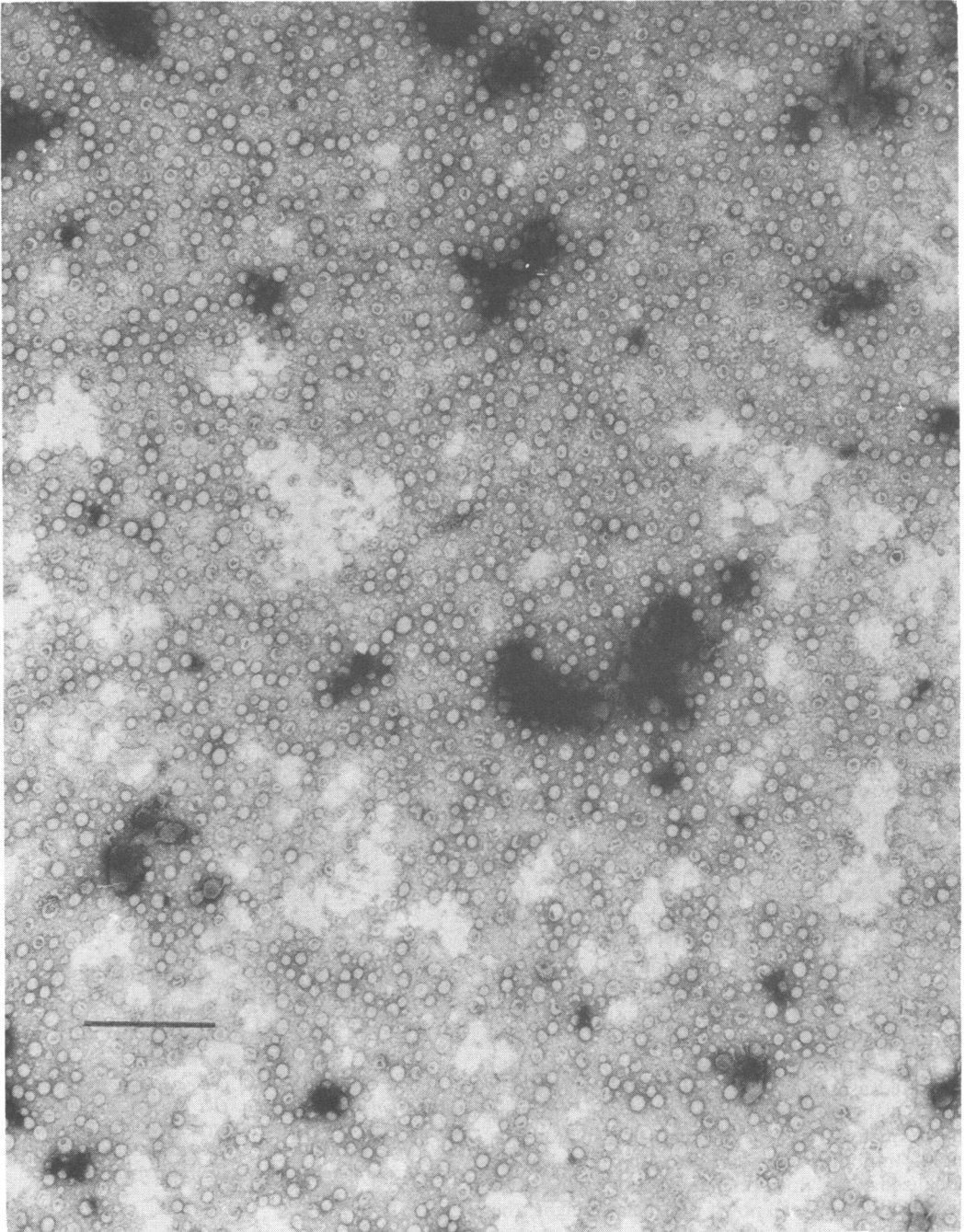
FIG. 3. *Electron micrograph of negatively stained staphylococcin (strain 414).* × *126,000. Maker represents* 0.2 μm.

itself. Strains 263 and 462 inhibited 25 and 6% respectively. Staphylococcin (414) has an activity spectrum identical to strain 414. Although the pattern of inhibition seems to be specific, adsorption of the bacteriocin seems to lack any specificity. Staphylococcin is adsorbed out of solution by both sensitive and resistant strains, by gram-negative organisms such as *E. coli*, and by such things as animal charcoal. Lysis does not occur in broth cultures killed with the bacteriocin.

The actual mode of action of the staphylococcins is under investigation and will be reported on separately.

In the course of this investigation, a large number of isolated colonies of *S. aureus* 414 were observed; not one failed to produce an inhibition of strain 698. In an attempt to derive isolates which had lost this ability, strain 414 was treated with acridine orange or neutral acriflavine, or grown at an elevated temperature of 43 C (32). The dye treatments resulted in an elimination of the bacteriocin production in greater than 90% of the colonies, whereas the elevated temperature had no effect. In contrast, when these procedures were applied to strains 263 and 462, no loss in bacteriocin production was noted. The inactive isolates of strain 414, grown under normal conditions, have not been observed to revert.

Immunization of rabbits with staphylococcin (414) elicited antibody formation since precipitates were observed when the ring test was performed by using staphylococcin and the homologous serum. However, none of the rabbits produced neutralizing antibody. The reasons for this are not clear and the matter is still under investigation. All three producing strains show alpha hemolysis when streaked on sheep blood-agar plates, but staphylococcin (414) does not cause hemolysis.

