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Chromosomal Synthesis of Staphylococcal Exfoliative Toxin

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Tox^+ staphylococcal strains, as opposed to Tox^- strains, produce epidermal exfoliation within 18 h after direct subcutaneous or intraperitoneal injection into newborn mice. The extracellular product responsible for exfoliation is termed exfoliative toxin (ET). When culture supernatant fluid from the plasmid-cured Tox^- substrains UT 0100 or UT 0111 or from six naturally occurring phage group 2 Tox^- strains was concentrated 20-fold and inoculated into newborn mice, ET activity could be detected. The Tox^-, cured derivatives produced ET at levels which were 32- and 64-fold lower than the amounts made by their Tox^+ parent strains. Since these Tox^-, cured substrains contained no plasmid deoxyribonucleic acid, it was postulated that the product possessing ET activity in strains UT 0100 and UT 0111 was made by chromosomal genes. This product has been isolated and purified from strain UT 0100 and appears as two faint bands after electrophoresis on polyacrylamide gels and corresponds in position to a heavy band of ET isolated from the Tox^+ strain UT 0007.

Phage group 2 strains of *Staphylococcus aureus* produce an extracellular toxin that is associated with a spectrum of clinical manifestations termed the staphylococcal scalded skin syndrome (SSS). SSS is characterized by exfoliation of superficial epidermis with the cleavage plane developing within the granular cell layer (7, 8, 9). The toxin responsible for epidermal exfoliation has been purified (1, 5, 9) and termed exfoliative toxin (ET) in this laboratory. The toxin has been reported to be antigenic, acid labile, and heat stable and to have a molecular weight of approximately 24,000 (4, 5). ET appears to be a glycoprotein since it is precipitated after interaction with concanavalin A (10). An experimental mouse model for the detection of ET was developed by Melish and Glasgow (7). Either ET-producing (Tox^+) *S. aureus* cells or unconcentrated culture supernatant fluids from these bacterial cells cause epidermal exfoliation after being injected either subcutaneously or intraperitoneally into newborn mice. Tox^- strains do not cause exfoliation under these conditions.

We have recently reported that most of the criteria commonly used to identify staphylococcal plasmids are characteristic of the genes controlling ET synthesis in the *S. aureus* strains UT 0001 and UT 0007 (11). The early and rapid accumulation of Tox^- variants during growth of the Tox^+ strains UT 0007 in either 6×10^{-6} M ethidium bromide, 0.003% sodium dodecyl sulfate, or at 44 C indicated that the gene(s) for ET synthesis was extrachromosomal (11, 12). Growth at 44 C, which yielded an elimination frequency of 98%, was more effective than growth in either ethidium bromide or sodium dodecyl sulfate in eliminating the ability of the Tox^+ strain to make ET (11). Furthermore, cured Tox^- substrains had no selective advantage over uncured Tox^+ strains during growth at 44 C or in ethidium bromide or sodium dodecyl sulfate. The plasmid deoxyribonucleic acid (DNA) associated with ET synthesis in *S. aureus* phage group 2 strain UT 0007 has been isolated and characterized as an unstable 56*S* covalently closed circular molecule with a molecular weight of 3.3×10^7 after cesium chloride-dye bouyant density centrifugation followed by analysis on 5 to 20% neutral sucrose velocity gradients (13). The purpose of this investigation was to determine if plasmid-cured Tox^- strains and naturally occurring plasmid-negative Tox^- strains made chromosomal products with exfoliative activity which were usually not detected by the standard assay procedure of Melish et al. (7, 9).

MATERIALS AND METHODS

Bacterial strains. Staphylococcal strains from phage groups 1, 2, and 3 were used in these studies. These strains are listed in Table 1. Phage group 2 strains UT 0001 and UT 0007 were isolated from clinical sources (11). The naturally occurring Tox^- strain 04081 was provided by E. Rosenblum. The

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Tox⁻, cured substrain UT 0100 was isolated in this laboratory after treatment of the Tox⁺ UT 0007 strain with ethidium bromide (12). The cured substrain UT 0111 was isolated in this laboratory after growing Tox⁺ strain UT 0001 at 44 C for 24 h (11). The remaining staphylococcal strains came from the culture collection of B. Wiley. Tox⁺ strains are classified by their ability to produce epidermal exfoliation in newborn mice after being tested by the assay procedure described by Melish et al. (7, 9),, whereas Tox⁻ strains produce no exfoliation in newborn mice if assayed by this procedure.

