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Phage Group II Staphylococcal Strains with Chromosomal and Extrachromosomal Genes for Exfoliative Toxin Production

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Staphylococcal phage group 2 strain UT0007 was previously shown to contain a high-molecular-weight plasmid containing genes for exfoliative toxin (ET) and bacteriocin production. Phage group 2 strains UT0002 and UT0003 (Tox^+Bac^-) underwent a twofold and ninefold loss of ET activity, respectively, after growth at 44 C for 18 h. Strain UT0002 also lost total bacteriocin activity. Both strains contained (i) a 56S plasmid that was lost from those substrains showing reduced ET activity and (ii) a 21S plasmid with a gene for cadmium resistance that could be transduced into two recipient strains. Since the ET plasmid-negative substrains still made ET, it was postulated that this residual toxin was made from chromosomal genes. In characterizing the plasmid species from strains UT0002 and UT0003, the 21S but little or no 56S plasmid deoxyribonucleic acid could be isolated after centrifugation of cleared lysates from these strains on dye-buoyant density gradients. Treatment of cleared lysates from strain UT0002 with ethidium bromide, Pronase, or sodium dodecyl sulfate, but not heat at 60 C, induced conversion of the 56S closed circular ET plasmid to a 38S open circular form as determined after centrifugation on 5 to 20% neutral sucrose gradients.

Most phage group 2 strains of *Staphylococcus aureus* produce an extracellular toxin and are associated with a spectrum of clinical manifestations known as the staphylococcal scalded skin syndrome. The disease is characterized by bulla formation, with the plane of cleavage occurring in the granular cell layer of the epidermis (7, 8). The toxin responsible for this epidermal exfoliation has been purified and termed exfoliative toxin (ET) (2, 4, 9). Recent investigations in this laboratory have shown that most of the criteria used to identify staphylococcal plasmids apply to the genes controlling ET synthesis in the *S. aureus* group 2 strains UT0001 and UT0007 (10, 11). For example, growth of the these Tox^+ strains is either 6×10^{-6} M ethidium bromide (EB) or 0.003% sodium dodecyl sulfate (SDS) or at 44 C resulted in the early and rapid accumulation of Tox^- variants (10). The ability of Tox^+ strains to make ET was most effectively eliminated, however, by growth at 44 C. More definitively, a single molecular species of plasmid deoxyribonucleic acid (DNA) associated with ET synthesis in strain UT0007 has been isolated and characterized as an unstable 56S covalently closed circular (CCC) molecule with a molecular weight of 3.3×10^7 after cesium chloride dye-buoyant density gradient centrifugation followed by analysis on 5 to 20% neutral sucrose velocity gradients (12). The ET plasmid was later found to control the production of a specific bacteriocin (Bac) active on staphylococcal strain 502A (11, 12). The instability of this 56S plasmid was evidenced by its spontaneous conversion to a 38S species that was assumed to represent the open circular (OC) form of the ET plasmid (12). Conversion of the ET plasmid from strain UT0007 to the OC form was enhanced after interaction with either SDS, Pronase, or alkali (R. Warren, M. Rogolsky, and B. Wiley, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, H95, p. 112). In a similar fashion certain *Escherichia coli*, colicin plasmids (Col E1, Col E2, Col E3, Col V2, and Col Ib), conjugative plasmids (F1), and R plasmids (R64, R28K, and R6K) can be induced to convert from the CCC form to the OC (relaxed) form when they interact with certain agents that affect protein structure (3, 5). Such plasmids have been designated as relaxation complexes. It has been suggested that a relaxation complex in the supercoiled state may possess a single-strand nick that is restrained by a protein which can be altered by treatment with relaxation agents (3). A more attractive explanation for this phenomenon is that both

strands of the plasmid are covalently closed, but one of the strands is tightly bound to a protein that includes an inactive endonuclease that can be activated after exposure to either detergents or proteases (3).

Only 2 (UT0001 and UT0007) out of our 12 Tox^+ phage group 2 strains of *S. aureus* could be successfully cured of their ability to make ET (10). The purpose of the present investigation was to carry out more thorough studies on the genetic factors controlling ET synthesis in two of these noncurable strains, UT0002 and UT0003, in order to determine whether ET in these strains is coded for by either plasmid genes, chromosomal genes, or both. If plasmid-controlled ET synthesis could be demonstrated, a second objective would then be to resolve whether the plasmids in strain UT0002 and UT0003 were similar in their physical and relaxation properties to the ET plasmid in strain UT0007.

