Nature of the Genetic Determinant Controlling Exfoliative Toxin Production in *Staphylococcus aureus*

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Phage group II *Staphylococcus aureus* has been identified as the etiological agent of the staphylococcal scaled skin syndrome. The development of an animal model system permitted fulfillment of Koch's postulates and recognition of exfoliative toxin (ET) as being responsible for some of the clinical manifestations of this syndrome. Initial studies directed toward associating a lysogenic phage with the genetic control of ET synthesis failed to support this hypothesis. Growth of two Tox+ strains at 44 C was more effective than growth in ethidium bromide or sodium dodecyl sulfate in eliminating the ability to produce ET. The early and rapid accumulation of ET-negative (Tox-) variants during growth of strain UT 0007 at 44 C, the lack of any selective advantage of the Tox- variants over Tox+ cells during growth at 44 C, and an enhanced elimination frequency at 44 C of 97.9% over the spontaneous frequency of loss strongly suggest that the gene for ET synthesis is extrachromosomal. Additional evidence suggests that this gene is located on a plasmid which is not associated with genes for penicillinase synthesis and cadmium resistance. Two Tox+ strains harbored lysogenic phage capable of transducing cadmium resistance, but not penicillin resistance, to specific Tox- recipients.

Phage group II *Staphylococcus aureus* has been associated with a spectrum of clinical disease entities termed the staphylococcal scaled skin syndrome (SSS). SSS is characterized by exfoliation of the superficial layer of the epidermis, with the cleavage plane developing within the granular cell layer (10, 11). The extracellular product responsible for epidermal exfoliation has been purified and termed exfoliative toxin (ET) (12). ET activity is stable upon storage at 4 C for 5 months and is heat stable to 56 C for 30 min (12). The toxin was found to be acid labile and antigenic, and to have a molecular weight of approximately 24,000 (7). Strains of phage group II staphylococci isolated from patients with SSS produce exfoliation in newborn mice after subcutaneous or intraperitoneal injection of an effective inoculum of staphylococci (10). The development of this experimental model system for the detection of ET makes it possible to score for the ET (Tox+) marker in genetic studies. As a result, the present investigation was initiated to determine whether the gene for ET is either under the genetic control of a bacteriophage or a gene located on a plasmid.

Melish et al. (12) speculated that ET synthesis might be under the control of a phage. Bacteriophage-induced bacterial toxins have been reported in *Corynebacterium diphtheriae* and group A streptococci. In both organisms, toxigenicity can be conferred only in the presence of a specific lysogenic phage and is lost in the absence of the phage (21). Prophage induction leading to autonomous phage replication can increase the phage-controlled streptococcal and diphtherial toxins to levels of 10 to 20 times above the amount made by the noninduced strain (21). Most staphylococci are lysogenic, but much less is known about the relationship of lysogeny to toxin production in *S. aureus* than to the production of streptococcal and diphtherial toxins. However, Blair and Carr (2) reported that the ability to produce alpha toxin was conferred upon certain non-toxinogenic staphylococcal cultures by lysogenization.

If ET production is not phage regulated, it is possible that toxin synthesis may be controlled by a plasmid-linked gene. Most staphylococcal strains probably carry one or more plasmids. These plasmids usually harbor one or more genetic determinants for resistance to inorganic ions or to antibiotics (16). For instance, staphylococcal penicillinase plasmids can carry, in addition to the genes that regulate penicillinase biosynthesis, genes for resistance to erythromycin, cadmium ions, mercury ions, arsenate ions,
and arsenite ions (14, 15). Another type of staphylococcal plasmid contains a gene for methicillin resistance and a gene for enterotoxin B (4). A third type of plasmid that controls the synthesis of a specific bacteriocin was found in a phage group II staphylococcal strain (16). Evidence for plasmids other than the bacteriocin plasmid has not been reported for phage group II staphylococci. Most staphylococcal plasmids are irreversibly lost from cells that carry them at rates greater than spontaneous mutation rates. Various physical and chemical agents can be used to enhance the frequency of plasmid elimination over that of spontaneous loss, but the frequencies of elimination will vary according to the strain and conditions used (16). The increased loss or curing of genetic markers after growth of some strains in curing agents or at elevated temperatures is indicative of plasmid location of the markers, but only if it can be shown that treatment did not select for cells from which plasmid markers were lost spontaneously (17). Possible selective effects can be ruled out (i) by determining the rate and time of appearance of negative variants during treatment of the wild-type culture, and (ii) by showing that growth at the elevated temperature or in the presence of the curing agent does not competitively favor plasmid-negative cells over plasmid-positive cells (9, 17).