Preparation of purified exfoliative toxin. Staphylococcal strains were grown in 250-ml flasks containing 100 ml of heart infusion broth (Difco). The flasks were incubated with shaking at 37 C on a New Brunswick model G76 gyrotory water bath shaker covered by a plexiglass hood that was flushed with 100% CO_2 twice daily. After 72 h of incubation, the cells were separated by centrifugation at 10,000 × g for 30 min and discarded. Ammonium sulfate was added to a liter of cell-free supernatant to a final concentration of 80%, and ET was purified from the resulting crude material by the electrofocusing procedure described by Melish et al. (9). Electrofocused ET gave a single band when subjected to electrophoresis on polyacrylamide gel, indicating that it was free of other contaminating staphylococcal extracellular products. Purified preparations of ET were shown to be free of alpha-toxin activity by their inability to lyse rabbit erythrocytes.

Assay of alpha-toxin activity in purified preparations of exfoliative toxin. Alpha hemolysin activity was determined by a micromethod developed in this laboratory. Twofold dilutions of electrofocused ET were made in phosphate-buffered (ph 7.2) 0.85% NaCl. An equal volume of a 2% suspension of washed rabbit erythrocytes was added to 50 μl of diluted ET in wells of a microtiter plate. The plates were incubated for 1 h at 37 C and then stored at 4 C overnight. Twenty-times the reciprocal of the highest dilution giving complete hemolysis was designated as the number of hemolytic units of alpha-toxin per milliliter.

Assay of exfoliative toxin. Strains were inoculated in heart infusion broth and incubated for 72 h in a CO_2 atmosphere at 37 C on a New Brunswick model G76 gyrotory water bath shaker. ET from Tox⁺ strains was then assayed by the procedure described by Melish and Glasgow (7). To detect ET activity in Tox⁻ strains, the procedure of Melish and Glasgow (7) was modified in the following manner. Crude culture supernatant fluids from Tox⁻ cells were obtained by the method described above and concentrated 20-fold by dissolving the material from 500 ml of supernatant into 25 ml of phosphate-buffered saline (8.5 g of NaCl, 87 ml of 0.67 M Na_2HPO_4, and 13 ml of 0.67 M KH_2PO_4 brought up to 1 liter in water) adjusted to pH 7.2 prior to subcutaneous injection into newborn mice. ET activity is expressed in exfoliative units, and the titer of a preparation in units is equivalent to 10 times the reciprocal of the highest dilution that gives a positive result when a standard dose of 0.1 ml is injected into newborn mice (9).

TABLE 1. *Assay of exfoliative toxin activity in strains of staphylococci by the standard[a] and modified procedures[b]*

Strain	Phage type	Exfoliative toxin activity as measured by the standard assay procedure[a]	Exfoliative toxin activity as measured by the modified assay procedure[b]
UT 1001	29-29A-31A-52-52A-79-80	−	+
UT 1002	29-29A-31A-52-52A-79-80	−	+
UT 0013	55/71	−	+
UT 0014	55/71	−	+
UT 0015	55/71	−	+
UT 0017	3B/3C/55/71	−	+
UT 0018	3A/3B/55/71	−	+
04081	55/71	−	+
UT 0111[c]	55/71	−	+
UT 0100[c]	55/3A/3C	−	+
UT 0007	55/3A/3C	+	+
UT 0001	55/71	+	+
UT 0639	7-47-53-75-77-81	−	−
UT 0645	7-74-53-54-75-77	−	−

[a] Standard assay procedure involves direct inoculation of staphylococci into newborn mice as described by Melish et al. (7, 9).

[b] Modified assay procedure involves the inoculation of 20-fold-concentrated culture supernatant fluid into newborn mice as described.

[c] Strains UT 0111 and UT 0100 are plasmid-cured substrains of UT 0001 and UT 0007, respectively.