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MATERIALS AND METHODS

Staphylococcal strains. Phage group 2 staphylococcal strains UT0002 (Tox^+Bac^+) and UT0003 (Tox^+Bac^-) were isolated from clinical sources as described previously (10). Strains UT0002-19 and UT0003-4 are substrains of strains UT0002 and UT0003, respectively, and were derived after overnight growth of the parent strains at 44 C. Both substrains have reduced levels of ET activity compared with their respective wild-type strains, and strain UT0002 does not produce the bacteriocin (Bac) active against staphylococcal strain 502A. Group 2 strain UT0017 used as a recipient in transduction experiments is the propagating strain for typing phage 3B. Strain RN11 carrying the R plasmid pI258 was provided by R. P. Novick, and the RN 1304 strain carrying the R plasmid T_{169} was provided by E. Rosenblum. The indicator strain for Bac activity, 502A, was supplied by B. F. Anthony.

Plasmid elimination. The ET plasmid was eliminated from strains UT0002 and UT0003 after growth of the strains at 44 C according to a method previously described in this laboratory (10). Cells from treated cultures were examined for loss of ET activity, penicillin resistance, cadmium resistance, and Bac activity where applicable.

Determination of ET and Bac activity. The assay for ET activity was the same as described previously (10) with the following exceptions. Cultures were grown in 250-ml shake flasks (Bellco) containing 25 ml of heart infusion broth. The flasks were incubated with shaking at 37 C on a New Brunswick model G76 gyratory water-bath shaker covered by a Plexiglas hood, which was flushed with 100% CO_2 twice daily. After approximately 48 h of incubation the culture suspension was diluted, and 0.1 ml of the dilutions was injected into the scapular area of a mouse under 5 days of age. Exfoliation at the site of injection occurred before 24 h. 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 the mouse.

Bac activity was determined by the method of Anthony et al. (1). Bac^+ colonies stabbed into heart infusion agar plates produced zones of clearing in a lawn of staphylococcal indicator strain 502A cells after overnight incubation at 37 C.

Isolation and characterization of plasmid DNA in the Tox^+ strains UT0002 and UT0003. Preparations of SDS-NaCl lysates and cleared lysates and analyses of these preparations by dye-buoyant density gradient centrifugation were accomplished as described previously (12), with the exception that strains containing ET plasmids were grown and labeled in CY medium lacking glucose before preparing SDS-NaCl or cleared lysates. The analyses of cleared lysates and CCC DNA isolated after dye-buoyant density gradient centrifugation on preformed 5 to 20% (wt/vol) neutral sucrose velocity gradients were exactly as described previously (12) with the exception that centrifugation was carried out in an SW41 Ti rotor at 40,000 rpm for 120 min when analyzing the ET plasmid and at 40,000 rpm for 150 min when analyzing the 21*S* plasmid for cadmium resistance. Reference markers consisting of either the penicillinase plasmid, pI258, or a plasmid determining tetracycline resistance, T_{169}, were cosedimented with plasmid DNA from the Tox^+ strains on neutral sucrose gradients. After centrifugation, fractions were collected from both types of gradients from the bottom of the centrifuge tubes and prepared for counting in a Nuclear Chicago 6848 scintillation spectrophotometer as described previously (12).

Relaxation of ET plasmid DNA. A 100-μl aliquot of a [methyl-^{3}H]thymidine (specific activity, 50 Ci/mmol)-labeled cleared lysate from strain UT0002 was suspended into 200 μl of TES buffer (0.05 M NaCl, 0.005 M sodium ethylenediaminetetraacetic acid, and 0.05 M tris(hydroxymethyl)aminomethane, pH 8.0). The DNA suspensions were then treated with either (i) 2 mg of Pronase (self-digested, 30 min at 37 C at a concentration of 10 mg/ml) for 15 min at 37 C, (ii) 0.3% SDS for 15 min at 30 C, (iii) 300 μg of EB per ml for 15 min at 30 C, or (iv) at 60 C for 15 min followed by quick cooling in an ice bath. The final volume of all reaction mixtures was 400 μl, and all agents used were dissolved in TES buffer. In other reaction mixtures cleared lysates were heated at 60 C for 15 min, quickly cooled, and then treated with either SDS or Pronase as described above. The 0.4-ml suspension containing the treated plasmid DNA was then loaded on top of a neutral sucrose gradient and cosedimented with a 0.4-ml sample of [methyl-^{14}C]-thymidine (specific activity, 50 mCi/mmol)-labeled pI258 DNA as described above. The gradients were then tapped and counted as described above.

Transduction. The method used to transduce a

plasmid determining cadmium resistance from Tox$^+$ strains into a UT0017 recipient is described in a previous paper from this laboratory (10).

