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Exfoliative Toxin Plasmids of Bacteriophage Group 2 *Staphylococcus aureus*: Sequence Homology

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The plasmid contents of seven exfoliative toxin-producing strains of phage group 2 *Staphylococcus aureus* were analyzed by agarose gel electrophoresis and deoxyribonucleic acid-deoxyribonucleic acid hybridization. All strains were found to contain a large plasmid with a molecular weight of 27×10^6 except for strain RW1005. A comparison of the restriction endonuclease cleavage products by agarose gel electrophoresis showed that the number and size distribution of the fragments of all these Tox plasmids were similar, except for pRW002, which appeared to contain two deletions. Deoxyribonucleic acid-deoxyribonucleic acid hybridization studies confirmed that these plasmids were related to a plasmid which carried the genes for exfoliative toxin B and bacteriocin R1 biosynthesis and that they shared some sequence homology with the penicillinase plasmid pI258 isolated from a phage group 3 *S. aureus*.

Localized infection with exfoliative toxin (ET)-producing strains of *Staphylococcus aureus* produces a broad spectrum of clinical diseases in newborns or immunosuppressed adults referred to as staphylococcal scalded skin syndrome (SSSS) (3–5). Biochemical and immunological investigations into the nature of ET have shown that at least two distinct forms of ET can be synthesized by toxigenic strains of *S. aureus* (9, 19).

Warren et al. (16) demonstrated that ET biosynthesis was lost from two strains after growth of Tox^+ strains in sodium dodecyl sulfate (SDS) or ethidium bromide or at elevated temperatures. In addition, the biosynthesis of a staphylococcin (BacR1) was co-eliminated at a high frequency from these strains. The authors concluded that both the genes for the synthesis of ET and BacR1 were on the same plasmid. Warren et al. (17) then demonstrated that a single large-molecular-weight plasmid (30×10^6) could be isolated from Tox^+ Bac^+ strains, but that this plasmid was absent from the cured substrains. This plasmid was shown to be unstable and rapidly converted to the open circular form after storage or treatment with protein-denaturing agents. The authors suggested that the ET plasmid had properties similar to those of the deoxyribonucleic acid (DNA)-protein relaxation complexes described for the ColE1 plasmid isolated from *Escherichia coli*.

In separate studies with two different strains of phage group 2 staphylococci, Rosenblum and Tyrone (13) were unable to correlate the loss of a large-molecular-weight plasmid with the reduction of ET. In addition, they demonstrated that a plasmid-free strain was capable of producing ET. These authors concluded that the biosynthesis of ET was under the control of chromosomal genes.

Later, Rogolsky et al. (12) showed that two strains (UT0002 and UT0003) had reduced titers of ET after growth of these strains in the presence of agents that eliminated ET production and plasmid DNA from strains UT0001 and UT0007. Under similar conditions strain UT0003 also lost the ability to produce BacR1. Plasmid analysis of the low ET-producing substrains of UT0002 (UT0002-19) showed that this strain had lost the large-molecular-weight plasmid. The authors concluded that ET biosynthesis could be under the control of both chromosomal and plasmid genes. Using these substrains of UT0002, Wiley and Rogolsky (19) demonstrated that UT0002 (Tox^+ $BacR1^+$) synthesized two different ET proteins, whereas UT0002-19 (Tox^+ $BacR1^-$) produced only one of the two ET proteins. Since these two proteins were shown to be antigenically distinct, it appears that there are two biologically and genetically distinct forms of the toxin.

Although plasmids with molecular weights of 27×10^6 have been isolated from the majority of ET-producing strains of phage group II *S. aureus*, the molecular relationships of these plasmids have not been studied. It is possible that these other plasmids may not be related to the ET plasmid. This paper examines the molecular relationship between the plasmids isolated from ET-producing strains of phage group II *S. au-*

reus and a plasmid responsible for ET biosynthesis in strain UT0001. These molecular relationships were examined by restriction endonuclease and DNA-DNA hybridization techniques.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1.

