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Isolation and Characterization of a Plasmid Involved with Enterotoxin B Production in *Staphylococcus aureus*

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Genetic analysis and molecular characterization of plasmid deoxyribonucleic acid (DNA) was performed in a toxigenic isolate of *Staphylococcus aureus* strain DU4916. Elimination, transduction, and transformation experiments provided us with a series of derivatives similar except for the presence or absence of genes mediating resistance to penicillin (*pen*^{r}), methicillin (*mec*^{r}), and tetracycline (*tet*^{r}) and enterotoxin type B (SEB) production (*entB*$^+$). The derivatives were examined for the presence of a plasmid species which encodes for SEB production. Two distinct species of covalently closed circular DNA of about 2.8×10^6 and 0.75×10^6 daltons were identified in an ethidium bromide-cured, penicillinase-negative (*pen*^{s}) isolate, SN109 (*mec*^{r} *tet*^{r} *emtB*$^+$). Further segregation of either methicillin resistance or tetracycline resistance or of both together resulted in the loss of SEB production and the disappearance of both plasmids. Transduction from strain SN109 showed that determinants for tetracycline resistance are carried by the 2.8×10^6-dalton plasmid. Transformation with covalently closed circular DNA from strain SN109 yielded *mec*^{s} *tet*^{r} *entB*$^-$ transformants harboring the tetracycline resistance plasmid alone and *mec*^{r} *tet*^{r} *entB*$^+$ transformants harboring both the tetracycline resistance and the 0.75×10^6-dalton plasmid. Further segregation of methicillin resistance in transformants was not associated with any change in plasmid DNA. The results indicate that a genetic determinant for SEB production is carried by the 0.75×10^6-dalton plasmid. It is possible, however, that this plasmid cannot be maintained in the host independently from the tetracycline resistance plasmid. Methicillin resistance in the strains examined could not be ascribed to any of the covalently closed circular DNA components resolved in strain DU4916.

Extrachromosomal elements responsible for drug resistance have been found in *Staphylococcus* (12) and many other genera (5, 8). Novick and Bouanchaud (12) used the cleared lysate method followed by cesium chloride-ethidium bromide gradient centrifugation to show the existence of covalently closed circular (CCC) deoxyribonucleic acid (DNA) in drug resistance strains. Dornbusch (7) presented genetic evidence indicating that *Staphylococcus aureus* DU4916 harbors at least two plasmids: one of them is responsible for penicillinase production and resistance to metallic ions, and the other is responsible for methicillin and cephalothin resistance together with enterotoxin type B (SEB) production. Lacey (10) could not demonstrate linkage of methicillin resistance to SEB production in an isolate of strain DU4916. Sjöström et al. (17) suggested chromosomal location for genetic determinants of methicillin resistance.

The present study was undertaken to determine the localization of genetic determinants for SEB production (*entB*$^+$) and the possible linkage of these genes to genes specifying methicillin resistance (*mec*^{r}) and tetracycline resistance (*tet*^{r}) in *S. aureus* DU4916. Genetic analysis was performed by studying coelimination, cotransformation, and cotransduction of these markers and was followed by centrifugal analyses and electron microscopy of plasmid DNA. Our genetic and biophysical evidence suggests the localization of a genetic determinant for SEB production on a separate plasmid of 0.75×10^6 daltons.

MATERIALS AND METHODS

Bacterial strains. The *S. aureus* DU4916 strains employed are listed in Table 1. Strain DU4916 was kindly supplied by H. O. Hallander, University of Uppsala, Sweden. Strains 8325 an 8325-4(ϕ11) were kindly provided by M. Lindberg, University of Uppsala, Uppsala, Sweden.

