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Isolation of Extrachromosomal Deoxyribonucleic Acid for Exfoliative Toxin Production from Phage Group II *Staphylococcus aureus*

RICHARD WARREN,[1] MARVIN ROGOLSKY,* BILL B. WILEY, AND LOWELL A. GLASGOW

Departments of Microbiology and Pediatrics, College of Medicine, University of Utah, Salt Lake City, Utah 84132

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The ability of phage group II staphylococcal strain UT 0101 to produce exfoliative toxin and bacteriocin could be eliminated at a high frequency after growth at high temperatures or in the presence of ethidium bromide or sodium dodecyl sulfate. Extrachromosomal deoxyribonucleic acid, associated with the genes for exfoliative toxin and bacteriocin production, was isolated from strain UT 0101 but was absent from an ethidium bromide-cured substrain. The molecular weight of the exfoliative toxin plasmid, determined by co-sedimentation with the penicillinase plasmid, PI_{258}, was 3.3×10^7. The 56*S* covalently closed circular form of the exfoliative toxin plasmid converted to a 38*S* open circular form after storage or exposure to sodium dodecyl sulfate. Plasmid deoxyribonucleic acid associated with penicillin resistance could not be identified in the penicillin-resistant Tox$^+$ strains, UT 0007 and UT 0001.

The ability of chemical agents or growth at elevated temperatures to enhance the rate of loss of genetic markers has been a useful test for the identification of plasmid genes (10). However, this test is only valid if the selection of a spontaneous negative variant during growth in the presence of chemical agents can be ruled out (7). Elimination of plasmid genes rather than the selection of plasmid-negative variants by chemical agents can be indicated by experiments showing the rapid and early appearance of the negative cells during the growth of the positive cultures in the presence of chemical agents (12), providing that the growth rates in the presence of the chemical agents are the same for a negative and positive strain (2).

It was previously reported from this laboratory that the ability to produce exfoliative toxin (ET) by two strains of phage group II staphylococci was lost at high frequencies after growth of these strains in the presence of ethidium bromide (EB), sodium dodecyl sulfate (SDS), or high temperatures (11, 16). The co-elimination of ET and bacteriocin (Bac) production from strain UT 0007 suggested that these genes were on the same plasmid (16). The selection of a spontaneous mutant which was unable to produce ET during growth at high temperatures or during treatment with either EB or SDS was ruled out by the rapid and early appearance of toxin negative (Tox$^-$) strains (11, 16). Furthermore, cured Tox$^-$ substrains had no selective advantage over Tox$^+$ strains during growth under these experimental conditions.

Although the data from our laboratory indicated that the genes for ET and Bac synthesis were extrachromosomal, the identification of plasmid genes based solely on their elimination with chemical agents suffered certain serious limitations (14). More definitive evidence for the extrachromosomal nature of genes was dependent upon associating these genes with plasmid deoxyribonucleic acid (DNA) that has been isolated and characterized by density gradient centrifugation. The purpose of the present study was to isolate the extrachromosomal DNA associated with ET and Bac production from a Tox$^+$ phage group II staphylococcal strain, UT 0007 (11, 16), and to demonstrate the absence of this extrachromosomal DNA in an EB-cured derivative of the Tox$^+$ strain.

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MATERIALS AND METHODS

Staphylococcal strains. Phage group II staphylococcal strains UT 0001, UT 0007, and UT 0008 were isolated from clinical sources (11). Two substrains were derived from growth of UT 0007 in the presence

[1] Present address: Laboratory of Molecular Biology, University of Wisconsin, Madison, Wis. 53706.

of EB (16). Substrain UT 0101 produces both ET and Bac, a substance which inhibits the growth of staphylococcal strain 502A. Substrain UT 0100 produces neither ET nor Bac. Staphylococcal strain RN 11, carrying the PI_{258} plasmid, was provided by R. P. Novick.