Ethidium bromide (EB) has proven to be a potent agent for eliminating penicillinase plasmids (3, 14, 17). Rubin and Rosenblum (17) observed that the curing frequencies for staphylococcal penicillinase plasmids were consistently greater with EB than with other curing agents. The presence of a high rate of spontaneous loss of the ability to make bacteriocin by group II staphylococci was enhanced by growth in EB, suggesting that bacteriocin synthesis in staphylococci was specified by a plasmid (16). A second chemical agent successfully used to eliminate staphylococcal plasmids was sodium dodecyl sulfate. Growth of two strains of S. aureus in 0.002% sodium dodecyl sulfate resulted in high rates of loss of ability to produce penicillinase (19). Loss of some, but not all, staphylococcal plasmids can be enhanced by growth at elevated temperatures (9, 16). A given plasmid can display a wide range of stability or sensitivity to growth at high temperatures depending upon the given carrier cell. Growth of an S. aureus strain at 44 C was shown to be very effective in eliminating a plasmid for tetracycline resistance but not the plasmid containing the genes for penicillinase synthesis (9).

When a specific plasmid is lost from a cell, all markers borne on this plasmid are concomitantly eliminated, and, when a plasmid is transduced into a recipient, all markers on the plasmid are usually jointly transferred. Irradiation of a generalized transducing phage with low doses of ultraviolet light enhances the frequency of transfer of chromosomal genes. Alternatively, transduction of extrachromosomal genes falls exponentially with linear increases in the dose of irradiation given to the donor phage. This phenomenon is known as the Arber experiment (16).

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MATERIALS AND METHODS

**Strains.** ET-producing (Tox+) strains listed in Table 1 were isolated from clinical sources by Melish and Glasgow (10, 11), except for Tox- strains UT 0011 and UT 0012 which were isolated by B. Wiley. All Tox+ strains belong to phage group II. Phage group II isolates of plasmid carriers were obtained by F-mediated transduction of penicillinase-negative strains (16). Strains from Table 2 were used in transduction experiments, was generously provided by E. Rosenblum. Substrains of UT 0007 were isolated after overnight growth (i) in 0.003% sodium dodecyl sulfate (SDS), designated at UT 1641 (Tox-) and UT 1643 (Tox+); (ii) in 6 x 10-4 M EB, designated as UT 0491 (Tox-) and UT 0495 (Tox+); and (iii) at 44 C, designated as UT 1784 (Tox-) and UT 1781 (Tox+). Four single-plaque isolates of phage from mitomycin C-induced lysates were propagated in strain UT 0015 to titers of 106 to 108 plaque-forming units (PFU)/ml. These phage isolates have been designated as PEV-1, PEV-2, PDM-1, and PDM-2.

**Media.** The medium used for growing cultures, phage propagation, transduction, and curing experiments was P brosh described by Friend and Slade (5). It consisted of brain heart infusion broth (Difco) supplemented with 0.2% yeast extract, 10 mg of dl-tryptophan per ml, and 10-4 M CaCl2. Solid media were prepared by adding 1.5% (P-hard agar) or 0.75% (P-soft agar) Difco agar to P broth. Mueller-Hinton agar (Difco) and P agar were used for disk diffusion tests to determine sensitivities to antibiotics and metal ions.

**Determination of antibiotic and metal ion resistance.** Resistance to penicillin, erythromycin, methicillin, and tetracycline was determined with the standardized single-disk test of Bauer et al. (1). Staphylococcal lawns for susceptibility tests were made by flooding an agar plate with 104 cells per ml, pouring off the excess broth, and rapidly drying the agar surfaces. Ten-unit penicillin disks (Difco) produced inhibition zones of 40 to 44 mm with sensitive strains and zones of 11 to 18 mm with resistant strains. Mercury sensitivity was determined by using the technique devised by Green (6). Screening tests for resistance to cadmium, arsenite, arsenate, and bismuth were carried out by the procedure of Novick and Roth (15), where blank 0.7-cm concentration
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EXOFLIATIVE TOXIN PRODUCTION