Polyacrylamide gel electrophoresis. The method of Davis (2) was followed for polyacrylamide disc gel electrophoresis. A vertical tube (75 by 7 mm) of polyacrylamide gel (7.5%) was loaded with 0.1 ml of an ET sample combined with bromophenol as the tracing dye and run at 3 mA/gel for 1.5 h. Electrophoresis was carried out in a buffer consisting of tris(hydroxymethyl)aminomethane (0.005 M) and glycine (0.038 M) (pH 8.3). After electrophoresis, the gels were removed from the vertical tubes and stained with Coomassie blue as described by Fairbanks et al. (3). Bands corresponding to ET were cut out of the gels and eluted with phosphate-buffered saline. The eluates were tested for ET activity by inoculating them into newborn mice.

RESULTS

Assay of ET activity in strain UT 0007 and its cured substrain UT 0100. When 0.1-ml cultures from strain UT 0007 and its cured derivative UT 0100 from which all plasmid DNA was eliminated (13) were inoculated into the scapular areas of newborn mice and tested for ET activity by the method of Melish and Glasgow (7), strain UT 0007 was Tox⁺ and UT

0100 was Tox⁻. When the extracellular supernatant fluid from strain UT 0100 was concentrated 20-fold prior to mouse injection, however, ET activity could be detected (Table 1). The biological activity of the ET in the 20-fold-concentrated material from strain UT 0100 was 200 U/ml compared to an activity of 6,400 U/ml from culture supernatant fluid concentrated 20-fold from strain UT 0007. When the ET in the concentrated material from both strains was purified by electrofocusing, the biological activity was 20 U/ml and 800 U/ml for strain UT 0100 and strain UT 0007, respectively.

When the concentrated culture supernatant fluid from strain UT 0100 was separated on polyacrylamide gels, two faint bands were observed in the material isolated from the plasmid-negative strain that corresponded in position to the heavy band of ET isolated from the material of the plasmid-positive Tox⁺ strain (Fig. 1). The eluted ET bands from both strains produced exfoliation in newborn mice. Biological activity could be detected in the dense ET band from strain UT 0007 after extracting one gel with PBS, but with strain UT 0100, the combined ET eluates from 35 gels had to be inoculated into a newborn mouse before exfoliation could be observed.

Assay of ET activity in strain UT 0001 and its cured substrain UT 0111. Strain UT 0111, a cured derivative of Tox⁺ strain UT 0001 (11) from which all plasmid DNA was eliminated (unpublished data, this laboratory), behaved similarly to strain UT 0100 in not showing any biological activity when tested by the assay procedure of Melish and Glasgow (7) but was shown to produce exfoliation when tested by the modified assay procedure (Table 1). The biological activity of the ET contained in the 20-fold-concentrated cell-free supernatant material obtained from strains UT 0001 and UT 0111 was 6,400 and 100 U/ml, respectively.

Assay of ET activity in naturally occurring phage group 2 Tox⁻ staphylococcal strains. Phage group 2 staphylococcal strains UT 0013, UT 0014, UT 0015, UT 0017, UT 0018, and 04081 are all Tox⁻ (11) when they are assayed for ET by the standard method (7). However, 20-fold-concentrated culture supernatant fluids from all of these strains produced epidermal exfoliation within 18 h after injection into newborn mice (Table 1). The biological activities of the concentrates from these strains ranged from 100 to 400. U/ml (5 to 20 U/ml in unconcentrated medium). In another experiment, the culture supernatant fluid from the strain UT 0017 was concentrated 20-fold and subjected to electrophoresis on a polyacrylamide gel, and ET activity was compared to culture supernatant fluid from the Tox⁺ strain UT 0007 that was also concentrated 20-fold. The material from strain UT 0017 also produced two faint bands on polyacrylamide gel (similar to the bands isolated from strain UT 0100) (Fig. 2). The combined ET bands from 35 gels were needed to produce exfoliation

