LYSOSTAPHIN: A NEW BACTERIOLYTIC AGENT FOR THE STAPHYLOCOCCUS

BY C. A. SCHINDLER* AND V. T. SCHUHARDT

DEPARTMENT OF MICROBIOLOGY, UNIVERSITY OF TEXAS, AUSTIN

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Interest in bacteriolytic agents of microbial origin has been manifest since Nicolle\(^1\) reported that filtrates of Bacillus subtilis had a lytic effect on "B. de Shiga, B. typhique and B. charbonneux." Fleming\(^2\) extended this interest to bacteriolytic agents from other biological sources by describing the bacteriolytic enzyme, lysozyme. Numerous investigators have utilized this enzyme to enrich our knowledge of cell wall and plasma membrane composition, and cell diffusion kinetics.\(^3\)

Many of the extracellular bacteriolytic substances have been derived from culture filtrates of the family, Actinomycetaceae.\(^4,5\) Extracellular bacteriolytic substances also have been reported from such diverse genera as Diplococcus,\(^6\) Micrococcus,\(^7\) Streptococcus,\(^8\) Bacillus,\(^9,10\) Flavobacterium,\(^11,12\) and Staphylococcus.\(^13,14\)

The literature dealing with bacteriolytic substances active against Staphylococcus pyogenes (aureus) has been reviewed by Elek.\(^15\) In addition to extracellular staphylo-lytic agents, the production of autolysins (endolysins) by staphylococci has been reported.\(^16-20\) A unique staphylococcal endolysin, virolysin, has been described.\(^21,22\)

Production of this endolysin requires induction by certain staphylococcal bacteriophages, and is only active against killed or stressed staphylococci. In addition to
exo- and endolysins, bacteriophages must be considered whenever new bacteriolytic phenomena are encountered.

During a series of attempted transduction studies with staphylococci, a small white colony surrounded by an area of growth inhibition was observed in the Staphylococcus aureus lawn. The isolate was given the code designation K-6-WI. It proved to be a gram-positive coccus with a specific antagonistic activity against strains of the genus Staphylococcus. The present paper deals with the characteristics of this microorganism and its spectrum of antagonistic action.

Methods.—Media: Unless otherwise stated, the media, reagents, and techniques used in identification of the K-6-WI isolate were those described by Shaw, Stitt, and Cowan. The organism was maintained, and surface growth characteristics were determined, on Trypticase-soy (Ts) broth (Baltimore Biological Laboratories, Baltimore, Md.) at pH 7.2-7.3 containing 2% (w/v) added agar (Difco, Detroit, Mich.). Gram staining characteristics were determined on 18-hr Ts broth and agar slant cultures. All agar surface antagonism experiments were performed by cross-streaking 18-hr Ts broth cultures of the test organisms on Ts agar plates.

Harvesting staphylocytic filtrates: Staphylocytic filtrates were recovered from Ts broth cultures of K-6-WI after incubation at 37° in 1-liter Fernbach or fermentation flasks on a reciprocating shaker operating with a 2.5-in stroke at a rate of 76 cycles/min. Preliminary studies indicated a maximum yield of staphylocytic substance when the pH, after an initial decrease from 7.3 to ca. 6.5, increased to pH 7.5. This usually occurred at 11-27 hr, depending upon both the size of the inoculum and quantity of growth medium, and was used as a determinant of harvest time. Longer incubation resulted in higher pH and lower yields. The cultures were pooled and centrifuged at 4° for 20 min at 13,200 g to sediment the cells. The supernates were filtered through cellulose membranes (Schleicher and Schuell, Bac-T-Flex, 0.5 μ pore diam, Scientific Products, Evanston, Ill.) and were stored at -20°. Hereafter, the staphylocytic factor will be referred to as lysostaphin.