Isolation of plasmid DNA. Plasmid DNA was isolated from *S. aureus* strains by a modification of the SDS-NaCl lysis used by Warren et al. (17). Basically, 200 ml of brain heart infusion was inoculated with a 1/50 dilution of an overnight brain heart infusion broth culture of cells. These cultures were incubated at 37°C with shaking for about 6 h. The cells were then harvested by centrifugation and resuspended in 10 ml of TE buffer [0.05 M tris(hydroxymethyl)aminomethane (pH 8.0)–0.005 M ethylenediaminetetraacetic acid]. The cells were harvested by centrifugation, and then they were resuspended in 13.5 ml of TE buffer with 25% sucrose. To this suspension of cells, 200 μl (2 mg/ml) of lysostaphin and 200 μl (20 mg/ml) of lysozyme were added. After 30 min of incubation at 37°C, 5 ml of 0.5 M ethylenediaminetetraacetic acid and 5 ml of 20% SDS were added. The cells were then incubated at 55°C. (At this point a clear solution should result). After 30 min of incubation, 5 ml of 5 M NaCl was added to the cell lysate. The chromosomal DNA and cell debris were precipitated by placing the cell lysates on ice for at least 18 h. The precipitate was removed by centrifugation (30 min, 15,000 × *g*, JA 20 rotor). The plasmid DNA was then concentrated by adding 0.313 volumes of 42% (wt/vol) polyethyleneglycol 6000 and by storing it on ice. After at least 6 h, the DNA was pelleted by centrifugation (10 min, 27,200 × *g*, JA 20 rotor). The DNA pellet was then resuspended in 1/10 SSC buffer. The plasmid was purified by cessium chloride-ethidium bromide density gradient centrifugation (21).

Restriction endonuclease digestion and agarose gel electrophoresis of plasmid DNA. Plasmid DNAs isolated from phage group 2 *S. aureus* were digested with either *Eco*RI or *Hin*dIII restriction endonucleases, and the DNA fragments were separated by electrophoresis through a 0.7 or 1.4% agarose gel as previously described (1, 15). The DNA bands were visualized by irradiation with ultraviolet light and were photographed using a Wratten no. 12 barrier filter. The plasmids from *E. coli* V517 (2) and the *Eco*RI fragments of plasmids pI258 (7) and pRR12 (1) were used as mobility and molecular weight standards for each gel. The molecular weights of plasmid DNAs or their restriction endonuclease fragments were calculated from a linear regression analysis of a graph of the log of the molecular weight versus the log of the mobility of the standards included in each gel (2).

DNA blot transfer. DNA fragments were transferred from the agarose gel to nitrocellulose paper by the method of Southern (14). In general, the agarose slab gel was treated with a 0.5 M NaOH–1.0 M NaCl solution. After neutralization with a solution of 3 M NaCl in 0.5 M tris(hydroxymethyl)aminomethane (pH 7.0), the DNA was transferred to nitrocellulose paper by 3× SSC buffer. Finally, the DNA was fixed to nitrocellulose paper by heating in a vacuum oven at 80°C for 2 h.

Nick translation of probe DNA. [α-^{32}P]Deoxyadenosine 5′-triphosphate was incorporated into the plasmid DNA probe by using a nick translation reagent kit purchased from Bethesda Research Laboratories, Inc. In general, the DNA was first treated with deoxyribonuclease I at 15°C in buffer containing 0.04 M Tris-hydrochloride (pH 7.8), 0.004 M $MgCl_2$, 0.01 M 2-mercaptoethanol, 118 μM deoxynucleoside triphosphate, 50 μCi [α-^{32}P]adenosine 5′-triphosphate and 0.01 mg of nuclease-free bovine serum albumin per ml. After 10 min of incubation, 2 U of DNA polymerase I was added to the reaction mixture. After an additional hour of incubation at 15°C, Na_2 ethyl-

TABLE 1. *Bacterial strains used in this work*

Strain designation	Phenotype	Relevant plasmid	Plasmid mol wt (10^6)[a]	Plasmid phenotype	Source (reference)
UT0001	Tox$^+$ BacR1$^+$	pRW003	27	Tox$^+$ BacR1$^+$	Rogolsky et al. (10)
UT0002	Tox$^+$ BacR1$^+$	pRW008	1.6	?	Rogolsky et al. (10)
		pRW010	2.4	Cd^r[b]	
		pRW002	27	Tox$^+$ BacR1$^+$	
UT0008	Tox$^+$ BacR1$^+$	pRW005	27	Tox$^+$ BacR1$^+$	Rogolsky et al. (10)
UT0101	Tox$^+$ BacR1$^+$	pRW001	29	Tox$^+$ BacR1$^+$	Warren et al. (16)
UT0100	Tox$^-$ BacR1$^-$				Warren et al. (16)
UT0010	Tox$^+$ BacR1$^-$	pRW006	27	Tox$^+$ BacR1$^-$	Rogolsky et al. (10)
RW1001	Tox$^+$ BacR1$^+$	pRW011	7.7	?	Children's Medical Center,
		pRW007	27	Tox$^+$ BacR1$^+$	Dayton, Ohio
RW1010	SM^r[c]				R. Warren
RW1005	Tox$^+$[d] Cd^r	pRW012	1.6	?	R. Warren
		pRW013	2.4	Cd^r	
RN11		pI258	18.8	Amp^r Erm^r Cd^r Asi^r	R. Novick

[a] Molecular weights of the supercoiled form of the plasmid DNA were determined by agarose gel electrophoresis. Plasmids from *E. coli* V517 were used as mobility and molecular weight standards (2).