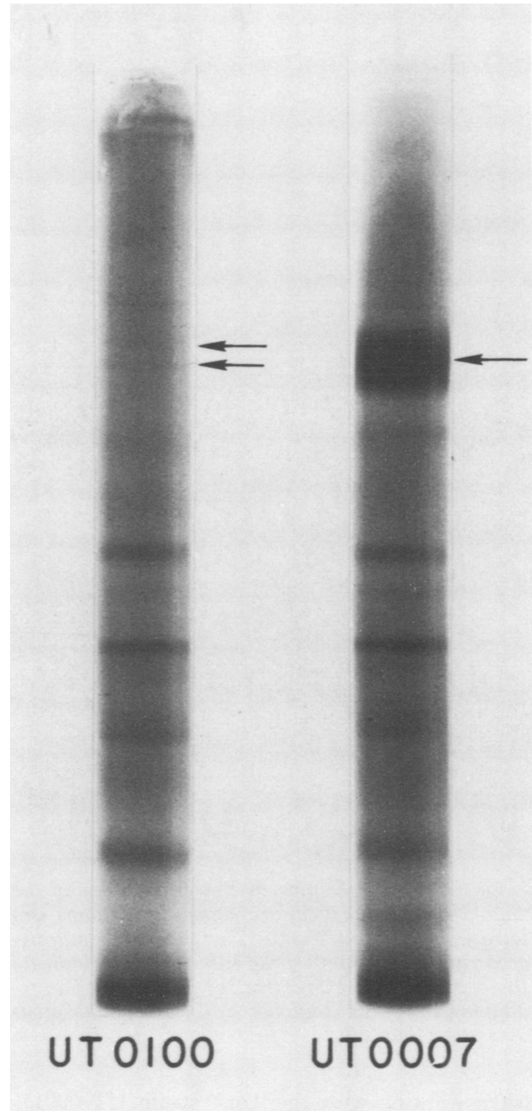

FIG. 1. *Polyacrylamide disc electrophoresis of the 20-fold-concentrated culture supernatant fluids isolated from Tox⁺ strain UT 0007 and its Tox⁻ plasmid-cured derivative UT 0100. Black arrows indicate those bands with exfoliative toxin activity. The remaining bands are staphylococcal extracellular products other than ET.*

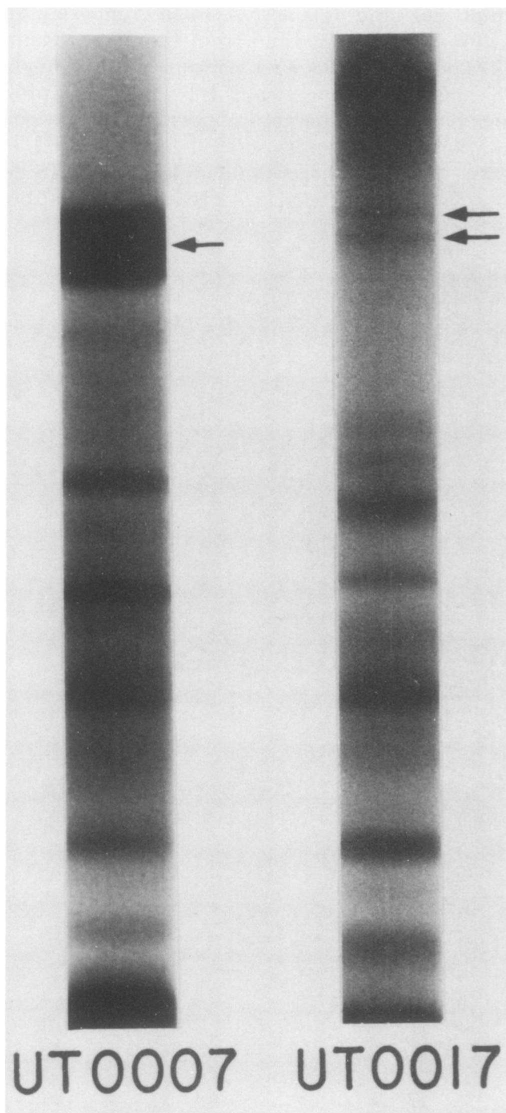

FIG. 2. *Polyacrylamide disc electrophoresis of the 20-fold-concentrated culture supernatant fluids isolated from strains UT 0007 (Tox⁺) and the naturally occurring Tox⁻ strain UT 0017. Black arrows indicate those bands with exfoliative toxin activity. The remaining bands are staphylococcal extracellular products other than ET.*

when working with the Tox⁻ strain UT 00017, whereas one ET band from Tox⁺ strain UT 0007 provided enough toxin to produce exfoliation.