Quantitative assay of lysostaphin: A rapid and reproducible assay was devised to compare the activity of different harvests of lysostaphin and to assess the contemplated purification procedures. The assay organism chosen was Staphylococcus aureus FDA 209P. The cells in an 18-hr, Ts broth, shake culture were sedimented at 13,000 g and washed twice with 0.05 M tris(hydroxymethyl)aminomethane-HCl (Tris) buffer at pH 7.5, containing 0.145 M NaCl (buffered saline). After adjusting the Klett Summerson photoelectric colorimeter (Klett) containing a 545 filter (500-570 μm) to give a reading of 110 with 0.001 M barium sulfate, the washed S. aureus cells were resuspended in buffered saline to give a Klett reading of 300. The addition of 1.5 ml of this S. aureus suspension to Klett tubes, containing sufficient buffered saline (with or without lysostaphin) to yield a total volume of 5 ml, gave initial Klett readings of 125 ± 5. The test and control preparations were incubated in a 37° waterbath, and Klett readings were made at 5-min intervals. One unit of lysostaphin was designated as being contained in that amount of test material which gave a 50% reduction in turbidity of the standard S. aureus suspension in 10 min at 37°. By constructing a standard curve, the unit value of any lysostaphin preparation could be established with a minimum of time and effort.

Cell wall and soluble cell substance (SCS) preparations: A concentrated suspension of Staphylococcus aureus FDA 209P cells was washed and resuspended in 0.145 M NaCl solution. Separate portions of these cells were used (1) unheated, (2) heated at 121° for 30 min, and (3) unheated and subjected to disintegration by no. 13 Ballotini beads in a Mickle Tissue Homogenizer (M.T.H.) operated at 4° for 50 min. The latter was used for cell-wall preparation. The disintegrated cell walls were washed and recovered as described elsewhere and were suspended in buffered saline.

To obtain SCS, 18-hr Ts broth cultures of K-6-WI were centrifuged, washed twice, and resuspended in Tris buffer. Separate portions of this concentrated cell suspension were subjected to disintegration in the M.T.H. operated at 4° for 20 min, and to ultrasonic vibration for 50 min in a water-cooled, 9-ke magnetostriiction oscillator (Raytheon Mfg. Co., Waltham, Mass.). After removal of glass beads by filtration through coarse sintered glass filters, the residual intact cells and cellular debris were sedimented by centrifugation at 13,200 g for 20 min at 4°.
Results.—(1) Characteristics of K-6-WI: Colonies of K-6-WI grown on Ts agar plates for 18 hr were 2–3 mm in diameter, opaque, white, circular, flattened convex, with a smooth glistening surface, entire edge, and a butyrous consistency. Stained preparations from 18-hr Ts broth and agar cultures revealed gram-positive cocci, 1.0–1.2 μ in diameter, dispersed singly, in pairs, short chains, tetrads, and as small clusters.

Under aerobic conditions, the organism produced acid from glucose, sucrose, lactose, D-mannose, and D(+) galactose. It failed to produce acid from mannitol, D(+) xylose, maltose, salicin, and glycerol. Using the anaerobic technique of Hugh and Leifson,26 acid was produced with the fermentation of glucose. The methyl red and the Voges-Proskauer tests were negative. Ammonia was produced, and nitrites were reduced to nitrites. Indole was not produced, and starch was not hydrolyzed. Gelatin liquefaction by the Frazier27 method was positive. Acid was produced in litmus milk, resulting in its coagulation. K-6-WI could not utilize ammonium dihydrogen phosphate as a sole source of nitrogen. Good growth of K-6-WI was obtained on Ts agar and in Ts broth containing 12 per cent (w/v) NaCl.

The K-6-WI organism produced catalase, but failed to produce coagulase. No hemolysins were produced for rabbit, sheep, or human erythrocytes. The organism was not susceptible to any of 26 Blair staphylococcal typing phages.

