

Biological Properties of α -Toxin Mutants of *Staphylococcus aureus*

J. K. McCLATCHY¹ AND E. D. ROSENBLUM

Department of Microbiology, The University of Texas Southwestern Medical School, Dallas, Texas

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ABSTRACT

McCLATCHY, J. K. (The University of Texas Southwestern Medical School, Dallas), AND E. D. ROSENBLUM. Biological properties of α -toxin mutants of *Staphylococcus aureus*. J. Bacteriol. 92:575-579. 1966.—Twenty nonhemolytic mutants of *Staphylococcus aureus* were isolated after treatment of a hemolytic strain with ultraviolet light or nitrous acid. Thirteen strains isolated were completely lacking in the synthesis of α toxin or immunologically related proteins, presumably the result of a single mutational event. Although the strains were nonhemolytic on rabbit blood-agar plates, six of them retained the dermonecrotic and lethal activities usually associated with staphylococcal α toxin, as well as slight hemolytic activity for rabbit erythrocyte suspensions. The active mutants and one inactive mutant produced a protein that reacted immunologically with antibody to α toxin. Mutations which alter the α toxin molecule can effect the lethal, dermonecrotic, and hemolytic activities separately or in varying ratios.

In recent years, several proteins have been reported to have multiple functions or catalytic activities. For example, the tryptophan synthetase enzymes produced by *Escherichia coli* and *Neurospora crassa* are able to catalyze three different reactions in the indole to tryptophan pathway (21). Mutations at different genetic sites can lead to the loss of one, two, or all of the enzymatic activities. Multifunctional enzymes have also been detected in the histidine biosynthetic pathway of *Salmonella typhimurium* (8). Several clostridial species produce toxins with multiple activities, including hemolytic, dermonecrotic, and lethal effects, in addition to well-defined enzymatic activities (15).

The α toxin of *Staphylococcus aureus* has recently been purified and has conclusively been shown to be multifunctional, possessing hemolytic, dermonecrotic, lethal, and leukocidal activities (2, 12, 13). Not only does α toxin have the above effects, but it can also lyse human platelets (3, 20), certain tissue culture cells (1, 10), and various bacterial spheroplasts and protoplasts (4). However, Bernheimer and Schwartz (4) suggested that the various substrates may contain the same or similar chemical compounds or groupings for which the toxin is active.

This work was initiated to determine whether

the unitarian hypothesis could be confirmed by the study of α -toxin mutants and whether these mutants might provide clues about the functional site(s) on the molecule. Nonhemolytic mutants of *S. aureus* were isolated and tested for dermonecrotic and lethal activities and for the presence of material cross-reacting immunologically with α toxin.

MATERIALS AND METHODS

Bacteria. Nonhemolytic mutants of *S. aureus* 233, previously described by Dowell and Rosenblum (7), were isolated after treatment with nitrous acid or ultraviolet light. In the latter method, approximately 10^8 colony-forming units (CFU) of strain 233 were irradiated for 25 sec with a 15-w General Electric germicidal lamp placed 53 cm above the bacterial suspension. After irradiation, appropriate dilutions were spread over the surface of BBL Trypticase Soy Agar (TSA) plates containing 7% (v/v) defibrinated rabbit blood. The plates were incubated at 37 C overnight in a 10% CO₂ atmosphere and examined for nonhemolytic colonies. Colonies were picked and streaked on rabbit blood-agar plates to test their stability.

To obtain nitrous acid-induced nonhemolytic mutants of strain 233, 1 ml (10^8 CFU) of a suspension was placed in a tube containing 9.0 ml of 0.2 M acetate buffer (pH 4.3) and 1.0 ml of 1.0 M NaNO₂. The reaction tube was incubated at 37 C with shaking for 10 min, and samples were diluted 10-fold in 0.2 M phosphate buffer (pH 7.2) for plating on blood-agar.

¹ Present address: Division of Research, The National Jewish Hospital, Denver, Colo.

Nonhemolytic variants were verified as above. The phage-typing patterns of colonies isolated by both procedures were identical to the pattern of strain 233.