[b] Resistant to 90 μg of Cd $(NO_3)_2$ per ml in brain heart infusion agar.

[c] Substrain of UT0100.

[d] Substrain of UT0003.

enediaminetetraacetic acid was added to a final concentration of 0.15 M to stop the reaction. The labeled DNA was separated from the unreacted [α^{32}P]adenosine 5'-triphosphate by ethanol precipitation. The DNA was recovered by ethanol precipitation. The DNA was resuspended in 100 μl of 1/10 SSC and denatured by boiling for 10 min just before the addition to the hybridization buffer.

Hybridization of ^{32}P-labeled probe DNA to nitrocellulose paper. In general, the hybridization reaction was carried out in a modification of PM buffer (14). This buffer contains 3× SSC, 0.02% Ficoll (molecular weight, 70,000), 0.02% polyvinylpyrrolidone (molecular weight, 360,000), 0.02% bovine serum albumin, and 0.02% SDS. The ^{32}P-labeled probe was prepared as described above and added to the PM buffer. The filter was incubated with the labeled DNA for 15 to 18 h at 65°C. The probe DNA was then removed, and the nitrocellulose paper was washed twice with PM buffer at 65°C and three times with 3× SSC at 65°C. The paper was dried and taped to a cardboard support. Then X-ray film was placed on top of the paper. After exposure, the film was developed and analyzed.

Densitometric analysis of autoradiograms. The Beckman model R-112 densitometer equipped with an integrator was used to determine the relative densities of each fragment.

RESULTS

Physical analysis of plasmid from toxin producing strains of *S. aureus*. Five of the six toxin-producing strains carried a large plasmid with a molecular weight of 27 × 10^6 (Table 1). In previous studies the loss of this plasmid from strains UT0101 and UT0001 was correlated with the loss of the strain's ability to produce ET and BacR1 (9, 17). In addition Rogolsky showed that strains UT0002 and UT0003 carried a small Cd ion resistance plasmid as well as the ET plasmid. However, a third plasmid was found in these strains with a molecular weight of 1.6 × 10^6. No phenotypic trait was assigned to this plasmid or to pRW011 (7.7 × 10^6 daltons) found in strain RW1001.

*Eco*RI cleaved the large plasmids (pRW001, pRW002, pRW006, and pRW007) into four fragments with molecular weights ranging from 10.7 to 3.0 × 10^6, but cleaved PRW011 only once (Fig. 1; Table 2). The small plasmids pRW012, pRW013, pRW008, and pRW010 were not cleaved by *Eco*RI. Inspection of the fragment molecular weights shows that their sum was less than the molecular weight determined for the supercoiled species of the large plasmid. Since the densitometric tracings of these bands showed that each fragment was a singlet, the variation in the measured molecular weight of these plasmids was probably due to the error in determining the size of the largest *Eco*RI fragment. In addition, pRW002's *Eco*RI fragments A and B were smaller than the corresponding fragments in the other Tox plasmids. Digestion of these plasmids with *Hin*dIII produced an even more complex pattern (Fig. 2). At least 15 fragments were found in the digest of pRW003; pRW002 had a similar pattern except it had lost DNA fragments D and G, and it had a new fragment C^+. Many of the *Hin*dIII fragments were doublets and triplets.

When pRW003 was used as the probe and hybridized against *Hin*dIII RN11, UT0001, RW1005, UT0002, UT0008, UT0010, RW1001,

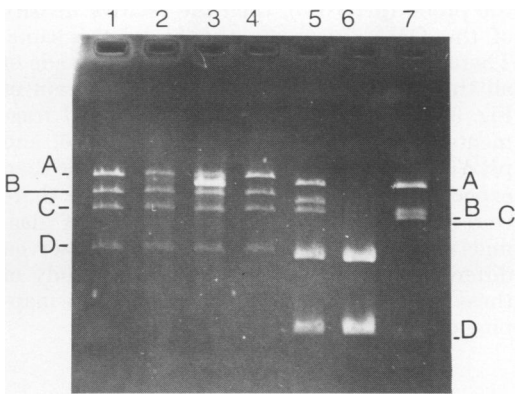

FIG. 1. *EcoRI digest of plasmids isolated from ET-producing strains of S. aureus. The plasmid DNAs were isolated from strains UT0008 (1), UT0010 (2), RW1001 (3), UT0101 (4), UT0002 (5), RW1005 (6), and RN11 (7), cleaved with EcoRI and electrophoresed through 0.7% agarose gel.*