Phages. Typing phage 29 was kindly supplied by D. Sampolinsky, Staphylococcus Reference Labora-

TABLE 1. *Strains of Staphylococcus aureus used*

Strain[a]	Relevant genetic markers	Derivation[b]
DU4916	*pen*^{r} *mec*^{r} *tet*^{r} *entB*$^+_{60}$[c]	Naturally occurring
SN161	*pen*^{r} *mec*^{r} *tet*^{r} *entB*$^+_{200}$	Single high SEB clone from DU4916
SN6		DU4916 after spontaneous elimination
SN6(ϕ11)		SN6 after lysogenization with phage ϕ11
SN2	*pen*^{r}	SN161 after SDS treatemnt
SN3		SN161 after SDS treatment
SN7		SN161 after ethidium bromide treatment
SN17	*pen*^{r}	SN161 after ethidium bromide treatment
SN109	*mec*^{r} *tet*^{r} *entB*$^+_{200}$	SN161 after ethidium bromide treatment
SN301		SN109 after spontaneous elimination
SN394	*mec*^{r}	SN109 after spontaneous elimination
SN411	*tet*^{r}	SN109 after spontaneous elimination
SN513	(ϕ11)*tet*^{r}	SN6(ϕ11) after transformation with SN109 DNA
SN518	(ϕ11)*tet*^{r} *entB*$^+_{200}$	SN6(ϕ11) after transformation with SN109 DNA
SN521	(ϕ11)*mec*^{r} *tet*^{r} *entB*$^+_{200}$	SN6(ϕ11) after transformation with SN109 DNA

[a] All strains are derivatives of strain DU4916.
[b] All strains except the original DU4916 were derived in our laboratory.
[c] The subscript indicates the level of SEB (micrograms/milliliter) synthesized in overnight cultures.

tory, Israel. Phage ϕ11 was isolated from 8325-4(ϕ11) and propagated on strain 8325. Phage stocks were grown using soft agar overlayer. Lysogens were isolated from turbid centers of spots of phage. Two criteria were employed to detect lysogeny: phage production and immunity to superinfection by ϕ11.

Chemicals and reagents. The suppliers of chemicals were as follows: lysostaphin (Schwarz/Mann, Orangeburg, N. Y.); tris(hydroxymethyl)aminomethane (Tris), ethidium bromide, and Brij 58 (Sigma Chemical Co., St. Louis, Mo.); *n*-dodecylamine (BDH Chemicals, Poole, England); CsCl (Merck, Darmstadt, Germany); polyethyleneglycol 20,000 (Fluka A. G., Switzerland); deoxyadenosine (Calbiochem, Calif.); [*methyl*-^{3}H]thymidine, 12.95 Ci/mmol (Nuclear Research Centre, Negev, Israel; and [2-^{14}C]thymidine, 59 mCi/mmol (Radiochemical Centre, Amersham, England).

Antibiotics. The following antibiotics were used: sodium methicillin, (a gift from Beecham Research Laboratories, Brentford, England); sodium penicillin, (Glaxo, Greenford, England); bacitracin (Teva, Israel); and chlorotetracycline, (Sigma Chemical Co.).

Media. The standard medium (CH medium) for growth of bacteria contained 4% casein hydrolysate (Sheffield), 0.2% K_2HPO_4, 0.005% $MgSO_4 \cdot 7H_2O$, 0.0001% thiamine, and 0.001% nicotinic acid. Plates were solidified with 2.0% agar (Difco). CH broth was used as medium for labeling of cells with [^{3}H]- and [^{14}C]thymidine by the method of Novick and Bouanchaud (12).

Production of SEB. Flasks (100 ml) containing 15 ml of CH broth were inoculated and incubated on a reciprocal shaker at 37°C for 24 h. The cultures were centrifuged, and the supernatant fluid was decanted and stored frozen until assayed. The Oudin method for quantitative determination of SEB was employed (22). SEB-negative supernatants were retested by reversed passive hemagglutination test as describe by Silverman et al. (16). Monovalent anti-SEB serum was prepared according to Silverman (15).

Screening procedure in genetic analysis. Methicillin-resistant (*mec*^{r}) and tetracycline-resistant (*tet*^{r}) colonies were scored on CH plates supplemented with 12.5 μg of methicillin per ml for *mec*^{r} and with 15 μg of tetracycline per ml for *tet*^{r}. Induced penicillinase synthesis by single colonies was detected as described by Baldwin et al. (2). Drug susceptibilities of all strains which appeared to have lost antibiotic resistance were retested by the disk method (6). SEB producer colonies were detected on CH agar supplemented with anti-SEB serum by the halo of precipitate formed. High-level SEB producers were selected by the diameter of halo of precipitate formed around the colonies. The distribution of unselected markers within a class of segregants, transformants, or transductants was determined by replicating from medium on which they were selected on CH plates selective for other marker(s) to be scored.