Production of α -toxin preparations. For production of crude α -toxin or cross-reacting material, the following medium was employed: Veal Infusion Broth (Difco), 25 g; sodium acetate, 11.6 g; Proteose Peptone No. 3 (Difco), 15 g; agar (Difco), 15 g; distilled water, 1,000 ml. This medium, a modification of that of Casman (5), was poured into petri dishes and allowed to solidify. A single layer of sterile dialysis membrane was placed over the surface and inoculated with 0.5 ml of a Trypticase Soy Broth cell suspension containing 2×10^8 CFU/ml. The cells were spread over the surface of the membranes with a moistened cotton swab, and the plates were incubated for 48 hr at 37 C in a 40% CO₂-60% O₂ atmosphere. Each membrane was then removed and washed with 3.0 ml of 0.2 M phosphate buffer (pH 7.2). The washings were centrifuged at $2,000 \times g$ for 30 min to remove the bacteria. The supernatant fluids, containing material washed from the membranes and dissociated from the bacteria, were sterilized by membrane filtration prior to use in the experiments reported. To detect reversions to wild-type toxin production, the sedimented bacteria were resuspended and appropriate dilutions were plated on blood-agar. Preparations found to contain hemolytic bacteria were discarded.

Determination of hemolytic activity. Twofold dilutions of the toxin preparations were made in 0.2 M phosphate buffer (pH 7.2) containing 0.85% (w/v) NaCl. An equal amount of a 2.0% suspension of washed rabbit erythrocytes was added to each tube, and the tubes were incubated at 37 C for 1 hr. The reciprocal of the highest dilution giving complete hemolysis was designated as the number (per milliliter) of hemolytic units (HU) of α toxin in each test material.

For measuring β -hemolysin activity, fresh sheep erythrocytes were employed; toxin-red cell mixtures were incubated for 1 hr at 37 C and then stored at 4 C overnight. The HU/ml were calculated as above.

Determination of dermonecrotic activity. The backs of adult male albino rabbits (without detectable α -toxin antibodies) were shaved and marked into 24 squares (3 by 3 cm). The center of each square was injected intradermally with 0.1 ml each of the toxin preparations from strain 233, the nonhemolytic mutants, and appropriate controls. The rabbits were observed daily for 7 days, and the areas of erythema, induration, and necrosis were recorded.

Determination of lethal activity. A 0.1-ml portion of each toxin preparation was injected intraperitoneally into each of five mice weighing 18 to 22 g each. Although all deaths occurred during the first 24 hr after injection, the surviving animals were observed for several days. The LD₅₀ of any of the above preparations showing lethal effects was then determined. Twofold dilutions of the lethal preparations were made in phosphate buffer, and 0.1 ml was injected intraperitoneally into each of five mice. The LD₅₀ was calculated by the method of Reed and Muench (18).

Mice surviving the initial injections were challenged 30 days later with 3.0 LD₅₀ of crude α toxin.

Determination of cross-reacting material. The α -toxin cross-reacting material was determined by the Ouchterlony double-diffusion agar-gel procedure (17); 8 ml of 1.0% Ionagar no. 2 (Oxoid) in 0.03 M phosphate buffer (pH 8.3) was placed onto each slide and allowed to solidify. Wells 1.5 cm apart were then cut in the agar layer, and the bottom of each well was sealed with a drop of melted agar. Twenty U.S. standard units of antitoxin was placed in the center well, the outer wells were charged with the test materials, and the slides were incubated in moist chambers at 37 C for 7 days. The slides were then soaked in 0.85% saline for 24 hr and then in distilled water for 48 hr. After drying overnight at 37 C, the slides were stained with 0.1% (w/v) Thiazine Red R in 1% acetic acid for 20 min and then destained for 5 min with 1% acetic acid in 70% ethyl alcohol (6).

The α toxin-antitoxin precipitin lines were located by adding red cells to the Ionagar to a final concentration of 5.0% before placing the agar on the slides; a precipitin line coincident with the boundary of hemolysis was assumed to be the toxin-antitoxin band. Bands continuous with the toxin-antitoxin band were assumed to be cross-reacting material.

Tests for coagulase, fibrinolysin, deoxyribonuclease, and lipase. Routine slide and tube coagulase tests were performed with reconstituted rabbit plasma (Hyland Laboratories, Los Angeles, Calif.). An adaptation of the plate method of Lack (14) was used for the demonstration of fibrinolysin. Human plasma to a final concentration of 12% (v/v) was added to melted TSA and held at 56 C for 20 min before pouring. This procedure gave an opaque medium on which fibrinolytic colonies produced clearing after overnight incubation at 37 C. Strain 233 and the nonhemolytic mutants were tested on DNase Test Agar (Difco) and Colbeck EY Agar Base plus Broth (Difco) for the production of deoxyribonuclease and lipase, respectively.