RESULTS

Partial elimination of ET activity from strains UT0002 and UT0003. It was previously reported in this laboratory (10) that growth at 44 C was effective in eliminating total detectable ET activity from strains UT0001 and UT0007 but not from strains UT0002 and UT0003. Further investigation, however, indicated that a large proportion of a UT0002 and a UT0003 population underwent twofold and ninefold reductions in ET activity, respectively, after growth at 44 C for 18 h. With strain UT0002, 40 out of 100 treated cells tested lost total Bac activity and 10 out of the 40 Bac$^-$ substrains tested had approximately 1,600 units of ET activity per ml compared with approximately 3,200 units of activity per ml for cells not losing the Bac marker. With strain UT0003 that was Bac$^-$, 5 out of 10 cells recovered after growth at 44 C produced approximately 200 units of ET activity per ml and the remaining cells had levels of ET activity like those of the wild type, which was calculated to be approximately 1,800 units/ml. As a result of these observations it was decided to examine whether partial reduction of ET activity was due to loss of an ET plasmid from the Tox$^+$ strains.

Isolation and characterization of plasmid DNA from strains UT0002 and UT0003. SDS-NaCl lysates from strains UT0002 and UT0003 were centrifuged to equilibrium in CsCl-EB buoyant density gradients, and the denser bands from these gradients were isolated and further analyzed by centrifugation through 5 to 20% neutral sucrose gradients. The penicillinase plasmid, pI258, with an *S* value of 47 and molecular weight of 1.8×10^7, was used as a reference marker. The dense band of CCC DNA isolated from strain UT0002 revealed three rapidly sedimenting bands of DNA in neutral sucrose gradients with *S* values of 56, 38, and 21 (Fig. 1). The 56*S* and 38*S* species most likely represent the CCC and OC forms of an ET plasmid similar to that observed with the ET plasmid isolated from strain UT0101 (12). Alternatively, the dense band of CCC DNA isolated from strain UT0003 contained only the 21*S* molecular species of DNA when analyzed on a neutral sucrose gradient. CCC DNA obtained after dye buoyant density centrifugation of cleared lysates from both strains UT0002 and UT0003 was found to contain only the 21*S* species. These observations were interpreted to indicate that the dye-buoyant density gradient centrifugation was somehow relaxing the 56*S* plasmid and could not be adequately

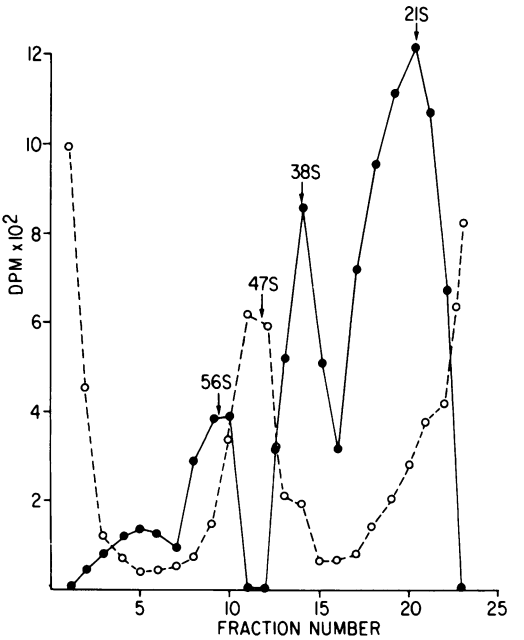

FIG. 1. *Neutral sucrose gradient of plasmid DNA from an SDS-NaCl lysate of [methyl-^{3}H]thymidine-labeled strain UT0002 (●) purified by CsCl-EB density gradient centrifugation. The fractions of the rapidly sedimenting band of DNA were pooled, dialyzed to remove EB and CsCl, and cosedimented with a [methyl-^{14}C]thymidine-labeled pI258 penicillinase plasmid (○) on a neutral 5 to 20% sucrose gradient. Recovery of added counts from the material layered on the gradients was greater than 90%.*

used to study this plasmid species. This conclusion was further supported after observing that cleared lysates from the two Tox$^+$ strains produced only the 56*S* and 21*S* species of DNA after direct centrifugation through neutral sucrose velocity gradients (Fig. 2).