Assay of ET activity in phage groups 1 and 3 staphylococci. Phage group 1 strains UT 1001 and UT 1002 and phage group 3 strains UT 0639 and UT 0645 are Tox⁻ when assayed for ET by the standard method. However, 20-fold-concentrated culture supernatant fluids from group 1 strains UT 1001 and UT 1002 were capable of producing exfoliation in newborn mice (Table 1). In contrast, 20-fold-concentrated extracellular material from group 3 strains UT 0639 and UT 0645 had no ET activity under given conditions of the assay (Table 1). These results indicate that ET production is not limited to phage group 2 strains of *S. aureus* and further suggest that certain of these Tox⁻ strains may in fact produce smaller quantities of ET that is identified only when the supernatant culture medium in which these strains have been grown is concentrated.

DISCUSSION

Tox⁺ staphylococcal strains as opposed to Tox⁻ strains produce epidermal exfoliation within 18 h after direct subcutaneous or intraperitoneal inoculation into newborn mice. However, if culture supernatant fluid from the Tox⁻, cured substrains, UT 0100 or UT 0111, or from the naturally occurring phage group 2 Tox⁻ strains, UT 0013, UT 0014, UT 0015, UT 0017, UT 0018 and 04081, was concentrated 20-fold and inoculated into newborn mice, ET activity could be detected (Table 1). The plasmid-cured Tox⁻ strain UT 0100 produced ET at a 32-fold lower level than its plasmid-positive parent Tox⁺ strain, UT 0007, and the plasmid-cured Tox⁻ strain, UT 0111, produced levels of ET 64-fold lower than its parent Tox⁺ strain, UT 0001.

A single molecular species of plasmid DNA associated with ET synthesis has been identified after dye-buoyant density gradient centrifugation followed by analysis on neutral sucrose density gradients in Tox⁺ strains UT 0001 and UT 0007 but not in their Tox⁻, cured substrains UT 0100 and UT 0111 (13; unpublished data, this laboratory). Since the cured strains contained no plasmid DNA it is believed that the product possessing ET activity in strains UT 0100 and UT 0111 is coded for by chromosomal gene(s). However, there is still a possibility that the ET found in Tox⁻ strains might have been made off of plasmid DNA that was not detected by our isolation procedures. Other investigations in this laboratory (unpublished data) have revealed that Tox⁻ strains UT 0017 and 04081 also lack any plasmid DNA, indicating that the production of ET in these strains may also be regulated by chromosomal loci. Whether the chromosomal and extrachromosomal products causing epidermal exfoliation are identical or regulated by the same genes has yet to be defined.

It is of interest to note that when plasmid DNA associated with ET synthesis is eliminated from strains UT 0007, the resulting cured derivatives behave as naturally occurring Tox$^-$ strains in producing minute amounts of a chromosomal product with ET activity. This product has been isolated and appears as two faint bands on polyacrylamide gels. Since two out of two Tox$^-$ phage group 1 strains and eight out of eight phage group 2 strains examined synthesize ET, it appears that many strains within these phage groups make ET that is probably coded for by chromosomal genes, but only rarely in amounts great enough to produce the clinical manifestations of the SSS after these organisms invade host tissue. Kondo and co-workers (5) have reported the isolation of several nonphage group 2 strains of *S. aureus* from patients with SSS, and it was observed in our own laboratory (H. Faden, J. Burke, J. Everett, and L. Glasgow, Pediatr. Res. **8**:424, 1974) that an outbreak of mild SSS in a nursery was associated with phage group 1 strains.

Our data appear to indicate that the ET made by Tox$^+$ strains UT 0001 and UT 0007 is largely synthesized by extrachromosomal genes, since cured derivatives of these strains are Tox$^-$ (10, 12). It is still possible, however, that Tox$^+$ strains other than UT 0001 and UT 0007 could produce most of their ET from chromosomal genes and lesser or nil amounts from extrachromosomal genes. This concept is supported by past investigations from this laboratory showing that only two (UT 0001 and UT 0007) out of twelve Tox$^+$ strains of staphylococci could be sucesssfully cured after treatment with either ethidium bromide or after growth at 44 C (11).

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