(2) The bacteriolytic spectrum of lysostaphin: The first indication that the antibacterial agent of the K-6-WI culture was actually bacteriolytic in nature was obtained when K-6-WI and various strains of Staphylococcus aureus were cross-streaked on Ts agar plates. Not only was the S. aureus growth adjacent to the K-6-WI culture inhibited, but with continued incubation the area of clearing of the S. aureus growth was extended (Fig. 1a and b). This could have been the consequence of staphylococcal endolysins coming into play after death due to a purely lethal action of the K-6-WI agent. To resolve this possibility, heat-killed (65°C) S. aureus cells were suspended in liquefied Ts agar, and overlays (ca. 2-in diam) were made on Ts agar plates. When the K-6-WI culture was streaked across the turbid agar overlay and incubated, a zone of decreased optical opacity, which increased in width with increased time of incubation, was observed adjacent to the K-6-WI growth.

Fifty-four viable strains of Staphylococcus aureus have been tested for susceptibility to lysostaphin by the cross streak and/or the tube method. Included were hemolytic (α and β) and nonhemolytic strains, coagulase positive and negative strains, 12 representative Blair bacteriophage propagating strains, 19 bacteriophage type 80/81 hospital strains, 11 antibiotic-resistant strains, and 2 mouse virulent strains. Also tested were 5 Staphylococcus epidermidis (saprophyticus) isolates, 4 of which produced penicillinase. All 59 of these Staphylococcus spp. cultures were attacked by the K-6-WI lytic factor. The S. epidermidis strains were lysed at a markedly slower rate than the S. aureus strains.

The bacteriolytic spectrum of lysostaphin against other bacterial species was determined using washed suspensions of viable cells in buffered saline. Klett readings on test and control (no lysostaphin) preparations were used to establish evidence of lysis. Staphylococcus aureus FDA 209P cell suspensions were included to be certain of the lytic activity of the lysostaphin used. The 27 nonstaphylococcal
species not lysed by lysostaphin are as follows: *Micrococcus lysodeikticus, Bacillus subtilis, Bacillus megaterium, Escherichia coli, Aerobacter aerogenes, Proteus morganii, Proteus rettgeri, Proteus vulgaris, Pseudomonas aeruginosa, Serratia marcescens, Lactobacillus plantarum, Gaffkya tetragena, Sarcina lutea, Corynebacterium diphtheriae (gravis),* C. *diphtheriae* (mitis), *Streptococcus lactis, Streptococcus fecalis, Streptococcus pyogenes, Bordetella pertussis, Brucella abortus, Klebsiella pneumoniae, Pasteurella pestis, Pasteurella tularensis, Salmonella paratyphi, Salmonella schottmuelleri, Salmonella typhosa, Vibrio cholerae (Inaba), V. cholerae (Ogawa), and Lysteria monocytogenes* (type 1). (*Micrococcus lysodeikticus and Bacillus megaterium* include cell walls and heat-killed cells; *Escherichia coli* includes heat-killed cells.) Additional tests on *B. subtilis, B. megaterium, S. lutea, S. marcescens, P. aeruginosa, A. aerogenes, and G. tetragena* indicated that the lysostaphin was neither bacteriocidal nor bacteriostatic for these organisms.

(3) **Lytic activity of lysostaphin against viable, heat-killed (121°C) cells, and cell-wall fragments:** *Staphylococcus aureus* cells were examined microscopically before and at intervals after the addition of a small quantity of lysostaphin. Samples (0.01 ml) of the *S. aureus* FDA 209P suspension were spread over circular areas (1 cm in diam) on a single, large microslide and stained by the gram method. Shortly after exposure to lysostaphin there was a decrease both in numbers and in the number of clusters of *S. aureus* cells. As the time of interaction between lysostaphin and cells increased, the remaining intact cells had less affinity for the gram stain (Fig. 1c and d). With passage of time, considerable gram-negative cell detritus appeared in the smears, and eventually all morphologically intact cells disappeared. Figure 2 illustrates the rapid decrease in viable *S. aureus* cells correlated with decreasing optical density of a test suspension.
The results presented in Figure 3 are not comparable on a substrate concentration basis since the Staphylococcus aureus suspension used for the cell-wall experiment was considerably more concentrated than were those used in the associated viable and autoclaved cell preparations. The viable-cell reduction of turbidity curve is typical of the results obtained with all preparations containing viable S. aureus cells and lysostaphin. The autoclaved S. aureus cells gave an initial rate of reduction of turbidity at least equal to that for viable cells. However, after 5 min the apparent lysis of the autoclaved cells decreased rapidly and attained only approximately 50 per cent of that observed for viable cells. This incomplete reduction of turbidity of the autoclaved cells was probably due to the presence of insoluble, coagulated proteins resulting from the heat treatment. S. aureus cells killed at lower temperatures and exposed to lysostaphin have given turbidimetric readings approaching that of viable cells. The apparent initial rate of lysis and the extent of lysis of the S. aureus cell-wall preparation were considerably less than that observed for viable cells, which may be a consequence of the greater substrate concentration and an incomplete attack by the lytic agent on cell walls. When K-6-WI SCS, prepared by either method of cell disintegration, was substituted for the lysostaphin, no lysis of the S. aureus cells occurred.