Plasmid elimination. For spontaneous elimination of antibiotic resistance and SEB production, colonies grown in the presence of antibiotics were inoculated into drug-free CH broth. The cultures were grown at 37°C for 24 h with vigorous shaking. Dilutions were plated on CH agar, incubated overnight, and replica plated to selective CH agar. Plasmid elimination by chemical treatment was performed by the same procedure except for growing cultures overnight in CH broth containing one of the following chemicals: 3.0×10^{-6} M ethidium bromide (3), 0.008% sodium dodecyl sulfate (SDS) (19), or 30 μg of 9-aminoacridine-Lycrochloride (6). The concentrations used were partial inhibitory for growth.

Transformation. The transforming plasmid DNA was isolated from cleared lysate by isopycnic centrifugation (details given below). DNA was assayed spectrophotometrically at 260 nm. The transformation procedure described by Stiffler et al. (21) was followed.

Transduction. Transductions were carried out as

described by Dornbusch et al. (6) using typing phage 29.

Isolation of circular plasmid DNA. The isolation of plasmid DNA from cleared lysates was done essentially by the method described by Clewell and Helinski (4) as modified by Novick and Bouanchaud for staphylococci (12). This procedure lysed only lysostaphin-sensitive strains, whereas most strains tested (Table 1) were lysostaphin insensitive. To improve lysis of lysostaphin-insensitive strains, a modified procedure was used: exponential broth cultures were treated with bacitracin (150 μg/ml) for 5 to 20 min, depending on strain, cooled in an ice bath, and harvested by centrifugation. The cell pellet was resuspended in 0.05 M Tris-2.5 M NaCl-0.05 M ethylenediaminetetraacetic acid (EDTA) (pH 7.0), to give 2% of original culture volume, and lysostaphin was added to 15 μg/ml final concentration. This mixture was diluted with an equal volume of cooled, saturated solution of *n*-dodecylamine preformed at 37°C (11) plus sucrose (40%, wt/vol) in TES buffer (0.05 M NaCl-0.005 M Na_2-EDTA-0.03 M Tris, pH 7.0). The mixture was frozen and kept at −70°C until further treatment. Lysis was achieved after thawing and incubation for 30 min at 37°C with slow shaking, cooling on ice, and addition of an equal volume of 0.05 M Tris containing BRIJ-58 (1%), sodium deoxycholate (0.04%), and 0.3 M EDTA (pH 8.0). The viscous crude lysate formed was centrifuged in 49,000 × *g* for 15 min. The supernatant, designated as cleared lysate, was subjected to centrifugal analyses and electron microscopy. All lysates were kept at −70°C until further use.

Dye-buoyant density equilibrium centrifugation. Cleared lysates (1 ml) were mixed with solid CsCl up to density of 1.54 g/cm³ (refractive index = 1.38470). The remainder of space in the centrifuge tube was filled by CsCl solution in TES at the same density. Stock solution of ethidium bromide in TES was added to 200 μg/ml final concentration. Centrifugation was performed on a Spinco model L3-50 ultracentrifuge in an SW50.1 rotor at 42,000 rpm at 19°C for 38 h. At the end of the run the tubes were observed for two visible colored bands, and the resulting gradient was collected by dripping into tubes or directly onto Whatman 3MM filters. The filters were dried, washed twice in cold (5%) trichloroacetic acid and twice in 96% ethanol, dried, and counted in a Packard liquid scintillation counter.

Electron microscopy. Peak fractions of CsCl-ethidium bromide gradients were prepared for electron microscopy without dialysis using spreading technique as described by Kleinschmidt (9). Electron micrographs were performed with a Jeol Jem 100 B electron microscope at a magnification of ×25,000 in bright field.