TABLE 2. *EcoRI endonuclease fragments of phage group 2 S. aureus plasmids*[a]

Fragment	Plasmid mol wt (10^6)					
	pRW001	pRW002	pRW005	pRW006	pRW007	pI258
A	10.7	8.4	10.7	10.7	10.7	8.5
B	6.7	6.0	6.7	6.7	6.7	4.7
C	5.2	5.2	5.2	5.2	5.2	4.2
D	3.0	3.0	3.0	3.0	3.0	1.4

[a] Molecular weights of the *Eco*RI fragments were determined by linear regression analysis based on the equation $y = b + mx$, where y = log molecular weight and x = log of relative mobility. *Eco*RI fragments of pI258 were used as molecular weight and mobility standards. The correlation coefficient was −0.99 for the standard curve.

and UT0101 (Fig. 3A, lanes 1–8, respectively), the distribution of radioactivity coincided with the distribution of the fluorescent bands seen in the agarose gels of all the *Hin*dIII fragments of each plasmid except pRW011 in strain RW1001. Hybridization to plasmids pRW008 and pRW010 in strain UT0002 and pRW012, and pRW013 in strain RW1005 was not expected. To determine the region of sequence homology between the small plasmids in RW1005 and the large Tox plasmid, we used the ^{32}P-labeled plasmids from strain RW1005 as a probe against *Hin*dIII digests of plasmid DNA targets from strains UT0101, RW1005, UT0010, UT0008, UT0002, RW1005, and UT0001 (lanes 1–7, respectively, Fig. 3B). As shown in Fig. 3B, the probe DNA hybridized to *Hin*dIII fragment G from all the Tox plasmids except for pRW002 (lane 5), which does not have a *Hin*dIII fragment G. The intensity of the G fragment indicated that the region of homology was small. It is possible that this region of homology was due to an insertion element common to all these plasmids and that this region was deleted from pRW002.

If these large plasmids were all homologous to the probe (pRW003), then the relative density of the DNA fragments should be the same. Therefore, densitometric tracings were made of all the target DNAs on the autoradiogram of Fig. 3A. As shown in Fig. 4, the *Hin*dIII fragments A and B from pRW002, pRW006, and pRW007 were less homologous to the probe than expected. These experiments suggest that there were many similar but not identical Tox plasmids in phage group 2 *S. aureus*. These observed differences predict that a systematic study of these various plasmids will be useful in the mapping of the Tox plasmids.

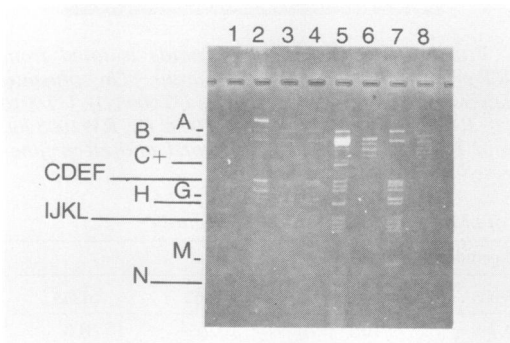

FIG. 2. *HindIII digest of plasmids isolated from ET-producing strains of S. aureus. The plasmid DNAs were isolated from strains UT0101 (1), RW1001 (2), UT0010 (3), UT0008 (4), UT0002 (5), RW1005 (6), UT0001 (7), and RN11 (8), cleaved with HindIII, and electrophoresed through a 1.4% agarose gel.*