Figure 4 records the results of an experiment designed to determine whether the activity of lysostaphin was modified as a consequence of the lytic reaction. For this experiment, Staphylococcus aureus FDA 209P cells were concentrated to the point that 0.2 ml in a total volume of 5 ml gave a Klett reading of 125. For the test preparation, a Klett tube received 4.55 ml of buffered saline, 0.2 ml of the concentrated S. aureus cell suspension, and 0.25 ml of the lysostaphin. The test preparation and a control without lysostaphin were incubated at 37°C, and Klett readings were recorded at 5-min intervals for 15 min, at which time ca. 80 per cent reduction in turbidity was observed. After an additional 15 min at 37°C, a second 0.2-ml sample of the concentrated S. aureus cells was added to the test preparation,
and the incubation and Klett readings were repeated. This procedure was repeated until 5 separate 0.2-ml samples of cells had been exposed to the original 0.25 ml of lysostaphin. No significant difference in either the rate or extent of lysis of the first 4 cell samples was observed in this experiment. A slight decrease in the rate of lysis was observed with the fifth cell sample. This decrease may have been due to one or a combination of the following factors: (a) the increased viscosity of the preparation, resulting from the liberated cell components, (b) a slight inactivation due to the prolonged incubation, or (c) the volume change of the test preparation effected by the repeated addition of cells. When 1 ml of lysostaphin was added to an identical S. aureus preparation, a significantly greater rate of lysis was observed, indicating that the prior results were not the consequence of an excess of lysostaphin in the original 0.25-ml test preparation. Also, the fact that the fifth S. aureus cell addition to the original 0.25 ml of lysostaphin was lysed at a significantly greater rate than the cells of another identical cell preparation exposed to 0.2 ml of the material, indicated that more than 80 per cent of the original lytic capacity was present after lysis of 4 aliquants of S. aureus cells.

Since serum inhibits certain bacteriolytic reactions, the lytic action of lysostaphin on Staphylococcus aureus cells suspended in human serum was compared with that of an equivalent amount of cells suspended in buffered saline. Just prior to each Klett reading the serum preparations received an equal volume of buffered saline, and the saline preparations received an equal volume of serum to compensate for the optical density of the serum. Figure 5 illustrates the results of this experiment which indicate that in the presence of human serum the rate of lysis of S. aureus cells by lysostaphin was reduced but the total lysis within 65 min was equivalent to that noted in the buffered saline preparation. This lag may be comparable to that noted for the fifth S. aureus sample in Figure 4, wherein the increased viscosity of the preparation appeared to be one possible explanation for the slightly decreased rate of lysis.
Discussion.—The gram-positive coccus (K-6-WI) was isolated from the lawn of a *Staphylococcus aureus* strain which had been exposed to the Blair typing phage no. 6. Based upon morphology, biochemical tests, and the organism's tolerance to high salt concentration, it is identified as a member of the genus *Staphylococcus*. The unique characteristic of this organism is its ability to produce a potent bacteriolytic agent, which has been proved active against more than 50 strains of staphylococci and inactive against 27 species of 19 other genera.