Sucrose density gradient. Neutral sucrose gradients (5 to 20%, wt/vol) were prepared in 0.05 M Tris plus 1 × SSC (1 × SSC = 0.15 M NaCl plus 0.015 M Na_3-citrate), pH 7.9. All centrifugations were carried out in an SW50.1 rotor under the conditions indicated. The sedimentation coefficient of plasmid DNA forms was determined by co-sedimentation with plaque-purified ^{14}C-labeled simian virus 40 (1) reference DNA (21*S*) kindly provided by S. Rozenblatt (the Weizmann Institute of Science, Rehovot, Israel).

RESULTS

Coelimination of *entB* and resistance markers. Elimination experiments were performed with strain DU4916 and with an isolate (SN161) from a single high-level SEB-producer clone identified by the large halo of precipitate formed on anti-SEB serum supplemented agar. Ethidium bromide, SDS, and 9-aminoacridine have been employed to eliminate resistance to penicillin, methicillin, and tetracycline. Attempts to detect elimination of *pen*^{r}, *mec*^{r}, and *tet*^{r} markers in strain DU4916 were unsuccessful. Several thousand survivor colonies from treated cultures were screened and showed the antibiotic resistance pattern of the parental strain. However, a spontaneous *pen*^{s} *mec*^{s} *tet*^{s} *entB*$^-$ derivative (SN6) of strain DU4916 was isolated from an *entB*$^-$ colony. Elimination of antibiotic resistance was accomplished in strain SN161 (Table 2). Three classes of "cured" derivatives were detected: *pen*^{r} *mec*^{s} *tet*^{s}, *pen*^{s} *mec*^{r} *tet*^{r}, and *pen*^{s} *mec*^{s} *tet*^{s}. The *entB*$^+$ marker was present in all *mec*^{r} *tet*^{r} derivatives (Table 2) and was lost in the two other classes of cured derivatives. Among the compounds employed, ethidium bromide was the most effective in elimination of antibiotic resistance.

Strain SN109 (*pen*^{s} *mec*^{r} *tet*^{r} *entB*$^+$), isolated from an ethidium bromide-treated culture of SN161, was further studied for spontaneous loss of antibiotic resistance markers. Cultures were grown in drug-free CH medium, and progeny colonies were screened for *mec*^{r}, *tet*^{r}, and *entB*$^+$. The following classes of segregants were detected among 10^4 colonies tested: *mec*^{s} *tet*^{r} *entB*$^-$ (10), *mec*^{r} *tet*^{s} *entB*$^-$ (8), and *mec*^{s} *tet*^{s} *entB*$^-$ (1).

TABLE 2. *Curing in strain SN161 (pen*^{r} *mec*^{r} *tet*^{r} *entB*$^+$*)*

Compound	No. of cured colonies			Total no. of colonies tested
	pen^r *mec*^{s} *tet*^{s} *entB*$^-$	*pen*^{s} *mec*^{r} *tet*^{r} *entB*$^+$	*pen*^{s} *mec*^{s} *tet*^{s} *entB*$^-$	
Ethidium bromide	154 (8.5)[a]	3 (0.16)	85 (4.7)	1,819
SDS	4 (0.18)	3 (0.13)	7 (0.3)	2,256
9-Aminoacridine	1 (0.27)	0	1 (0.27)	372

[a] Numbers in parentheses indicate percentage.

Thus, coelimination of *entB*$^+$ with either *mec*^{r} or *tet*^{r} appeared to indicate interaction, but not necessarily physical linkage, between genetic elements carrying determinants for either methicillin or tetracycline resistance and such an element carrying the determinant for SEB production (see also plasmid DNA analysis of cured derivatives detailed below).

Cotransduction of *entB* and resistance markers. Drug-sensitive and enterotoxin-negative derivatives of strain SN161 were used as recipients in most transduction experiments. Transducing lysates were prepared by growing phage 29 on strains DU4916, SN161, and SN109. In transduction using *pen*^{r} recipients, *tet*^{r} and *entB*$^+$ were cotransduced jointly with *mec*^{r} selection at a frequency of 18 and 2.6% from strains SN161 and DU4916, respectively. Cotransduction of *tet*^{r} alone with *mec*^{r} selection was not observed (Table 3). In transduction from strain SN161, using *pen*^{s} recipients and methicillin selection, penicillin resistance was cotransduced at a frequency of about 35%, and penicillin resistance, tetracycline resistance, and enterotoxin production were jointly cotransduced at a frequency of 4% (Table 3). Higher transduction frequencies were obtained with lysates from strain SN161 than with lysates from strain DU4916 (Table 3). The level of enterotoxin production in transductants corresponded to the level of the respective donor strain. From strain SN109, tetracycline resistance was transduced at a frequency of 3.5×10^{-6}, but neither methicillin resistance nor enterotoxin production were cotransduced with tetracycline selection (Table 3). No transduction was observed from this strain with methicillin selection (Table 3).