TABLE 1. Phenotypic properties of exfoliative toxin-producing staphylococci

<table>
<thead>
<tr>
<th>Strain</th>
<th>Previous designation</th>
<th>Phage type</th>
<th>Presence of a lyso-</th>
<th>Marker pattern*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>genic phage active</td>
<td>Phage Pen Cad</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>on strain UT 0015</td>
<td>PDM-1</td>
</tr>
<tr>
<td>UT 0001</td>
<td>DM (10)</td>
<td>55/71</td>
<td>+</td>
<td>R R R</td>
</tr>
<tr>
<td>UT 0002</td>
<td>EV (10)</td>
<td>55/71</td>
<td>+</td>
<td>R R R</td>
</tr>
<tr>
<td>UT 0003</td>
<td>TA (10)</td>
<td>71</td>
<td>+*</td>
<td>R R R</td>
</tr>
<tr>
<td>UT 0004</td>
<td>CH (10)</td>
<td>71</td>
<td>+</td>
<td>R R R</td>
</tr>
<tr>
<td>UT 0005</td>
<td>PM (10)</td>
<td>71</td>
<td>-</td>
<td>R R R</td>
</tr>
<tr>
<td>UT 0006</td>
<td>SA (10)</td>
<td>71</td>
<td>+*</td>
<td>R R R</td>
</tr>
<tr>
<td>UT 0007</td>
<td>DI (10)</td>
<td>55/3A/3C</td>
<td>+</td>
<td>R R R</td>
</tr>
<tr>
<td>UT 0008</td>
<td>KG (10)</td>
<td>71</td>
<td>+</td>
<td>R S R</td>
</tr>
<tr>
<td>UT 0009</td>
<td>JD (10)</td>
<td>55/71</td>
<td>+</td>
<td>R R R</td>
</tr>
<tr>
<td>UT 0101</td>
<td>TG (10)</td>
<td>55/71</td>
<td>+</td>
<td>R R R</td>
</tr>
<tr>
<td>UT 0011</td>
<td>853</td>
<td>3A/3B/3C/55</td>
<td>-</td>
<td>R R R</td>
</tr>
<tr>
<td>UT 0012</td>
<td>854</td>
<td>3A/3B/3C/55</td>
<td>+</td>
<td>R R R</td>
</tr>
</tbody>
</table>

* R, Resistant; S, sensitive; Pen, penicillin; Cad, cadmium.
* Capable of transducing the cad marker into Tox+ strains UT 0017 and 04081.

TABLE 2. Phenotypic properties of phage group II staphylococci not producing exfoliative toxin

<table>
<thead>
<tr>
<th>Strain</th>
<th>Previous designation</th>
<th>Phage type</th>
<th>Presence of a lyso-</th>
<th>Marker pattern*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>genic phage active</td>
<td>Phage Pen Cad</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>on strain UT 0015</td>
<td>PDM-1</td>
</tr>
<tr>
<td>UT 0013</td>
<td>850</td>
<td>55/71</td>
<td>-</td>
<td>S S R</td>
</tr>
<tr>
<td>UT 0014</td>
<td>851</td>
<td>55/71</td>
<td>-</td>
<td>S S R</td>
</tr>
<tr>
<td>UT 0015</td>
<td>852</td>
<td>55/71</td>
<td>-</td>
<td>S S R</td>
</tr>
<tr>
<td>UT 0016</td>
<td>3A*</td>
<td>3A/3B/3C/55</td>
<td>-</td>
<td>R S S</td>
</tr>
<tr>
<td>UT 0017</td>
<td>3B*</td>
<td>3B/3C/55</td>
<td>-</td>
<td>S S S</td>
</tr>
<tr>
<td>UT 0018</td>
<td>3C*</td>
<td>3A/3B/3C/55</td>
<td>-</td>
<td>S S S</td>
</tr>
<tr>
<td>UT 0019</td>
<td>DR003</td>
<td>55/71</td>
<td>-</td>
<td>S S R</td>
</tr>
</tbody>
</table>

* R, Resistant; S, sensitive; Pen, penicillin; Cad, cadmium.
* Propagating strains for the respective phages.

disks (Difco) were impregnated with 0.02 ml of a salt solution, dried, and placed on a P agar plate seeded with the test organism. To determine the loss of penicillin resistance or cadmium resistance during curing, or the gain of these markers through transduction, cells were plated directly or replica-plated onto P agar containing the maximal subinhibitory concentration of either penicillin or Cd(NO₃)₂. The concentration of penicillin was 1.6 U/ml and ranged between 60 and 90 µg/ml for Cd(NO₃)₂, depending on the specific strain being used.