## DISCUSSION

As pointed out earlier, our knowledge of some of the bacteriocins, particularly the colicins, has accumulated at an accelerated pace and some truly fascinating questions have come out of this work. For example, how do different colicins affect different biochemical targets while staying at the cell surface (34), or, even more interesting, how do different but related colicins attaching to the same receptor sites affect completely different biochemical targets (35)? Not only are the bacteriocins intriguing substances in themselves, but they may also be of value in studying related areas such as the genetics, structure and function of cell membranes (29, 35, 36). If such work is ultimately going to be expanded to include gram-positive organisms, as it should be, then a greater effort needs to be made to isolate, purify and characterize the bacteriocins from gram-positive microorganisms.

The work presented here represents the first step towards a desired goal of fully characterizing several staphylococcins. The screening procedure was carried out with this in mind, and three different strains were isolated which differ markedly in their specificity (Tables 2 and 4). The studies with strains 263 and 462 have just been

TABLE 4. *Spectra of inhibition shown by three bacteriocinogenic strains of Staphylococcus aureus by using the stab plate procedure*

| Microorganism tested | Bacteriocinogenic strains | | |
|---|---|---|---|
| | 263 | 414 | 462 |
| *Micrococcus freudenreichii*... | —[a] | + | — |
| *M. flavus*.................... | — | + | + |
| *M. conglomeratus*........... | — | — | — |
| *Bacillus cereus*.............. | — | — | + |
| *B. megaterium*.............. | + | + | — |
| *B. subtilis*.................. | — | + | — |
| *Sarcina lutea*............... | — | + | + |
| *Brevibacterium linens*........ | + | + | — |
| *Lactobacillus acidophilus*..... | — | + | — |
| *Streptococcus lactis*.......... | — | + | + |
| *S. faecalis* var. *liquefaciens*.. | + | — | — |
| *S. bovis*.................... | — | + | — |
| *Escherichia coli*............. | — | — | — |
| *Aerobacter aerogenes*........ | — | — | — |
| *Proteus vulgaris*............. | — | — | — |
| *Serratia marscescens*........ | — | — | — |
| *Pseudomonas fluorescens*..... | — | — | — |
| *Alcaligenes viscolactis*....... | — | — | — |

[a] No observable zone of inhibition; + denotes inhibition.

initiated, and the bacteriocins produced by these strains have not been isolated. The specificities involved and the precautions taken to rule out phage during the screening procedures lead us to believe that we are dealing with true bacteriocins in all three cases, but this remains to be proven for strains 263 and 462.

The isolation experiments demonstrated that high titers of staphylococcin (414) could be obtained by fragmenting the cells at high pressure in a Ribi cell fractionator. Why the staphylococcin is not found in the supernatant fluid of an old broth culture is not clear, but it may be that the bacteriocin is quickly inactivated under these circumstances. Lachowicz (25) also reported that he was unable to recover staphylococcin from broth supernatant fluids. He obtained the bacteriocin from the agar on which the producer had grown. His material is difficult to compare with ours since no dry-weight activities or chemical analyses were given. However, the bacteriocin was said to be insensitive to trypsin and pepsin and could not be precipitated with ethanol or the salts of heavy metals. Barrow (1) isolated an inhibitory substance from *S. aureus* type 71 by growing the microorganism in dialysis tubing and harvesting the surrounding medium. Attempts to separate the active principal from the medium by a variety of procedures were unsuccessful. The inhibitor was said to be inactivated

by trypsin, but was essentially unaffected by pepsin. There are a number of other reports dealing with inhibitory substances produced by staphylococci (13, 22, 26, 38, 42), but these are difficult to assess because the investigators were more concerned with the biological implications than with the chemical characterization of the inhibitors. For the most part, these workers believed they were dealing with small diffusible polypeptides.

Staphylococcin (414) was examined in the electron microscope and no phage or phagelike structures were seen (Fig. 3). The small round objects observed are reminiscent of vesicles which form in some preparations of cell membranes (43). Indeed, the chemical composition agrees very well with the values found with membrane preparations of S. aureus prepared by Mitchell and Moyle (33). Upon isolation, then, staphylococcin appears to be a lipoprotein-carbohydrate complex with a molecular weight greater than 200,000. The complex can be dissociated in SDS into smaller subunits which still give positive results when qualitatively checked for the presence of protein, lipid, and carbohydrate. The bacteriocin is still active in the small subunit form or becomes active through reaggregation upon removal of the SDS. Further experiments are planned to resolve this point. The fact that trypsin or Pronase can destroy biological activity suggests that the latter resides in the protein moiety of the molecule.

Although mitomycin C and ultraviolet light were ineffective under the conditions employed, it is entirely possible that some agent could be found which would induce the cells to produce an unconjugated bacteriocin protein. Certainly this seems to be true for colinin K; the bacteriocin is normally produced as a protein-lipopolysaccharide complex, but under the influence of mitomycin C, the cells produce a considerable amount of unconjugated colicin K protein (44).

Staphylococcin (414) has a somewhat broader range of activity than would normally be expected from a bacteriocin such as a colicin. However, Hamon and Péron (14) made the following generalizations concerning the bacteriocins from gram-positive bacteria. (i) The activity spectra of these agents are much broader than those from gram-negative organisms, often crossing intergeneric lines, but never inhibiting gram-negative organisms. (ii) Frequently, the bacteriocin-producing strain is as sensitive to its products as heterologous strains are to its products. (iii) Their activity is expressed through the cell membrane. (iv) They appear to be protein in nature. Other tests with staphylococcin (414) have clearly

shown that it is not a hemolysin like the enterococcin (type 1) produced by Streptococcus faecalis var. liquefaciens (2) nor is it a lytic enzyme such as lysostaphin (3) or megacin A (37).

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