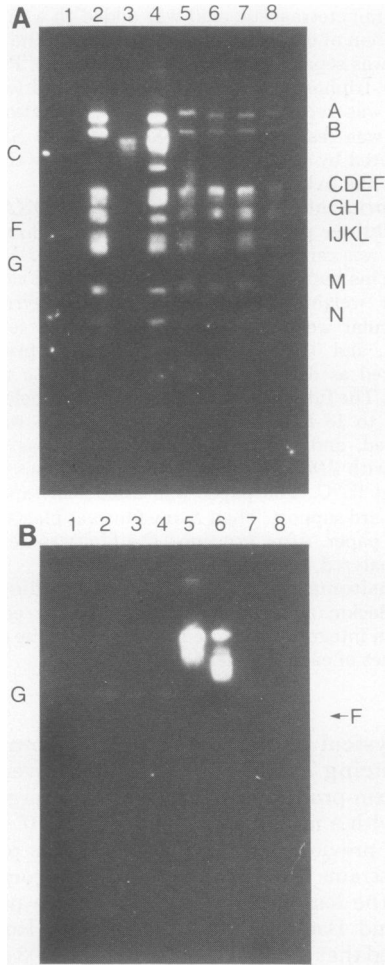

FIG. 3. *(A) Autoradiogram of pRW003 probe DNA hybridized to HindIII digests of plasmid DNAs isolated from strains (1) RN11, (2) UT0001, (3) RW1005, (4) UT0002, (5) UT0008, (6) UT0010, (7) RW1001, and (8) UT0101. (B) The plasmids isolated from RW1005 were used as a probe DNA and hybridized against plasmids isolated from (1) UT0101, (2) RW1005, (3) UT0010, (4) UT0008, (5) UT0002, (6) RW1005, (7) UT0001, and (8) RN11.*

Sequence homology between pI258 and plasmids of phage group 2 *S. aureus*. In the previous experiment, the plasmid pI258 was an internal molecular weight marker. However, upon examining the autoradiograms (Fig. 3A) we detected homologous sequences in the *Hin*dIII fragments C, F, and G of pI258 (lane 1). In addition, when the plasmids from RW1005 were used as the probe DNA, sequence homology was detected for *Hin*dIII fragment F (Fig. 3B, lane 8). The nature of these regions of sequence homology between pI258 and the plasmids of phage group II *S. aureus* is unknown.

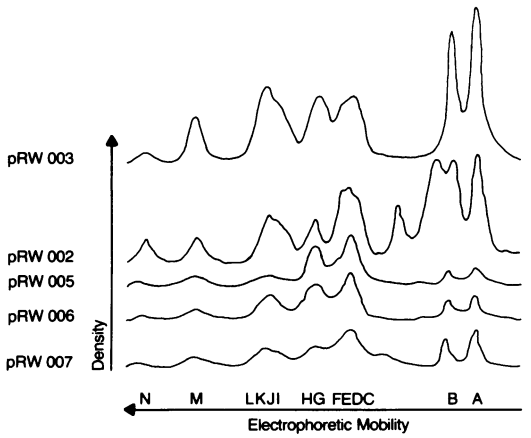

FIG. 4. *Densitometric tracings of autoradiogram in Fig. 3A.*

However, with the extensive physical and genetic map available for pI258 it may be possible to identify these common sequences (6–8).

DISCUSSION

Previous physical analysis of the plasmids from toxin-producing strains of *S. aureus* (9, 13, 17) showed that some of these strains had a large plasmid with a molecular weight of 26×10^6. These plasmids were shown to carry the genes for BacR1 and ETB synthesis (17). In addition, Rogolsky (9) reported that strain UT0002 also contained a small plasmid (21S, 2.6×10^6 daltons) that carried the genes for Cd ion resistance. In these studies, the plasmid content of several different strains of phage group 2 *S. aureus* was examined by agarose gel electrophoresis. In general, the results were similar to earlier findings. Restriction endonuclease digests with *Eco*RI or *Hin*dIII showed that the large plasmids had very similar patterns and were probably closely related. In addition, another small plasmid was found in strain UT0002 with a molecular weight of 1.6×10^6 (pRW008).

When plasmid DNAs isolated from toxin-producing strains were examined for sequence homology by using DNA-DNA hybridization techniques, homology was detected between the large Tox plasmids and surprisingly between some of the small plasmids in strains UT0002 and RW1005. Since these plasmids may coexist in the same host, recombination between them should occur. Such recombination could generate small plasmids that carry the *tox* genes, large plasmids that have picked up the cadmium resistance genes, or deletions of the large Tox plasmid. The deletions observed in pRW002 may have resulted from such deletion recombination events. Since the Tox plasmids also share some sequence homology with the penicillinase plasmid pI258, it is also likely that recombination of these two plasmids may occur. The advent of such a naturally occurring recombinant plasmid molecule would have a significant effect on the clinical management of SSSS simply by increasing the antibiotic resistance of this phage group 2 *S. aureus*. In addition, the apparent restriction modification barrier (9) between phage group 2 and phage group 3 *S. aureus* would be overcome by these regions of sequence homology, which could provide more virulent strains of *S. aureus* with a potential for ET production.

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