Preparation of SDS-NaCl lysates. A modification of the method of Hirt (5) to isolate polyoma DNA was adapted to isolate the extrachromosomal DNA from staphylococci. The cultures were grown at 37 C with shaking in 30 ml of CY medium (8) to approximately 180 Klett units (no. 66 filter), in the presence of [*methyl*-^{3}H]thymidine (specific activity, 50 Ci/mmol) or [*methyl*-^{14}C]thymidine (specific activity, 50 mCi/mmol). The cells were then centrifuged and washed twice with a buffer (TES) consisting of 0.01 M tris(hydroxymethyl)aminomethane, 0.01 M ethylenediaminetetraacetate, and 0.5 M sodium chloride (pH 8.0). These cells were then resuspended in 1 ml of 2.5 M NaCl-TES (pH 8.0) with 200 μg of lysostaphin. After incubation at 37 C for 15 min, 1.5 ml of 1.67% SDS was added to the spheroplasts. The lysed cells were stored overnight at 4 C and then centrifuged at 27,000 $\times$ *g* in a Sorvall RC2B centrifuge for 30 min. The supernatant from the SDS-NaCl lysates was decanted and stored at −70 C until used.

Preparation of cleared lysates. The method of Novick and Bouanchaud (8) was used to prepare cleared lysates. The DNAs were labeled with [*methyl*-^{3}H]thymidine or [*methyl*-^{14}C]thymidine. The cells were washed twice in TES and resuspended in a 2.5 M NaCl-TES buffer (pH 8.0) with 200 μg of lysostaphin. After incubating the cells for 15 min at 37 C, 1.5 volumes of lysing cocktail (1% Brij-58, 0.4% deoxycholate, and 0.2 M ethylenediaminetetraacetate) were added to lyse the cells. The lysed cells were centrifuged immediately in a Sorvall RC2B centrifuge for 30 min at 27,000 $\times$ *g*. The cleared lysates were decanted and stored at −70 C until used.

CsCl-ethidium bromide dye-buoyant density gradients. The dye-buoyant density centrifugations were performed on a Spinco L2-65-B ultracentrifuge in a 50 Ti rotor at 35,000 rpm for 40 to 48 h at 20 C. CsCl-EB gradients were prepared by adding 8.5 ml of CsCl (60% wt/wt in TES) to 3.4 ml of cleared lysate. Immediately before the centrifuge tubes were placed into the 50 Ti rotor, 0.12 ml of EB (10 mg/ml) was added. The fractions containing the denser peak from the CsCl-EB gradients were mixed and dialyzed to remove the EB (14) before centrifugation through a neutral 5 to 20% sucrose gradient.

Neutral sucrose velocity gradients. Plasmid DNA isolated by SDS-NaCl lysates, cleared lysates, and CsCl-EB dye-buoyant density gradients were further analyzed on neutral sucrose velocity gradients. Centrifugation of neutral sucrose gradients was performed in the 50 Ti rotor at 35,000 rpm for 90 min at 20 C. Preformed 5 to 20% (wt/vol) linear sucrose gradients were prepared at room temperature. Samples of 0.4 ml were layered on top of the sucrose gradients and immediately centrifuged. The penicillinase plasmid, PI_{258}, was used as a reference marker (13). No difference in $s_{20,w}$ values was observed when using a SW41 rotor in place of the 50 Ti rotor.

Counting of radioisotopes. Fractions were collected from the bottom of the centrifuge tubes of the CsCl-EB and neutral sucrose gradients, and 0.1 ml of each fraction was placed on Whatman GF/A filters. The filters were dried, washed twice with ice cold 5% trichloroacetic acid, once with cold 95% ethanol, once with cold anhydrous ether, and dried again. The filters were added to 10 ml of a 1,4-di[2-(5-phenyloxazolyl)benzene, 2,5-diphenyloxazole, toluene scintillation cocktail and counted on a Nuclear Chicago 6848 scintillation spectrophotometer. Some fractions were collected directly into scintillation vials and NCS (Amersham-Searle) was added to the scintillation cocktail to solubilize the aqueous fractions.

RESULTS

Isolation and characterization of the ET plasmid from strain UT 0007. SDS-NaCl lysates of substrains UT 0101 and UT 0100 were pooled and centrifuged to equilibrium in CsCl-EB dye-buoyant density gradients (Fig. 1). A denser band of DNA corresponding to extrachromosomal DNA (9) was demonstrated for substrain UT 0101 (Tox$^+$, Bac$^+$), whereas no plasmid DNA was demonstrated for the EB-cured substrain, UT 0100 (Tox$^-$, Bac$^-$). Although these data confirmed the existence of extrachromosomal DNA in substrain UT 0101 (Tox$^+$, Bac$^+$), the DNA isolated could represent one or more different plasmids.