Penicillinase assay. Penicillinase was determined by the microiodometric assay (13). Penicillinase production was preinduced with 0.45 µg of methicillin per ml, and enzyme activity was measured in a Coleman colorimeter. To test for penicillinase in colonies on agar plates, colonies were replica-plated from P agar to Mueller-Hinton plates with 0.2% soluble starch and incubated at 37°C overnight. The colonies were then tested for enzyme by using the starch-iodine test of Workman and Farrar (20). Zones of decolorization appeared around penicillinase producers.

Assay of ET. Strains were streaked onto P agar and incubated overnight in a CO₂ atmosphere at 35°C. Cells were suspended into saline to a final concentration of approximately 10⁶ cells per ml. A 0.1-ml amount of the saline suspension was injected into the scapular area of a mouse under 7 days of age. Eighteen to 24 h after injection of ET-producing staphylococci, the epidermal area at the site of injection remained wrinkled after gentle stroking, producing evidence of the Nikolsky sign which is characteristic of the SSS. The affected epidermal area could then be easily rubbed loose to reveal underlying erythematous, moist, glistening tissue.

Plasmid elimination. The method of Bouanchaud et al. (3) was used for plasmid elimination in EB. An initial inoculum of 10⁵ cells per ml was incubated with shaking for 24 h at 37°C in P broth containing 6 × 10⁻⁴ M EB, and plated onto P agar. The resulting colonies were examined for loss of ET, cadmium resistance, and penicillin resistance. The method of Sonstein and Baldwin (19) was used for plasmid elimination with SDS. An initial inoculum of 10⁵ cells per ml was incubated with shaking at 37°C for 18 h in P broth containing 0.003% SDS. The cultures were then plated on P agar and subsequently examined for loss of ET. The given concentration of EB and SDS represent maximal subinhibitory concentrations for Tox+ cells growing in P broth. To test for plasmid
elimination at high temperatures, 10^{-7} colony-forming units per ml were inoculated into P broth and incubated for 24 h in a water-bath-shaker set at 44 C, and plated onto P agar. High cell inocula were used prior to growth at 44 C or in EB to overcome a lengthened lag period. In all curing experiments, cell suspensions were exposed to some oscillation during serial dilutions to break up cell clusters which might have contained a mixture of Tox+ and Tox- types. Large numbers of colonies resulting from the experiments designed to eliminate plasmids could rapidly and easily be examined for the loss of penicillinase and cadmium resistance by replica plating onto agar test plates as described above. However, since the Tox+ phenotype could only be detected in the animal model, the numbers of colonies that could be tested for the loss of ET were limited to the number of mice that could be reasonably handled during a given period of time.

**Phage elimination.** A procedure similar to that described by Seidman et al. (18) was used to eliminate lysogenic phage, except that EB was used in place of acidicine orange (AO). EB has proven to be more successful than AO in eliminating staphylococcal extrachromosomal elements (16). Tox- cultures were grown for 3 h in 20 ml of P broth, centrifuged, and suspended into 20 ml of a tris(hydroxymethyl)aminomethane (Tris)-succinate-magnesium acetate (TSM) buffer, 6 \times 10^{-4} M (final concentration) EB, and 3 \mu g of mitomycin C per ml. After 20 min of incubation with shaking at 37 C, the culture was washed with TSM buffer, resuspended into P broth containing a final concentration of 6 \times 10^{-4} M EB, allowed to shake at 37 C overnight, and plated onto P agar. Colonies derived from treated and untreated cells growing on P agar were replica-plated onto new P agar plates which were incubated overnight at 37 C. The resulting colonies were irradiated for 8 s with an ultraviolet lamp calibrated at 1,000 ergs per s per cm^2 and overlaid with a soft agar suspension of strain UT 0015 cells. Zones of clearing appeared around colonies from which lysogenic phage active on the sensitive strain were liberated. Absence of zones of clearing around colonies indicated a lack of the temperate phage active on strain UT 0015. However, this method was not completely accurate in determining the loss of lysogenic phage since a small percentage of cells taken from colonies showing no zones of clearing could still be induced with mitomycin C to yield temperate phage. Therefore, it was possible to select for the loss of phage by the soft agar overlay method, but the actual loss of phage had to be confirmed by spotting a mitomycin C-induced lysate of the strain onto a lawn of UT 0015 cells. Some of the strains tested did not respond to mitomycin C, but none was resistant to the drug.