Thus, using strains DU4916 and SN161 as donors, *entB*$^+$ and *tet*^{r} were cotransduced jointly with methicillin selection; methicillin resistance was much more frequently transduced either alone or linked to *pen*^{r} than cotransduced jointly with *tet*^{r} and *entB*$^+$. Moreover, transduction of *tet*^{r} alone was observed using strain SN109 as donor. From this series of experiments we concluded that the cotransduction observed is not a manifestation of true genetic linkage between determinants for antibiotic resistance and the determinant for enterotoxin production.

Isolation of plasmid DNA. The following experiments were performed to identify the plasmid species carrying the genetic determinant(s) for enterotoxin production. Since plasmids are present as CCC duplex DNA, cleared lysates of strains DU4916, SN161, SN109, and SN6 were analyzed on CsCl-ethidium bromide gradients (14). Both strains DU4916 and SN161 contained CCC DNA, and no such DNA molecules could be detected in the drug-sensitive, enterotoxin-negative strain SN6. CsCl-ethidium bromide

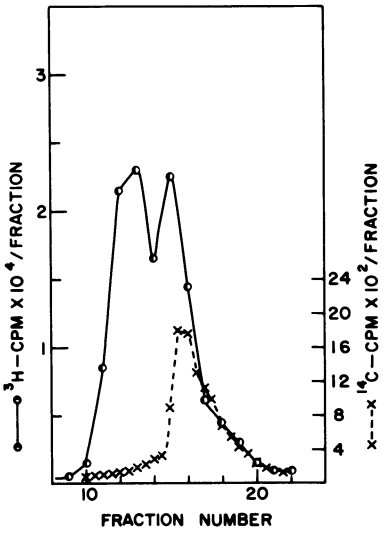

FIG. 1. *Dye-buoyant density gradient analysis of plasmid DNA of strain SN109. Parallel exponential cultures (40 ml) of strain SN109 (pen*^{s} *mec*^{r} *tet*^{r} *entB*$^+$*) and strain SN6 (pen*^{s} *mec*^{s} *tet*^{s} *entB*$^-$*) were labeled with [methyl-*^{3}*H]thymidine (1.0 μCi/ml) and [2-*^{14}*C]thymidine (0.2 μCi/ml), respectively, for 120 min. Strain SN109,* ^{3}*H (●); strain SN6,* ^{14}*C (×). The recovery of culture radioactivity in lysates was 3.96% of* ^{3}*H counts per minute and 1.30% of* ^{14}*C counts per minute, whereas the recovery of radioactivity in density gradients was 3.48 and 0.65% of culture radioactivity, respectively.*

TABLE 3. *Transduction of antibiotic resistance and SEB production*[a]

Donor strain	Recipient strain	Selective marker	Transduction frequency	Cotransduction
DU 4916	SN2	*mec*^{r}	6.0×10^{-7}	*tet*^{r} + *entB*$^+$ (2.6%)
SN161	SN2	*mec*^{r}	4.4×10^{-4}	*tet*^{r} + *entB*$^+$ (18%)
SN161	SN3	*mec*^{r}	2.3×10^{-4}	*pen*^{r} (26%); *pen*^{r} + *tet*^{r} + *entB*$^+$ (4.5%)
SN161	SN7	*mec*^{r}	1.0×10^{-3}	*pen*^{r} (50%); *pen*^{r} + *tet*^{r} + *entB*$^+$ (3.4%)
SN109	SN6	*tet*^{r}	3.5×10^{-6}	<0.05%
SN109	SN6	*mec*^{r}	$<10^{-8}$	

[a] The multiplicities of infection ranged from two to four plaque-forming phage 29.

gradients of cleared lysates of strain SN109 (*pen*^{s} *mec*^{r} *tet*^{r} *entB*$^+$) showed the usual dense peak composed of CCC DNA and a less dense peak containing linear chromosomal DNA and open circular (OC) DNA (Fig. 1).