DNA of different molecular weights and topographical configurations may be resolved by velocity sedimentation through a linear sucrose gradient (15). To determine the number of

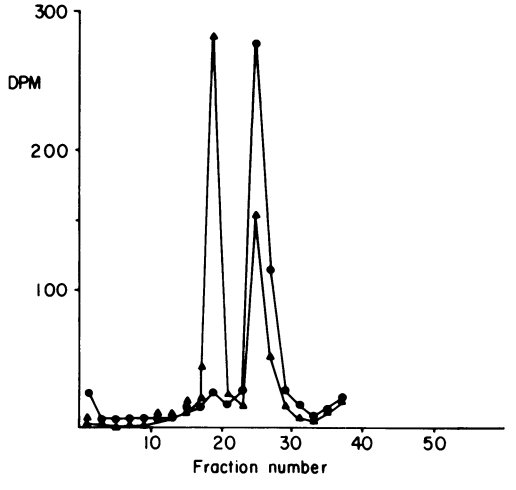

FIG. 1. *CsCl-EB dye-buoyant density gradient analysis of SDS-NaCl lysates pooled from [methyl-^{3}H]thymidine-labeled UT 0101 (▲) and [methyl-^{14}C]thymidine-labeled UT 0100 (●). Recovery of added counts from the material layered on the gradient was greater than 90%.*

molecular species of plasmid DNA in strain UT 0101, the denser DNA band from a dye-buoyant density gradient (fractions 17 to 23) from substrain UT 0101 was pooled, dialyzed against TES buffer (pH 8.0), and centrifuged through a neutral sucrose gradient (Fig. 2). Two species of DNA molecules with sedimentation coefficients of 56 and 38*S* were observed. The penicillinase plasmid, PI_{258}, with an *S* value of 47 and a molecular weight of 1.8×10^7 (13) was used as a reference marker. The *S* value of PI_{258} was determined by co-sedimentation with polyoma 20*S* DNA (15) on a 5 to 20% neutral sucrose density gradient. The PI_{258} DNA prepared under identical conditions also produced two species of DNA, 47 and 34*S*. These data suggested that the method of preparation of the DNA resulted in partial breakdown of the covalently closed circular (CCC) PI_{258} DNA to open circular (OC) DNA. It further suggested that the 38*S* species of DNA isolated from substrain UT 0101 represented the slower sedimenting (OC) form of the 56*S* CCC ET plasmid. A molecular weight of 3.3×10^7 was calculated for the 56*S* species by using the equation $s_{20,w} = 0.034\ M^{0.428}$ (1). The *S* value of the OC form of a CCC DNA with a molecular weight of 3.3×10^7 should be approximately 38*S* (6).

A second series of experiments was designed to avoid conditions that would cause the partial conversion of CCC DNA to the slower sedimenting OC DNA form. In these experiments, cleared lysates of substrain UT 0101 containing the ET plasmid and RN 11 containing the penicillinase plasmid, PI_{258}, were immediately analyzed on 5 to 20% sucrose gradients (Fig. 3). Only a single, rapidly sedimenting band of 56*S* DNA corresponding to the CCC ET plasmid was produced from the cleared lysate of strain UT 0101. The PI_{258} plasmid that was co-sedimented with the ET plasmid also produced a single 47*S* CCC DNA species. No OC plasmid DNA was recovered with this method of plasmid isolation. Storage of the cleared lysates at −70 or at 4 C prior to centrifugation through a sucrose gradient resulted in a partial conversion of the 56*S* species to the 38*S* species. These conditions had no effect on the PI_{258} plasmid.