Strain UT0002-19 is a substrain of strain UT0002 (Tox$^+$Bac$^+$) that lost half of its ET activity and total Bac activity after growth at 44 C. Strain UT0003-4 is a substrain of strain UT0003 (Tox$^+$Bac$^-$) that underwent a ninefold reduction of ET activity after growth at 44 C. When cleared lysates from strain UT0002 and its substrain UT0002-19 were mixed and analyzed on neutral sucrose gradients, no extrachromosomal DNA corresponding to either the OC or CCC forms of the ET plasmid was identified from the DNA isolated from strain UT0002-19 (Fig. 3A), but a peak of DNA corresponding in position to the ET plasmid was distinctly observed in cleared lysates made from strain UT0002. Similarly, when mixed cleared lysates from strains UT0003 and UT0003-4 were cosedimented through neutral sucrose, DNA corresponding to the ET plasmid was identified in

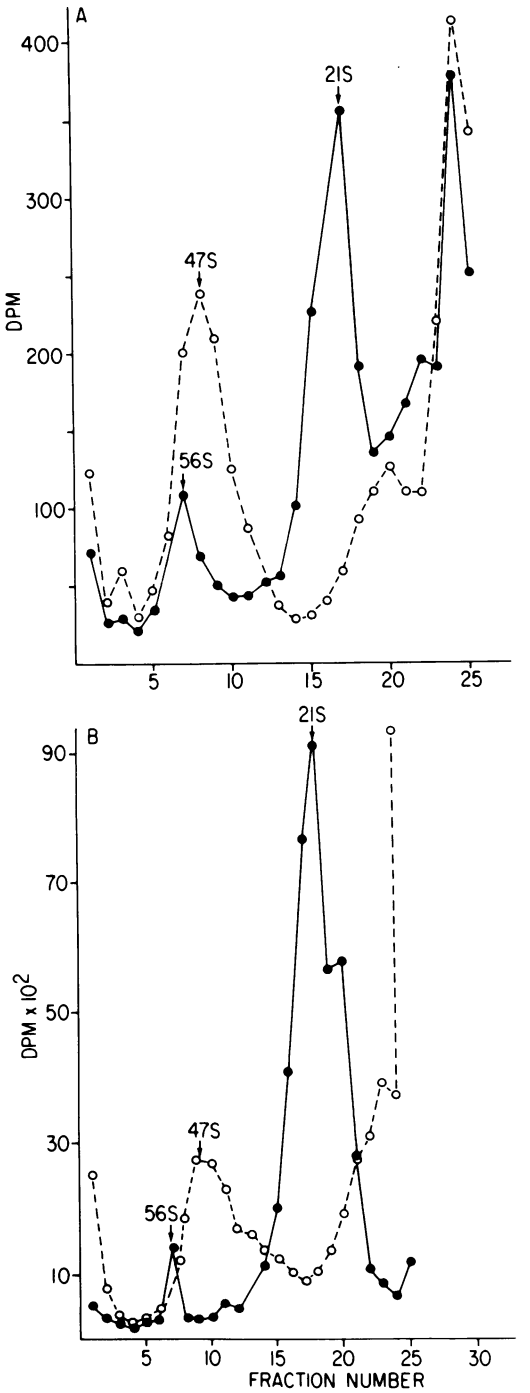

FIG. 2. *Direct neutral sucrose gradient analysis of cleared lysates isolated from (A) [methyl-³H]thymidine-labeled strain UT0002 (●) and (B) [methyl-³H]thymidine-labeled strain UT0003 (●). Both cleared lysates were cosedimented with a [methyl-¹⁴C]thymidine-labeled pI258 penicillinase plasmid (○). Recovery of added counts from the material layered on the gradients was greater than 90%.*

the former but not latter strain (Fig. 3B). These observations indicate that the partial reduction of ET activity in strains UT0002-19 and UT0003-4 could be directly related to a loss of the ET plasmid from these strains.

Conversion of the CCC form of the ET plasmid from strain UT0002 to the OC form. The *56S* form of the ET plasmid from strain UT0002