Cleared lysates were further analyzed by zonal sedimentation in neutral sucrose gradients to isolate individual plasmids. The sedimentation profile of strain SN109 exhibited two distinct peaks with sedimentation coefficients of 21*S* and 14*S* (Fig. 2). Pooled fractions (12–15) from the denser peak on CsCl-ethidium bromide gradients (Fig. 1) were rerun in neutral sucrose gradients. The resedimented SN109 CCC DNA formed two distinct peaks with sedimentation coefficients of 21*S* and 14*S*, respectively. The sedimentation profile of DNA from

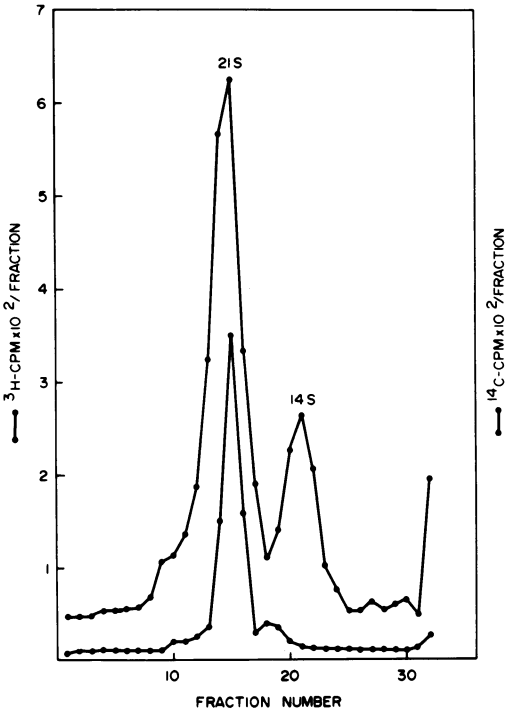

FIG. 2. *Zonal rate sedmientation analysis of plasmid DNA of strain SN109. Cleared lysate was dialyzed against 1× SSC-0.05 M Tris (pH 7.9) and concentrated against polyethyleneglycol 20000 (30%, wt/wt). A 100-μl sample was laid upon a preformed 5-ml linear 5 to 20% (wt/vol) sucrose gradient in 1× SSC-0.05 Tris (pH 7.9). Reference ^{14}C-labeled simian virus 40 CCC DNA was used as internal standard (1). The gradients were centrifuged in a Spinco type SW50.1 rotor at 45000 rpm for 210 min at 4°C. Thirty-two fractions (about 7 drops each) were collected and counted on Whatmann 3MM filters as described. The recovery of ^{3}H in the two plasmid peaks was 2.26% of culture radioactivity. Strain SN109, ^{3}H (◐); simian virus 40 CCC DNA, ^{14}C (●).*

strain SN161 exhibited three distinct peaks, 37*S*, 21*S*, and 14*S* (Fig. 3). The sedimentation profile of strain SN17 (*pen*^{r} *mec*^{s} *tet*^{s} *entB*$^-$) exhibited only one distinct peak, with a sedimentation coefficient of 37*S* (Fig. 3). The 37*S* peak can, therefore, be assigned to the penicillinase plasmid. The *mec*^{s} *tet*^{s} *entB*$^-$ (SN301), *mec*^{r} *tet*^{s} *entB*$^-$ (SN394), and *mec*^{s} *tet*^{r} *entB*$^-$ (SN411) segregants of strain SN109 exhibited no distinct plasmid peaks in either CsCl-ethidium bromide (data not shown) or neutral sucrose gradients (Fig. 3C) of at least three independent cleared lysates from each segregant. This latter finding may indicate that either *tet*^{r} or *mec*^{r} but not *entB*, can be transpositioned to the chromosome.

A *mec*^{s} *tet*^{r} *entB*$^-$ transductant obtained by transducing strain SN6 with phage grown on SN109 lysate contained a single 21*S* peak (data not presented). The 21*S* peak was thus assigned as the tetracycline resistance plasmid (21).