Isolation of plasmid DNA by the SDS-NaCl lysing procedure should selectively precipitate the larger molecular weight chromosomal DNA

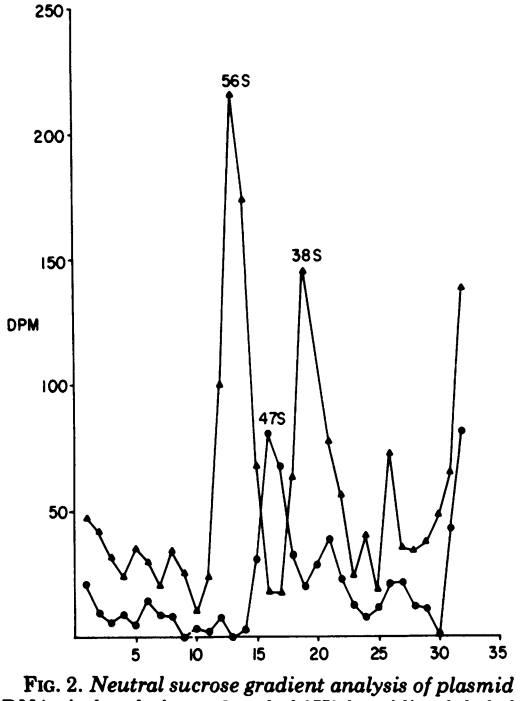

FIG. 2. *Neutral sucrose gradient analysis of plasmid DNA isolated from [methyl-³H]thymidine-labeled UT 0101 (▲) by CsCl-EB density centrifugation. Fractions 17 to 23 of Fig. 1 were pooled, dialyzed, and then co-sedimented with the penicillinase plasmid from [methyl-¹⁴C]thymidine-labeled RN 11 (●). Recovery of added counts from the material layered on the gradient was greater than 90%. Numbers on the ordinate represent fraction numbers.*

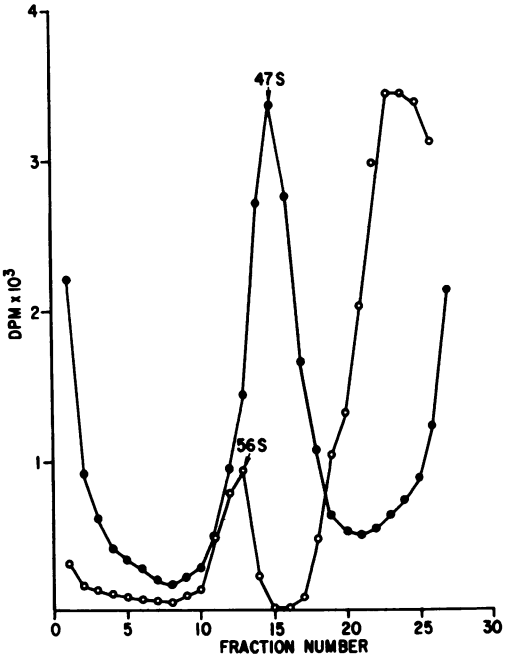

FIG. 3. *Direct neutral sucrose gradient analysis of cleared lysates pooled from [methyl-³H]thymidine-labeled UT 0101 (○) and [methyl-¹⁴C]thymidine-labeled RN 11 (●). Recovery of added counts from the material layered on the gradient was greater than 90%.*

from the smaller molecular weight plasmid DNA (4, 5). The major advantage of this technique was that it did not require the CCC form of the plasmid DNA for isolation (4). To determine if other plasmid forms were present in strain UT 0101, direct centrifugation of SDS-NaCl lysates through a neutral sucrose gradient was performed (Fig. 4). The PI_{258} plasmid isolated from strain RN 11 produced a single 47*S* species. However, the DNA isolated from strain UT 0101 produced a 56 and a 38*S* species. Since the 56*S* DNA spontaneously converted to the 38*S* form upon storage, the 38*S* species was again believed to represent the OC form of the ET plasmid DNA. Neutral sucrose gradient analyses of cleared lysates from strains UT 0101 and RN 11 occasionally revealed small slower sedimenting peaks, as seen in Fig. 2 and 4. Since such peaks were not consistently observed and strain RN 11 is known to contain only one plasmid (8), it was assumed that these slower sedimenting bands represented uneven banding of chromosomal DNA.