RESULTS

Hemolytic activities of the membrane preparations. The hemolytic effects of the toxin preparations of the parent organism (strain 233) and of the mutants on washed rabbit erythrocytes are shown in Table 1. Strain 233 produced 640 HU/ml, whereas the titers of the mutant preparations varied from 0 to 20 HU/ml. Although several of the mutant preparations had slight hemolytic activities, these strains were nonhemolytic when grown on blood-agar plates. A 1-hr incubation period followed by overnight storage at 4 C failed to increase the hemolytic titers of the preparations. Hemolysis was not detectable if the preparations were heated at 60 C for 30 min. The heat lability of the hemolysin appears to rule out the possibility of a contaminating δ hemolysin, which is a heat-stable substance.

Dermonecrotic activities of the toxin preparations. Several of the toxin preparations of the

TABLE 1. *Properties of strain 233 and its α -toxin mutants*

Mutant	Mutagenic treatment	Hemolytic activity	Dermonecrotic activity ^a	Lethal activity ^b	Cross-reacting material	Bound coagulase	Fibrinolysin	β -Hemolysin activity
233	(parent)	HU/ml 640	Necrosis-15	8.9	+	+	+	HU/ml 20
140a	HNO ₂	ND ^c	0	—	—	+	+	10
140b		ND	Slight erythema-5	—	—	+	—	10
140c		ND	0	—	—	+	—	10
140d		ND	0	—	—	+	—	10
144g	UV ^d	ND	Erythema-7	—	—	+	+	ND
152a		ND	0	—	—	+	—	20
152c		ND	0	—	—	+	—	10
152d		ND	0	—	—	+	+	ND
152f		ND	0	—	—	—	—	40
152g		ND	0	—	—	+	—	20
152i		ND	0	—	—	+	+	10
152j		ND	0	—	—	+	—	20
152k		ND	0	—	—	+	—	ND
152e	UV	ND	0	—	+	+	—	20
142f	HNO ₂	20	Necrosis-10	6.2	+	+	+	40
144h	UV	20	Necrosis-15	5.7	+	+	+	40
144i		10	Necrosis-11	4.2	+	+	+	20
144m		10	Necrosis-15	3.1	+	+	+	40
152h		ND	Necrosis-5	0.8	+	—	—	320
152l		20	Necrosis-10	8.0	+	—	+	40

^a Reaction observed and diameter of area (millimeters) 96 hr after intradermal injection.

^b Expressed as LD₅₀/0.1 ml of preparation.

^c Not detectable in undiluted toxin preparations.

^d Ultraviolet irradiation.

mutant strains, in addition to the wild-type preparation, were dermonecrotic (Table 1). Frequently, however, the areas of necrosis were not as large and the reactions not as severe as those seen with the parent strain. Two of the mutant preparations (140b, 144g) caused slight erythematous reactions without the development of necrosis. No reaction was noted when the rabbits were injected with heated toxin preparations; thus, the necrosis was produced by a heat-labile substance, most probably α toxin.

Lethal activities of the toxin preparations. Table 1 shows the LD₅₀/0.1 ml of the toxin preparations. All of the deaths occurred within 24 hr after injection of the test materials, suggesting that α toxin was responsible for the deaths. Heated preparations had no lethal effects. The wild-type preparation killed all of the mice injected, as did the toxin preparations from mutants 142f, 144h, 144i, 144m, and 152l. Mutant 152h preparations had diminished lethal activity compared to the wild type.

Thirty days after the initial injections, surviving mice were injected with 3.0 LD₅₀ of the wild-type

α toxin. Prior injection of those preparations (1:10 of 233 and mutant 152h) with some lethal activity seemed to protect the survivors against subsequent challenge with toxin. Mice which survived injection with preparations lacking lethal activity were all susceptible to challenge with wild-type toxin.

Detection of α toxin cross-reacting material. The observation that some of the nonhemolytic mutants retained their dermonecrotic and lethal effects suggested that these mutants might be producing an altered α -toxin protein. The production by these mutants of a protein immunologically related to α toxin was determined by the agar gel diffusion technique. When gel diffusion slides were prepared with standard antiserum and the membrane preparations, three to five precipitin bands were usually visible. The α toxin-antitoxin band was found to be the innermost line by the use of slides containing red cells. When erythrocytes were present, a hemolytic zone was observed; this zone extended from the toxin-containing well to the innermost precipitin band. Table 1 summarizes these data.