**Lysogenization.** Phage isolates PEV-1, PEV-2, PDM-1, and PDM-2 were propagated on strain UT 0015 to titters of 10^{14} PFU/ml. A specific phage strain was then mixed with either UT 0013, UT 0014, or UT 0015 cells at a multiplicity of infection of 1:10 and incubated at 37 C for 3 h. The cells were washed twice in 0.02 M sodium citrate to eliminate contaminating free phage, and plated onto P agar. The resulting colonies were replica-plated onto fresh P agar plates and then tested for lysogeny as described above. A substrain was considered lysogenic for one of the four phages if it produced a phage active on the nonlysogenic parent and was immune to the lysogenic phage.

**Transduction.** Strains UT 0003 and UT 0006 were grown in P broth for 3 h, centrifuged, and suspended in 10 ml of TSM buffer with a final concentration of 3 \mu g of mitomycin C per ml. After 30 min of incubation with shaking at 37 C, the cultures were washed, suspended into P broth, and allowed to shake at 37 C until maximal lysis occurred. The donor lysates were sterilized through 0.45-\mu m membrane filters (Millicore Corp.). The resulting phage concentration was approximately 10^7 to 10^{14} PFU/ml. Transduction was performed by the method of Sonstein and Baldwin (19) with the exception of P broth being used in place of Trypticase soy broth, and P agar without CaCl_2 in place of yeast extract-Trypticase soy agar. The selective concentration of Cd(NO_3)_2 was 90 \mu g/ml for recipient strain UT 0081 and 60 \mu g/ml for recipient strain UT 0017, and the selective concentration of penicillin was 1.6 U/ml for both recipients. In the Arber test, donor lysates were irradiated with increasing doses of ultraviolet light to produce a 10 to 99% reduction in PFU/ml and, subsequently, used to transduce the cadmium-resistance marker.

**RESULTS**

**Phenotypic properties of Tox+ and Tox- strains.** The phenotypic properties of phage group II Tox+ (Table 1) and Tox- (Table 2) strains were found to be markedly different. Whereas 10 of 12 Tox- strains could be induced with mitomycin C to yield a phage active on strain UT 0015, none of the Tox- strains contained such a phage, indicating that a lysogenic phage might be controlling toxin production. Whereas all Tox+ strains were resistant to phage PDM-1, six of seven Tox- strains were sensitive to the phage. All Tox+ strains except strain UT 0008 were resistant to penicillin (Table 1), but all Tox+ types were sensitive to the antibiotic (Table 2). All ET producers and three of seven non-toxin producers were resistant to cadmium. Tox+ and Tox- strains showed no significant differences in response to inhibitory effects to erythromycin, methicillin, tetracycline, mercury, arsenate, arsenite, and bis-muth.

**Elimination of phage and ET production.** Strains UT 0001 and UT 0002 contain a lysogenic phage active on Tox- strain UT 0015, which can be eliminated by treatment with mitomycin C and EB. Twenty of 206 treated UT 002 derivatives and 14 of 198 treated UT 0001 derivatives were cured of phage capable of lysing strain UT 0015 (Table 3). Seven of the 20 phage-cured UT 0002 derivatives and 8 of the 14
Table 3. Elimination of lysogenic phage and exfoliative toxin production with mitomycin C and ethidium bromide

<table>
<thead>
<tr>
<th>Strain</th>
<th>Total colonies</th>
<th>Colonies nonlysogenic for phage active on strain UT 0015</th>
<th>Nonlysogenic Toxic^− colonies^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>UT 0002</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>278</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Treated</td>
<td>206</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>UT 0001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>280</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Treated</td>
<td>198</td>
<td>14</td>
<td>8</td>
</tr>
</tbody>
</table>

^a Tox^− strains lost neither cadmium resistance, penicillin resistance, nor resistance to phage PDM-1.