Finally, SDS-NaCl lysates from substrains UT 0101 (Tox^+, Bac^+) and UT 0100 (Tox^-, Bac^-) were analyzed directly on a 5 to 20% neutral sucrose gradient (Fig. 5). No plasmid DNA was demonstrated in substrain UT 0100, but the 56 and 38*S* species were distinctly observed in the lysate made from the Tox^+ strain. Therefore, the SDS-NaCl lysing technique provided a simple and rapid method for isolating both CCC and OC forms of plasmid DNA in staphylococci. Plasmid DNA other than the 56 and 38*S* species could not be demonstrated in DNA lysates made from substrain UT 0101. The isolation of a single plasmid from substrain UT 0101 (Tox^+, Bac^+) and the loss of this plasmid in the EB-cured substrain UT 0100 (Tox^-, Bac^-) confirmed that the genes for exfoliative toxin and bacteriocin production were located on the same plasmid.

Isolation of ET plasmid DNA from strains UT 0001 and UT 0008. Some phenotypic traits of strains UT 0001, UT 0007, and UT 0008 were compared in Table 1. The substrains of UT 0007 (UT 0100 and UT 0101) have been added to Table 1 for comparative purposes. Although strains UT 0001, UT 0007, and UT 0008 were all lysed by group II phages, they had different

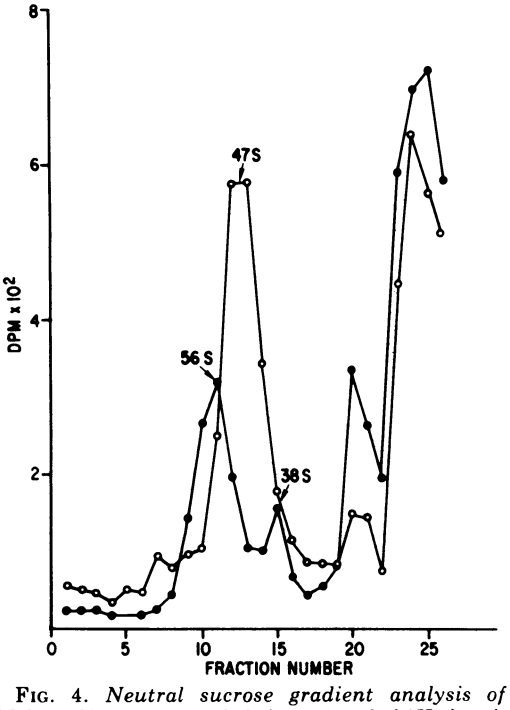

FIG. 4. *Neutral sucrose gradient analysis of SDS-NaCl lysates pooled from [methyl-³H]thymidine-labeled UT 0101 (●) and [methyl-¹⁴C]thymidine-labeled RN 11 (○). Recovery of added counts from the material layered on the gradient was greater than 90%.*

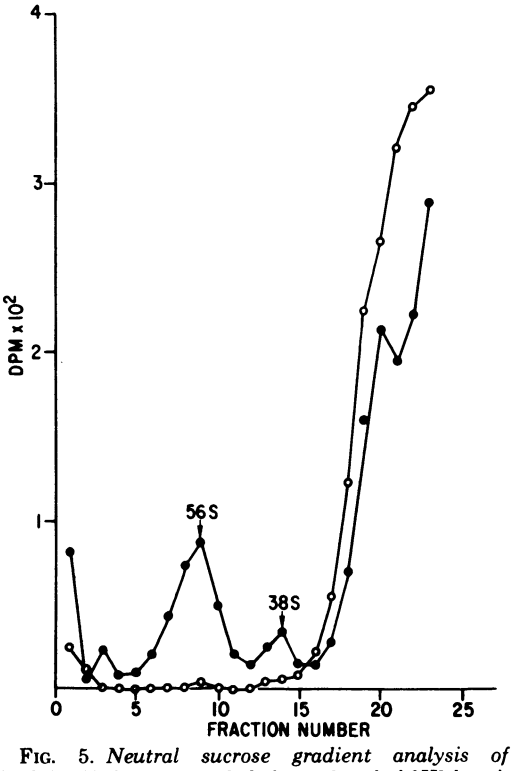

FIG. 5. *Neutral sucrose gradient analysis of SDS-NaCl lysates pooled from [methyl-³H]thymidine-labeled UT 0101 (●) and [methyl-¹⁴C]thymidine-labeled UT 0100 (○). Recovery of added counts from the material layered on the gradient was greater than 90%.*