Other biological characteristics of the mutants. Table 1 shows the effects of mutations affecting hemolytic activity on some other staphylococcal characteristics commonly associated with virulence. All of the mutants were similar to the parent strain in the production of lipase, deoxyribonuclease, free coagulase, and pigment, and in the fermentation of mannitol. Differences among the mutants were observed in the production of bound coagulase, fibrinolysin, and β hemolysin.

deWaart et al. (Antonie van Leeuwenhoek J. Microbiol. Serol. 29:324, 1963) reported a double lysogenic conversion leading to loss of the capacity to produce β hemolysin and acquisition of the ability to synthesize fibrinolysin. Although alterations were not observed in the lysogenic states of the mutants (16), one mutant, 152h, was synthesizing β lysin in amounts much greater than the wild-type strain and was nonfibrinolytic. There was no correlation between increase in β -toxin production and loss of fibrinolytic activity; most of the other nonfibrinolytic mutants had unaltered β -lysin activities.

DISCUSSION

The recent isolations of staphylococcal α toxin by several workers (2, 9, 12) have provided strong direct evidence for the unitarian theory which holds that the hemolytic, dermonecrotic, lethal, and leukocidal activities are actually properties of a single protein species. The work reported here lends further support to this theory, since a presumably single mutational event has caused a loss of all biological activity attributable to the α toxin in the majority of the mutants studied. The remainder of the nonhemolytic mutants are lethal for mice and dermonecrotic for rabbits, suggesting that there may be two or more sites on the molecule, each with activity for a different substrate.

If the unitarian hypothesis is correct, then the six mutants showing some degree of biological activity usually associated with α toxin may be synthesizing a protein altered in such a manner that only the hemolytic property is markedly affected. This indeed seems to be the case, since all of the nonhemolytic mutants with residual α -toxin activities were producing an immunologically related protein or cross-reacting material. One of the mutants with no toxic activity was also producing cross-reacting material.

The mutants can be grouped according to their α toxin-producing capacity. One group of 13 mutants does not produce cross-reacting material and has no biological activity. Mutant 152e, although producing cross-reacting material, is also not reactive in the various in vivo and in vitro

tests. Toxin preparations from five other mutants had minimal hemolytic activities, but were both dermonecrotic and lethal. However, these mutants are nonhemolytic when grown on rabbit blood-agar. Mutant 152h is dermonecrotic, produces cross-reacting material, has only very slight lethal activity, and is without detectable hemolytic activity. The three mice surviving initial challenge with this preparation were resistant to subsequent challenge with 3 LD₅₀ doses of wild-type toxin. The altered toxin apparently can induce formation of protective antibodies.

The mutants that do not produce cross-reacting material do not produce protein with any detectable α -toxin properties. Some members of this group may conceivably produce a protein so altered that it cannot be recognized by the detection technique employed. This group may also include regulator mutations that prevent the expression of the structural gene for α toxin.

The mutants that do produce cross-reacting material have relatively minor lesions, since the gene product retains immunological specificity. These mutants probably represent a small proportion of the possible alterations of α toxin, since mutants with partial activity on rabbit blood-agar would not be detected because of the difficulty in distinguishing them from members of the parental population. Nor would mutants be selected that were deficient in dermonecrotic or lethal activity but still retained their hemolytic activity.

The data are insufficient to differentiate between the possibilities of one active site or several active sites with different specificities. Mutant 152e produces cross-reacting material, but has no detectable activity, suggesting that a single mutational event has destroyed all biological activity. The other mutants which produce cross-reacting material have marked alterations in the ratios of hemolytic, dermonecrotic, and lethal activities. This alteration may result from a change in the primary structure of the protein having a differential effect on several active sites. The wide range of α -toxin substrates may be more apparent than real, since Robertson (19) suggested that all biological membranes have a similar basic molecular pattern and that the occurrence of the same or similar molecular groupings in various cell membranes can explain their sensitivity to α toxin. The structural change might thus have altered the affinity of a single active site on the toxin for its various substrates, as in the "induced fit" theory of Koshland, Yankeelov, and Thoma (11).

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