Phage-cured UT 0001 derivatives also lost the ability to make ET. Since many substrains that have lost phage still retain the capacity to produce ET, it is unlikely that a temperate phage of UT 0001 or UT 0002 active on strain UT 0015 is controlling toxin production. Also, among the treated UT 0001 cells, five substrains were isolated that lost the ability to produce toxin but still harbored the phage. Lysogenic conversion to toxin production in Tox^− cells could not be demonstrated when phages PDM-1, PDM-2, PEV-1, and PEV-2 were used for lysogenization after being propagated on strain UT 0015. The activity of phage-controlled toxins is enhanced after phage induction (21), but attempts to enhance ET activity after phage induction of Tox^+ strains were unsuccessful.

Elimination of ET production with EB and SDS and at 44 C. Loss of the ability of a number of Tox^+ strains to make ET after growth in 0.003% SDS, in 6 x 10^-4 M EB, or at 44 C was examined (Table 4). Elimination of the ability to make toxin was detected after growth in the presence of both agents and at 44 C, although treatment at the elevated temperature resulted in the highest curing frequencies. Both strains UT 0001 and UT 0007 lost the capacity to synthesize ET at a frequency of 98% after growth at 44 C for 18 h (Table 4). Elevated temperatures were ineffective in curing any of the other Tox^+ strains listed in Table 1. Detectable frequencies of curing with SDS were observed with strains UT 0001, UT 0004, and UT 0007 (Table 4), but not with any of the additional Tox^+ strains listed in Table 1. All Tox^−-cured substrains were stable and all those examined maintained resistance to phage PDM-1. Among 150 of the cured Tox^− substrains examined, only one, strain UT 0111, lost both resistance to penicillin and cadmium, whereas the remaining substrains remained resistant to both agents. Penicillinase assays demonstrated that, except for strain UT 0111, no difference in induced enzyme levels existed in cured substrains as opposed to their wild-type parent strains, indicating that the genes for penicillinase synthesis and ET production were not co-eliminated.

Appearance of ET-negative variants during growth of the Tox^+ culture at 44 C. One culture of strain UT 0007 was grown for 24 h at 44 C, and a second culture for 24 h at 37 C. At various times, samples were plated and the resulting colonies tested for loss of ET. Although there was no lag during growth at 44 C, the concentration of viable cells per milliliter was consistently higher during growth at 37 C than at 44 C (Fig. 1). ET-negative variants were detectable neither in culture at 0 h nor at any time interval during growth at 37 C. Alternatively, Tox^− cells were initially detected after 3 h of growth at 44 C, and within 1 h the number of negatives increased 25-fold, whereas the total population increased only twofold (Fig. 1). At 4 h, 3.9 x 10^-7 out of 7.2 x 10^7 viable cells per ml, or 54% of the total population, lost the ability to make ET. The percentage of elimination continued to increase until about 8.5 h, after which the fraction of negative cells remained constant at about 88 to 98% of the total population (Fig. 2). A possible explanation for the observed population changes is that parental Tox^+ strains do not grow at 44 C and are overgrown by spontaneously produced Tox^- types. However, when strain UT 0007, a Tox^- heat-cured

Table 4. Elimination of exfoliative toxin production with ethidium bromide, sodium dodecyl sulfate, and elevated temperatures

<table>
<thead>
<tr>
<th>Strain</th>
<th>EB^b (6 x 10^-4 M)</th>
<th>SDS^c (0.003%)</th>
<th>44 C</th>
<th>Control^d</th>
</tr>
</thead>
<tbody>
<tr>
<td>UT 0001</td>
<td>10.5</td>
<td>1.9</td>
<td>98</td>
<td>0</td>
</tr>
<tr>
<td>UT 0002</td>
<td>3.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>UT 0004</td>
<td>0</td>
<td>2.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>UT 0007</td>
<td>42</td>
<td>10</td>
<td>98</td>
<td>0.1</td>
</tr>
</tbody>
</table>

^b Based upon the examination of at least 100 colonies for each recorded value.
^c EB, Ethidium bromide; SDS, sodium dodecyl sulfate.
^d Control values were determined by averaging the frequency of spontaneous loss for each experiment with each strain.
or 2% of the total population (Fig. 4). Within
1 h the Tox− variants increased 14.3-fold com-
pared to a 2.7-fold increase for the total popula-
tion. At 9.5 h the number of negatives attained
a maximal level of $1.2 \times 10^9$ cells per ml and
maintained a plateau. Growth of strain UT 0007
in SDS was slightly slower than the growth of
untreated cells over the first 5 h of incubation,
but after this time the growth curves of both
cultures were similar with each attaining the
same maximal level of viable cells per ml (Fig.