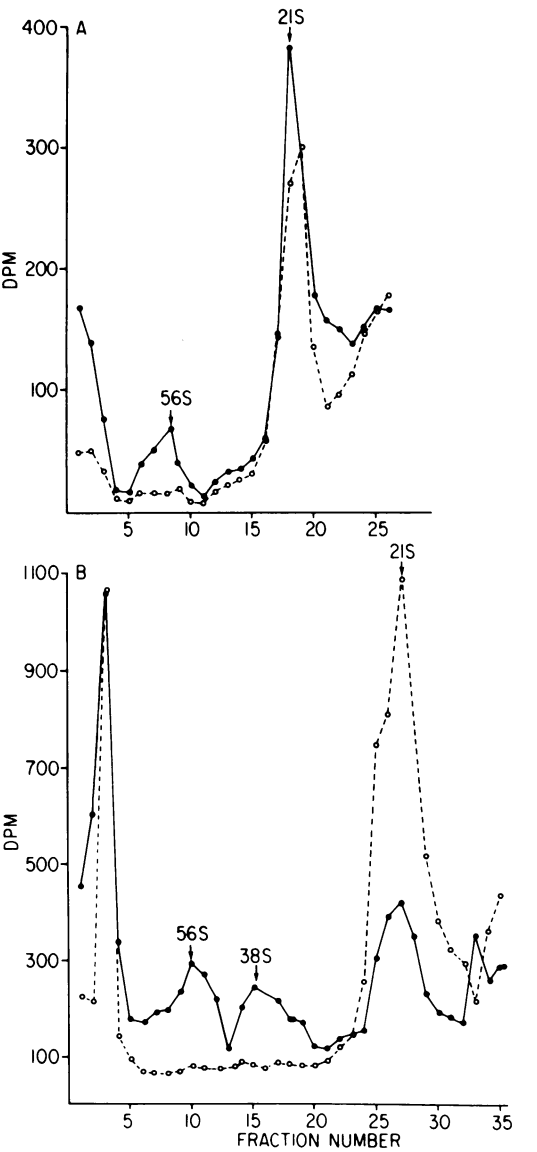

FIG. 3. *Neutral sucrose gradient analyses of mixed cleared lysates of (A) [methyl-³H]thymidine-labeled strain UT0002 (●) and [methyl-¹⁴C]thymidine-labeled strain UT0002-19 (○) and (B) [methyl-³H]thymidine-labeled strain UT0003 (●) and [methyl-¹⁴C]thymidine-labeled strain UT0003-4 (○). Recovery of added counts from the material layered on the gradients was greater than 90%.*

was very unstable in that it relaxed to the 38S form upon isolation (Fig. 1) or during storage. Investigations were therefore pursued to determine whether the ET plasmid behaved like the DNA-protein relaxation complexes studied in *E. coli* (3, 5). Three 0.1-ml aliquots from a freshly prepared cleared lysate from strain UT0002 were immediately treated with either Pronase, at 60 C for 15 min, or at 60 C for 15 min followed by exposure to Pronase and centrifuged through neutral sucrose gradients. Whereas the ratio of 56S DNA to 38S DNA did not change after incubation of the cleared lysate at 60 C (Fig. 4B), a substantial fraction of the 56S species was relaxed after interaction with Pronase (Fig. 4C). Similar results were observed when SDS was used in place of Pronase in that treatment with the detergent but not at 60 C relaxed the 56S species.

The properties of the R28K and Col E2 relaxation complexes from *E. coli* (5) resemble those of the ET plasmid from strain UT0002 in that they relax in the presence of SDS and Pronase but not when exposed to high temperatures. They differ, however, in that the heat-treated R28K and Col E2 complexes become insensitive to relaxation by SDS and Pronase (5), whereas the ET plasmid is still relaxed by Pronase (Fig. 4D) and SDS (Fig. 5) after incubation at 60 C for 20 min.

Since the ET plasmid could not be successfully isolated on CsCl-EB dye-buoyant density gradients, it was decided to test whether EB caused relaxation of the ET plasmid. Treatment