TABLE 1. *Some phenotypic traits of phage group II staphylococcal strains*

Strain	Phage type	Marker pattern[a]				
		Pen	Cad	Bac	Pigment	ET
UT 0001	55/71	R	S	+	Y	+
UT 0007	55/3A/3C	R	S	+	Y	+
UT 0008	71	S	S	+	Y	+
UT 0101[b]	55/3A/3C	R	S	+	Y	+
UT 0100[b]	55/3A/3C	R	S	-	Y	-

[a] R, Resistant; S, sensitive; Y, yellow; Pen, penicillin; Cad, cadmium; Bac, bacteriocin; Pigment, color of strain grown on milk agar for 48 h; and ET, exfoliative toxin.

[b] Substrains of strain UT 0007 (16).

phage patterns. All strains were sensitive to 80 μg of cadmium sulfate per ml and produced yellow pigment on milk agar (3), ET, and Bac with the exception of UT 0100. UT 0008 was sensitive to penicillin whereas UT 0001 and UT 0007 were resistant to penicillin. Since ET plasmids were identified in strains UT 0007 and UT 0101, strains UT 0001 and UT 0008 were also assayed for the presence of this plasmid. Cleared lysates were prepared from the two strains. The DNA was centrifuged to equilibrium in CsCl-EB gradients. The denser DNA band from each strain was pooled, dialyzed against TES buffer, and further analyzed on a 5 to 20% sucrose gradient (Fig. 6 and Fig. 7). Both staphylococcal strains UT 0001 and UT 0008 contained 56 and 38S plasmid DNA. Therefore, extrachromosomal DNA of similar sedimentation rates was isolated from three phenotypically different staphylococcal strains, UT 0001, UT 0007, and UT 0008.

Penicillin resistance could not be eliminated from strains UT 0001 and UT 0007 by growth in the presence of EB or growth at high temperatures, suggesting a chromosomal location for this trait (16). Strain UT 0008, a naturally occurring penicillin-sensitive staphylococcus, contained only the 56 and 38S species of the ET plasmid. Plasmid DNA other than the 56 and 38S species could not be demonstrated in the penicillin-resistant staphylococci, UT 0001 and UT 0007. The failure to demonstrate a species of plasmid DNA associated with penicillin resistance suggested further that penicillin resistance was controlled by chromosomal genes in phage group II staphylococci.

DISCUSSION

In previous investigations, this laboratory showed that ET and Bac production were lost at a high frequency after growth at high temperatures or in the presence of EB or SDS (11, 16). The early and rapid accumulation of Tox$^-$ and Bac$^-$ variants during treatment suggested the elimination of a plasmid rather than the selection of a spontaneous negative variant. The Tox$^+$ and Bac$^+$ markers were co-eliminated at a frequency of 92% (16). These data suggested that the genes for ET and Bac synthesis were extrachromosomal and were on the same plasmid DNA.

Caution must be used when interpreting the ability of a chemical agent or growth conditions to enhance the loss of genes as evidence for the extrachromosomal location of those genes. Pigment production (3) and methicillin resistance (14) are examples of genetic traits that were lost at high frequencies after various treatments, for which no plasmids have been isolated. Therefore, definitive proof for the presence of extrachromosomal genes was dependent upon the association of these genes with plasmid DNA.

The present studies have shown that extrachromosomal DNA associated with ET and Bac production could be identified by its behavior in CsCl-EB density gradients. Although the