Fig. 1. Appearance of exfoliative toxin-negative
variants (Δ) during growth of UT 0007 (▲) at 44 C.
Growth of UT 0007 (●) at 37 C.

UT 0007 substrain (UT 1784), and a third Tox+
UT 0007 substrain (UT 1781), which was iso-
lated after 18 h of growth at 44 C, were grown
separately at 44 C, the Tox+ and not the Tox−
strain had a slight selective advantage at the
raised temperature (Fig. 3). The growth
curves of these three strains at 37 C were very
similar. The rapid rate of appearance of nega-
tive variants at 44 C, the lack of any selective
advantage of Tox− cells at 44 C, and an en-
hanced elimination frequency at 44 C of 97.9%
over the spontaneous frequency of loss strongly
suggest that the gene for ET is extrachromo-
somal.

Appearance of ET-negative variants du-
ring growth of the Tox+ culture in SDS.
Strain UT 0007 was grown in the presence of
0.003% SDS for 24 h. At given time intervals,
samples were plated onto P agar plates and the
resulting colonies were tested for loss of ET. In
an untreated culture, no negative variants were
detected up to 24 h of growth. In SDS, Tox−
cells were initially detectable after 5 h of
growth, where they numbered $7 \times 10^3$ cells per
ml or 2% of the total population (Fig. 4). Within
1 h the Tox− variants increased 14.3-fold com-
pared to a 2.7-fold increase for the total popula-
tion. At 9.5 h the number of negatives attained
a maximal level of $1.2 \times 10^9$ cells per ml and
maintained a plateau. Growth of strain UT 0007
in SDS was slightly slower than the growth of
untreated cells over the first 5 h of incubation,
but after this time the growth curves of both
cultures were similar with each attaining the
same maximal level of viable cells per ml (Fig.

Fig. 2. Frequency of elimination of the ability to
synthesize exfoliative toxin by strain UT 0007 (●)
during growth at 44 C. The spontaneous frequency of
loss is 0.1%.

Fig. 3. Growth of UT 0007 (Tox+), UT 1781 (Tox+)
and UT 1784 (Tox−) at 44 C. Symbols: ●, UT 0007;
■, UT 1781; □, UT 1784.
4). When strain UT 0007, an SDS-cured UT 0007 Tox- substrain (UT 1641), and an SDS-treated UT 0007 Tox+ substrain (UT 1643) were grown separately in the presence of 0.003% SDS, their growth curves were parallel (Fig. 5), suggesting no selective advantage of the cured derivative.

Appearance of ET-negative variants during growth of the Tox+ culture in EB. Strain UT 0007 was grown in the presence of $6 \times 10^{-8}$ M EB over a 24-h period, and samples were plated on P agar to determine the number of Tox- variants. Curing at a frequency higher than the spontaneous frequency of loss was not observed until h 6 of incubation (Fig. 6). During the subsequent 2 h of growth, Tox- cells increased 15-fold compared to a 6-fold increase of the total population. By h 8 the growth curve of the Tox- variants paralleled that of the total population, and both cell populations reached a maximal level of viable cells per ml at 13.5 h (Fig. 6). No Tox- variants were isolated after growth of UT 0007 in the absence of EB. Strain UT 0007, an EB-treated, cured derivative (UT 0491) and an EB-treated, uncured derivative (UT 0495) were grown separately in $6 \times 10^{-8}$ M EB, and strain UT 0491 lacked any selective advantage.