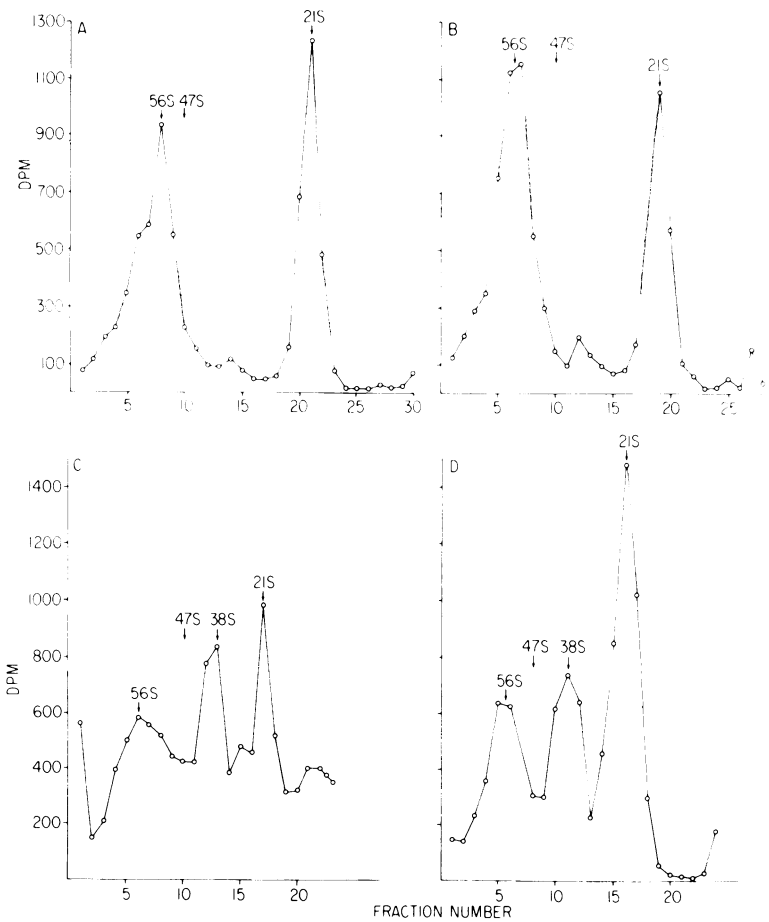

FIG. 4. *Neutral sucrose gradient analysis of a cleared lysate from strain UT0002 after treatment at (A) 37 C for 30 min (control) and at (B) 60 C for 15 min with (C) 2 mg of Pronase for 15 min at 37 C and at (D) 60 C for 15 min followed by incubation in 2 mg of Pronase for 15 min at 37 C. The penicillinase plasmid, pI258, with a sedimentation coefficient of 47S was used as a reference marker. Recovery of added counts from the material layered on the gradients was greater than 90%.*

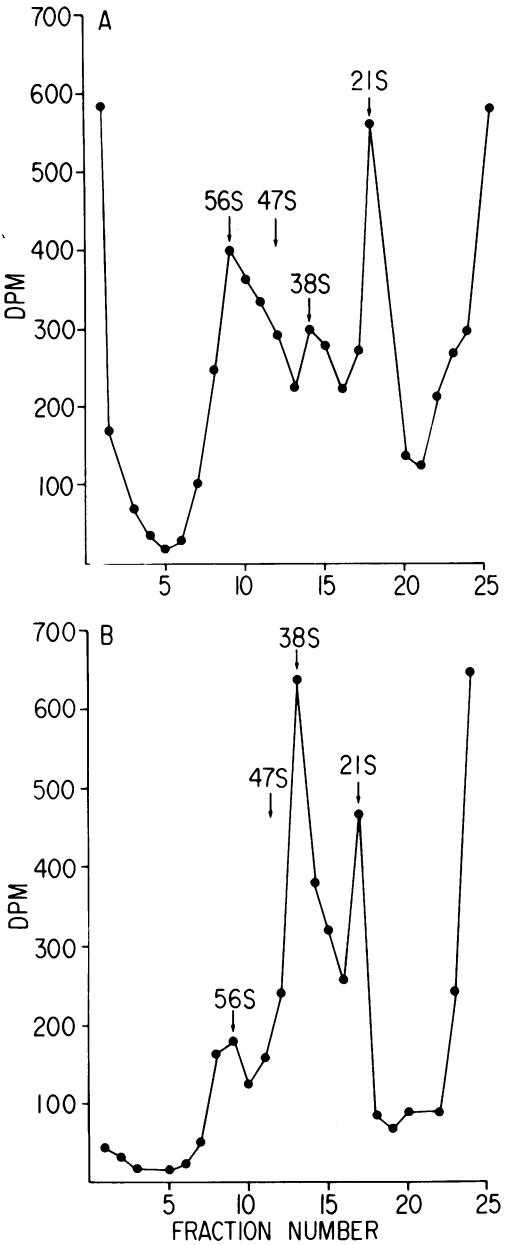

FIG. 5. *Neutral sucrose gradient analysis of a cleared lysate from strain UT0002 after treatment at (A) 30 C for 30 min (control) and at (B) 60 C for 15 min followed by incubation in 0.3% SDS for 15 min at 30 C. The penicillinase plasmid, pI258 (47S), was used as a reference marker. Recovery of added counts from the material layered on the gradients was greater than 90%.*

of a cleared lysate from strain UT0002 with a final concentration of 300 μg of EB per ml distinctly produced a substantial conversion of 56S DNA to the 38S form (Fig. 6). Since conversion of the 21S plasmid was never observed in any of the experiments described above, it is unlikely that a contaminating endonuclease contained in a cleared lysate of strain UT0002 was responsible for nicking the 56S plasmid.

Identification of the 21S species of plasmid DNA in strains UT0002 and UT0003. Neutral sucrose gradient analyses of DNA from strains UT0002 and UT0003 consistently revealed a