The possibility that the K-6-WI lytic factor might be a bacteriophage is eliminated by the following observations: (a) the rapidity of lysis is greater than that caused by virulent bacteriophage; (b) the lytic factor is active against all of a wide variety of phage types of *Staphylococcus aureus* as well as 5 strains of *S. epidermidis*; and (c) there is no appreciable change in the lytic capacity of *S. aureus* preparations subsequent to their lysis. The latter also excluded lysis from without by an avirulent bacteriophage.

The possibility that lysostaphin is a lysozyme of microbial origin is excluded. It does not share the same antibacterial spectrum of action with lysozyme, either for gram-positive organisms (i.e., *Micrococcus lysodeikticus*) or gram-negative organisms rendered susceptible to lysozyme by heat treatment and although lysozyme attacks certain strains of staphylococci, only coagulase negative strains are susceptible. Lysostaphin is active against all strains of *Staphylococcus aureus* tested regardless of coagulase production or other known strain differences.

In a subsequent paper, we will present procedures used in the partial purification and concentration of lysostaphin with additional characteristics of this material. Also preliminary results on the lysostaphin treatment of mice infected with *Staphylococcus aureus* will be reported.

Summary.—A gram-positive coccus (code designation K-6-WI), assigned to the genus *Staphylococcus*, has been shown to produce extracellularly a lytic factor active against all of 59 other staphylococcus strains tested. The K-6-WI lytic factor lysostaphin, was inactive against both viable and heat-killed K-6-WI cells and cells of 27 other species, many of which have been reported to be attacked by previously described bacteriolytic agents of microbial origin. Lysostaphin lysed both viable and heat-killed *S. aureus* cells. It is a new antibacterial agent for the staphylococci. A rapid assay for quantitating lysostaphin has been devised.

* Present address: Armed Forces Institute of Pathology, Washington 25, D. C.
STUDIES ON LABILE DEOXYCYTIDYLATE HYDROXYMETHYLASES FROM ESCHERICHIA COLI B INFECTED WITH TEMPERATURE-SENSITIVE MUTANTS OF BACTERIOPHAGE T4*

BY JOHN S. WIBERG† AND JOHN M. BUCHANAN

DIVISION OF BIOCHEMISTRY, DEPARTMENT OF BIOLOGY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY

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The present studies were undertaken as an attempt to demonstrate altered properties in the enzyme, dCMP hydroxymethylase, formed on infection of E. coli B by genetically altered bacteriophage T4. An earlier report from this laboratory demonstrated that this enzyme is undetectable in extracts of E. coli B infected with a T4 mutant, am N122; the site of mutation of this strain has been located by genetic mapping in gene 42. Among the temperature-sensitive (ts) mutants of T4 isolated by Edgar and Lielausis are some that map in this same gene. The dCMP hydroxymethylases formed in E. coli B by two of these, ts G25W and ts L13, have been studied in some detail. The results demonstrate that each of the two mutant enzymes is more temperature-sensitive than the wild type, and that the sensitivity of one differs qualitatively from that of the other. An interesting property of the wild-type enzyme is reported, namely, its ability under certain conditions to regain most of its activity after heat inactivation at 40°C.

Methods and Materials.—The sources of the following materials were: folic acid (C grade), d-cytidine, and dCMP, California Corp. for Biochemical Research; C14-labeled formaldehyde, New England Nuclear Corp. and Volk Radiochemical Co.; 2-mercaptoethanol (Eastman grade) and the disodium salt of EDTA, Eastman Kodak Co.; Tris, Sigma Chemical Co. The preparation of dHMP has been described. Reagent-grade hydroxylamine hydrochloride was obtained from Baker Chemical Co.

The method of isolation of the ts mutants has been described. T4D, a revertant of am NS2 (gene 44), was used as the wild-type phage and is referred to as T4ts+. Mutant ts A41 was included in most experiments as a second reference phage; this mutant maps genetically in cistron 39, the same cistron in which am N116 maps, and presumably contains the genetic information