Transduction. All 12 Tox+ lysates were inducible with mitomycin C. Two of these lysates from strains UT 0003 and UT 0006 were capable of transducing cadmium resistance (cad), but not penicillin resistance (pen), to Tox- recipients UT 0017 and 04081 (Table 1). Transduction frequencies were approximately eight recombinants per $10^8$ PFU per ml. The cadmium-resistant recombinants remained sensitive to the donor lysates, indicating that the transferred trait was not a result of lysogenic conversion. None of 150 transduced recombinants examined received either the pen or Tox+ markers. Transduction of the pen or Tox+ markers was never observed. It was not possible to
propagate the transducing phage on any Tox+ strain since they were all resistant to the phage. Typing phages 55 and 71 could be propagated on specific Tox+ strains (Table 1), but none of these donor lysates was capable of transducing any strain used as a recipient. When transducing phages were irradiated with ultraviolet light to produce a 10 to 99% reduction in PFU per milliliter and then used for transduction, the transduction frequency for cad decreased with corresponding increases in irradiation dose, indicating that the cad marker is extrachromosomal.

**DISCUSSION**

The establishment of viral influence on the production of bacterial toxins (21) raised the possibility that ET production might also be phage mediated. This possibility seemed unlikely in view of the inability to demonstrate any association between specific lysogenic phages of Tox+ strains and ET production. However, since staphylococci are usually multitysogenic and can carry defective phage, it is possible that a phage other than those examined could control ET synthesis. Lysogenic phage and the ability to make ET could be eliminated by combined mitomycin C and EB treatment, but the ability to eliminate the ET synthesis by treatment with EB alone was the first indication that the gene for ET might be extrachromosomal.

Important criteria that have proven useful for the identification of a plasmid include a high spontaneous rate of loss of a genetic marker, the effect of specific chemical agents such as EB or SDS on enhancing this rate of loss, and the effect of growth at elevated temperatures on enhancing the rate of spontaneous loss (16). These procedures are valid only if it can be shown that plasmid-negative variants are produced and not selected during treatment of the positive culture. Growth at high temperatures was more effective than growth in EB or SDS in eliminating the ability of strains to make ET. Whereas rates of elimination were as high as 98% for strains UT 0001 and UT 0007, the other Tox+ strains lost the ability to make ET at frequencies of 0 to 3.5%, meaning that elimination was strain specific. The early and rapid rate of appearance of Tox- cells during growth of the Tox+ culture in either EB or SDS, or at 44 C, and the percentage of Tox- variants appearing in the population with time indicate that the selection and growth of a small number of spontaneously produced Tox- cells could not account for the high frequencies of negative variants which appeared during the early intervals of growth during treatment. This is supported further by the observation that a cured substrain has no selective growth advantage over a similarly treated but uncured derivative when both substrains are grown again in either EB or SDS, or at 44 C. Similar data have been presented to provide evidence for the extrachromosomal nature of other staphylococcal genes (9, 14, 16, 17, 19).

The 98% elimination frequency for the ability to synthesize ET by strains UT 0001 and UT 0007 after growth at 44 C suggests an interesting possibility about the nature of the gene for ET synthesis in these strains. If the gene for ET synthesis is localized on a plasmid, the replication rate for this plasmid is probably equivalent to the chromosomal replication rate at 37 C; but at 44 C, plasmid replication becomes defective. Therefore, during growth at the nonpermissive temperature, the gene for ET becomes rapidly diluted out of the growing population, accounting for the high frequency of elimination. The frequency and time of loss of an extrachromosomal staphylococcal gene for tetracycline resistance from a culture growing at 44 C (9) simulate the frequency and time of loss of the ability to make ET by UT 0007 cells growing at
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44 C (Fig. 1). A possible mechanism for the elimination of staphylococcal plasmids by EB was discussed by Rubin and Rosenblum (17), and the mechanism for SDS was discussed by Sonstein and Baldwin (19).

Why the cad but not the pen marker can be transduced from Tox* to Tox- recipients is poorly understood. The results from the Arber test indicated that the cad marker is extrachromosomal and not chromosomal. The possibility exists that cad and pen are harbored by the same plasmid, but this plasmid is too large to be packaged by the transducing phage. Strains UT 0003 and UT 0006 could house fragments of penicillinase plasmids which contain the cad marker and are small enough to be packaged by the transducing phage. The transduction of the gene for ET synthesis has not been possible, largely for want of an adequate method to select Tox* recombinants. Therefore, it has not been possible to apply the Arber test to the marker associated with ET production. However, even without these criteria, enough evidence has been presented to suggest strongly that ET synthesis in staphylococci is specified by a plasmid.

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