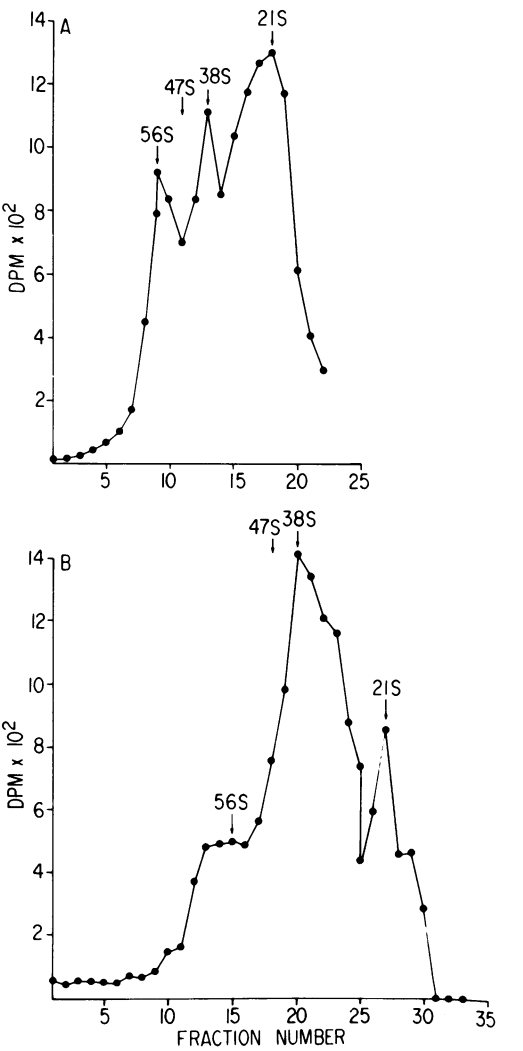

FIG. 6. *Neutral sucrose gradient analysis of a cleared lysate of strain UT0002 after treatment at (A) 30 C for 15 min and with (B) 300 μg of ethidium bromide per ml for 15 min at 30 C. The penicillinase plasmid, pI258 (47S), was used as a reference marker. Recovery of added counts from the material layered on the gradients was greater than 90%.*

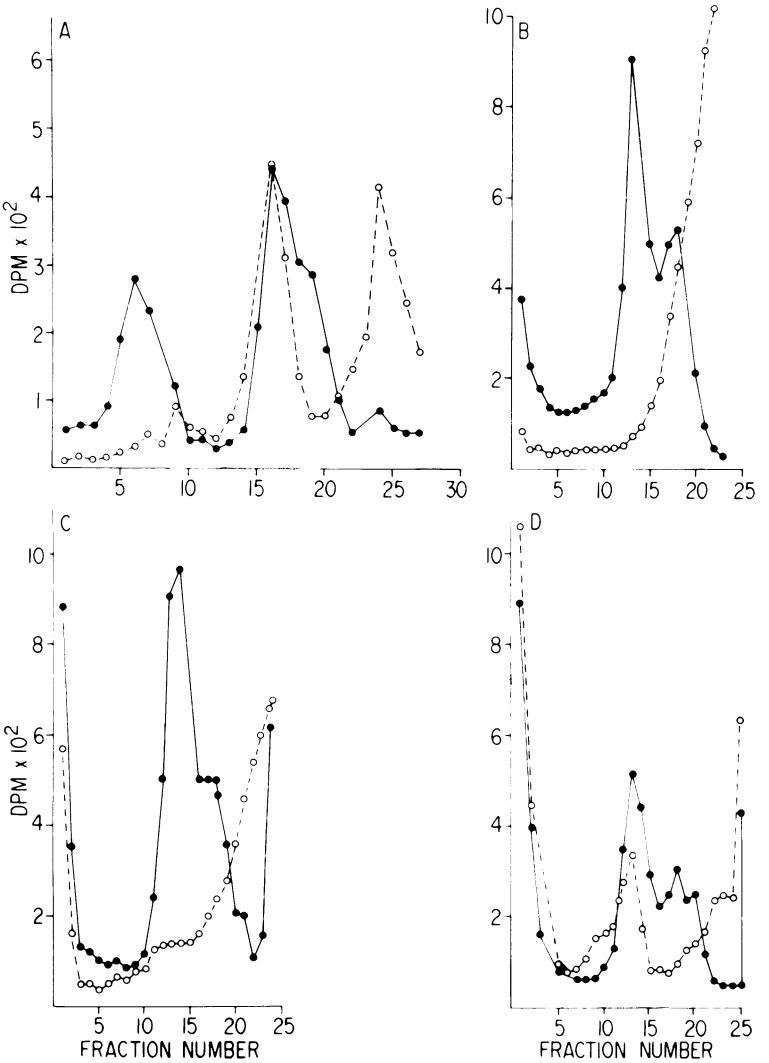

FIG. 7. *Neutral sucrose gradient analysis of cleared lysates from the donor (UT0002), recipient (UT0017), and recombinant (UT0017-T3) strains used for transduction of the 21S plasmid carrying a gene for cadmium resistance. The T_{169} plasmid from strain RN1304 was used as a reference marker. (A) Analysis of mixed cleared lysates of [methyl-³H]thymidine-labeled strain UT0002 (●) and [methyl-¹⁴C]thymidine-labeled strain RN1304 (○) carrying the T_{169} plasmid. (B) Analysis of mixed cleared lysates of [methyl-³H]thymidine-labeled strain RN1304 (●) and [methyl-¹⁴C]thymidine-labeled strain UT0017 (○). (C) Analysis of mixed cleared lysates of [methyl-³H]thymidine-labeled strain UT0017-T3 (●) and [methyl-¹⁴C]thymidine-labeled strain UT0017 (○). (D) Analysis of mixed cleared lysates of [methyl-³H]thymidine-labeled strain UT0017-T3 (●) and [methyl-¹⁴C]thymidine-labeled strain RN1304 (○). Recovery of added counts from the material layered on the gradients was greater than 90%.*