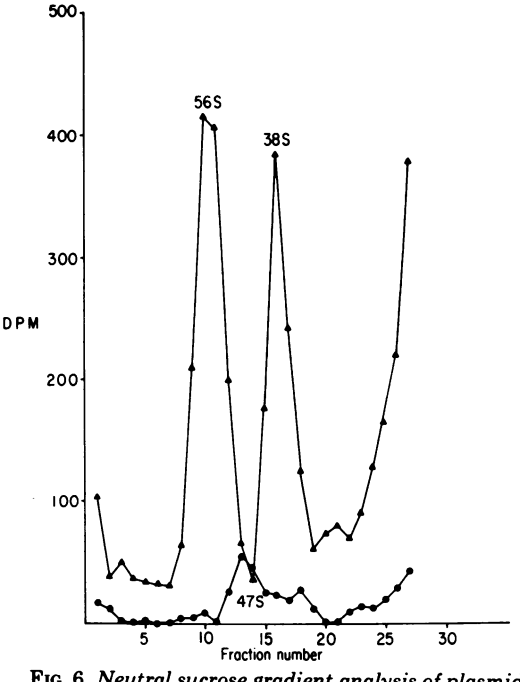

FIG. 6. *Neutral sucrose gradient analysis of plasmid DNA isolated from [methyl-^{3}H]thymidine-labeled UT 0001 (▲) by CsCl-EB density centrifugation. The denser peak of the CsCl-EB centrifugation was pooled, dialyzed, and then co-sedimented with a [methyl-^{14}C]thymidine-labeled cleared lysate from strain RN 11 (●). Recovery of added counts from the material layered on the gradient was greater than 90%.*

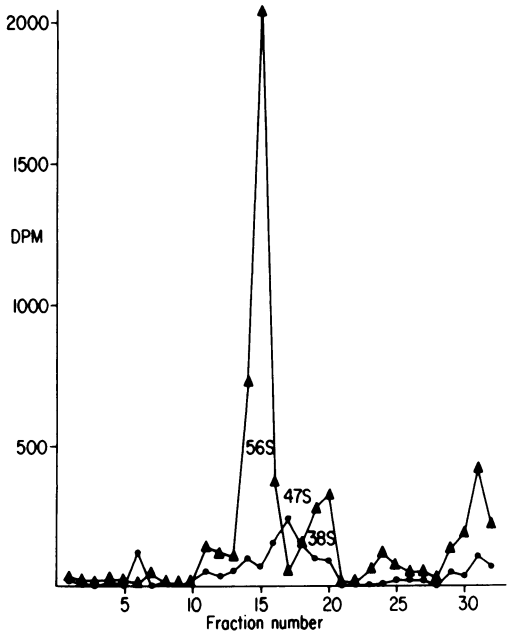

FIG. 7. *Neutral sucrose gradient analysis of plasmid DNA isolated from [methyl-^{3}H]thymidine-labeled UT 0008 (▲) by CsCl-EB density centrifugation. The denser peak of the CsCl-EB centrifugation was pooled, dialyzed, and then co-sedimented with a [methyl-^{14}C]thymidine-labeled cleared lysate from strain RN 11 (●). Recovery of added counts from the material layered on the gradient was greater than 90%.*

denser peak from these gradients contained only CCC DNA, further centrifugation of the fractions containing this peak through a neutral sucrose gradient resulted in two molecular species for the penicillinase plasmid, PI_{258}. The slower sedimenting species was believed to be the OC form of the 47*S* CCC DNA. Similar results were reported by Stiffler et al. (14) for the PI_{524} penicillinase plasmid. Since the penicillinase plasmid, PI_{258}, used as a reference marker was partially converted to the OC form by these manipulations, it was assumed that the 38*S* species was the OC form of the 56*S* ET plasmid DNA isolated from substrain UT 0101. Only a single 56*S* species was found in freshly prepared, cleared lysates. However, upon storage at 4 C or freeze-thaw, the 38*S* species was generated. Isolation of plasmid DNA from substrain UT 0101 by SDS-NaCl lysis also produced both 56 and 38*S* DNA.

Since the 56 and 38*S* species of DNA were the only plasmid DNAs demonstrated by three isolation techniques, it was concluded that the ability to produce ET and Bac were located on the same plasmid. The 56*S* ET plasmid was also isolated from two other Tox$^+$ strains of *Staphylococcus aureus*, UT 0001 and UT 0008. Both the 56 and 38*S* species of DNA were absent from a Tox$^-$, Bac$^-$-cured substrain, UT 0100.

In summary, a single plasmid has been associated with the ability to produce exfoliative toxin and bacteriocin in *Staphylococcus aureus*. The ET plasmid had a molecular weight of 3.3 $\times 10^7$. The single ET plasmid was demonstrated for three Tox$^+$ staphylococcal strains. No plasmid associated with penicillin resistance could be demonstrated.

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