rapidly sedimenting band of DNA with an *S* value of 21. Since substrains of UT0002 and UT0003 cured of the 56*S* plasmid still produced residual ET, it was possible that the 21*S* plasmid contained a gene(s) for ET synthesis. All attempts to cure the 21*S* plasmid from the two Tox⁺ strains failed. In previous investigations from this laboratory, it was shown that strain UT0003 harbored a lysogenic phage capable of transducing an extrachromosomal gene for cadmium resistance into strain UT0017 and strain 04081 recipients (10). To determine whether the 21*S* plasmid was carrying a gene for cadmium resistance, phage lysates were made from strains UT0002 and UT0003 after induction with mitomycin C (10), and the Cad marker carried by these strains was transduced into the cadmium-sensitive strain UT0017 re-

cipient. Transduction frequencies averaged approximately 10 recombinants per 10^9 plaque-forming units per ml. Cleared lysates from donor, recipient, and recombinant strains were analyzed on neutral sucrose gradients. These experiments distinctly elucidated that cadmium resistance was associated with the 21S plasmid in both UT0002 and UT0003. The 21S Cd plasmid from strain UT0002 sedimented at the same rate in neutral sucrose as a plasmid for tetracycline resistance (T_{169}) that had a mass of 2.66 megadaltons (6) (Fig. 7A).

In a neutral sucrose gradient analysis of a cleared lysate of the cadmium-sensitive recipient strain UT0017, no rapidly sedimenting peaks of DNA were detected (Fig. 7B). Alternatively, sucrose gradient analyses of a cleared lysate from a recombinant strain, UT0017-T3, receiving the Cad marker from strain UT0002 showed a heavy DNA band sedimenting at the same rate as the T_{169} plasmid (Fig. 7C, D). The 21S plasmid from strain UT0003 was identical in all respects to the 21S plasmid isolated from strain UT0002. Cadmium-resistant recombinant strains received neither genes for ET synthesis nor genes for pencillin resistance. Pencillin resistance was not associated with either the 56S or 21S plasmids isolated from penicillin-resistant strains UT0002 and UT0003. This evidence supports previous data from our laboratory (12) to indicate that penicillin resistance is a function of chromosomal genes in phage group 2 staphylococci.

DISCUSSION

In previous investigations directed toward eliminating genes for ET synthesis from phage group 2 strains, only 2 (UT0001 and UT0007) out of 12 Tox^+ strains could be cured (10). One of these strains, UT0007, was shown to contain a 56S plasmid with a molecular weight of 3.3×10^7 that controlled the synthesis of ET and Bac (12). This 56S plasmid was unstable in that it spontaneously converted to the OC form (12), and this conversion could be enhanced by treatment with Pronase, SDS, and alkali but not with heat or ribonuclease (R. Warren et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, H95, p. 112).

The present studies indicated that the reason why some Tox^+ strains could not be cured is that they contain both chromosomal and extrachromosomal genes for ET synthesis. This assumption is based on the observation that treatment of Tox^+ strains UT0002 and UT0003 at 44 C yielded substrains that lost both a 56S plasmid and partial ET activity. Alternatively, the genes for Bac synthesis in strain UT0002 seem to be entirely extrachromosomal since loss of the 56S plasmid consistently resulted in total loss of Bac activity. The ET plasmid found in strains UT0002 and UT0003 is probably the same as the one found in strain UT0007 (12) since they both have the same molecular weight and relax after treatment with Pronase and SDS but not heat. The relaxation properties of the ET plasmid are similar to those reported for the *E. coli* R28K and Col E2 complexes with the exception that the staphylococcal molecule remains sensitive to Pronase and SDS after heat treatment. The reason for the difficulty in isolating the ET plasmid on dye-buoyant density gradients could be best explained by EB causing the relaxation of the DNA-protein complex. Since cleared lysates of strains UT0002 and UT0003 show no heavy band of DNA on dye-buoyant density gradients, it is believed that all of the 56S plasmid DNA in these strains exists as a relaxation complex. Therefore one should be aware of the limitations in using dye-buoyant density gradients to analyze specific plasmids when these plasmids have the possibility of being relaxation complexes.

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