Thus, three distinct plasmid entities were identified by sedimentation analysis of derivatives of strain DU4916: the 37*S* penicillinase plasmid, the 21*S* tetracycline resistance plasmid, and the 14*S* plasmid possibly carrying a genetic determinant for enterotoxin production (see transformation analysis given below).

Electron micrography of plasmids. Peaks of heavy DNA from CsCl-ethidium bromide gradients were prepared for electron micrography. Figure 4 illustrates the two different size plasmids that could be isolated from strain SN109. Contour length measurements of OC molecules are presented in Table 4. The presence of circular molecules of about 2.8×10^6 and 0.75×10^6 daltons was clearly established. A minimal estimate of 16 to 18 plasmid copies for each of the two different size plasmids was calculated (12) from the percentage (2.26) of culture radioactivity that was recovered and the distribution of the label in the 21*S* and 14*S* peaks, respectively (ratio 4:1). Larger forms were observed in SN161 and SN109. These larger molecules were much more abundant in strain SN161 and may represent the penicillinase plasmid. No further attempt was made in this work to characterize this plasmid species.

Plasmid-specific transformation. The transformation experiments were performed by the procedure of Stiffler et al. (21). Strains SN6, SN6(ϕ11), and 8325-4(ϕ11) were used as recipients. CCC DNA from strain SN109 was isolated from the denser peak on CsCl-ethidium bromide gradients. No transformation occurred with *mec*^{r} selection. Transformation occurred when *tet*^{r} was selected (Table 5). As expected (17), lysogenization of strain SN6 with phage ϕ11 enhanced transformation. In this strain

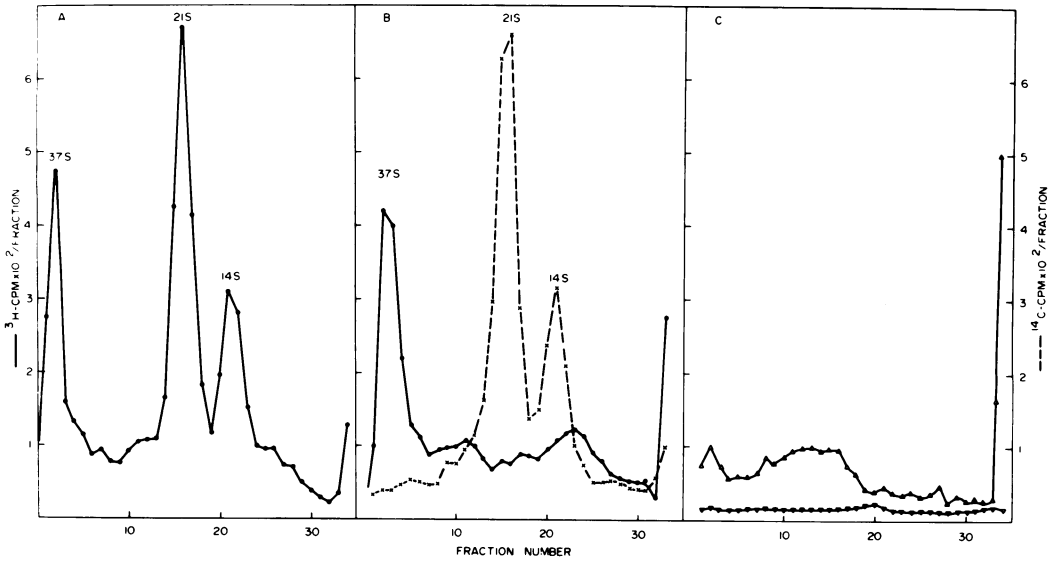

FIG. 3. *Zonal rate sedimentation analyses of plasmid DNA of cured strains. Cleared lysates (^{3}H-labeled) were prepared and centrifuged for 160 min as described in the legend to Fig. 2. Reference ^{14}C-labeled DNA of strain SN109 lysate (×) was used an internal standard. Counts were normalized to culture label. (A) Strain SN161 (●); (B) strain SN17 (◑); (C) strain SN394 (△); strains SN301 and SN411 (▽). The latter two strains were centrifuged in parallel and showed similar distribution of radioactivity in the gradients.*

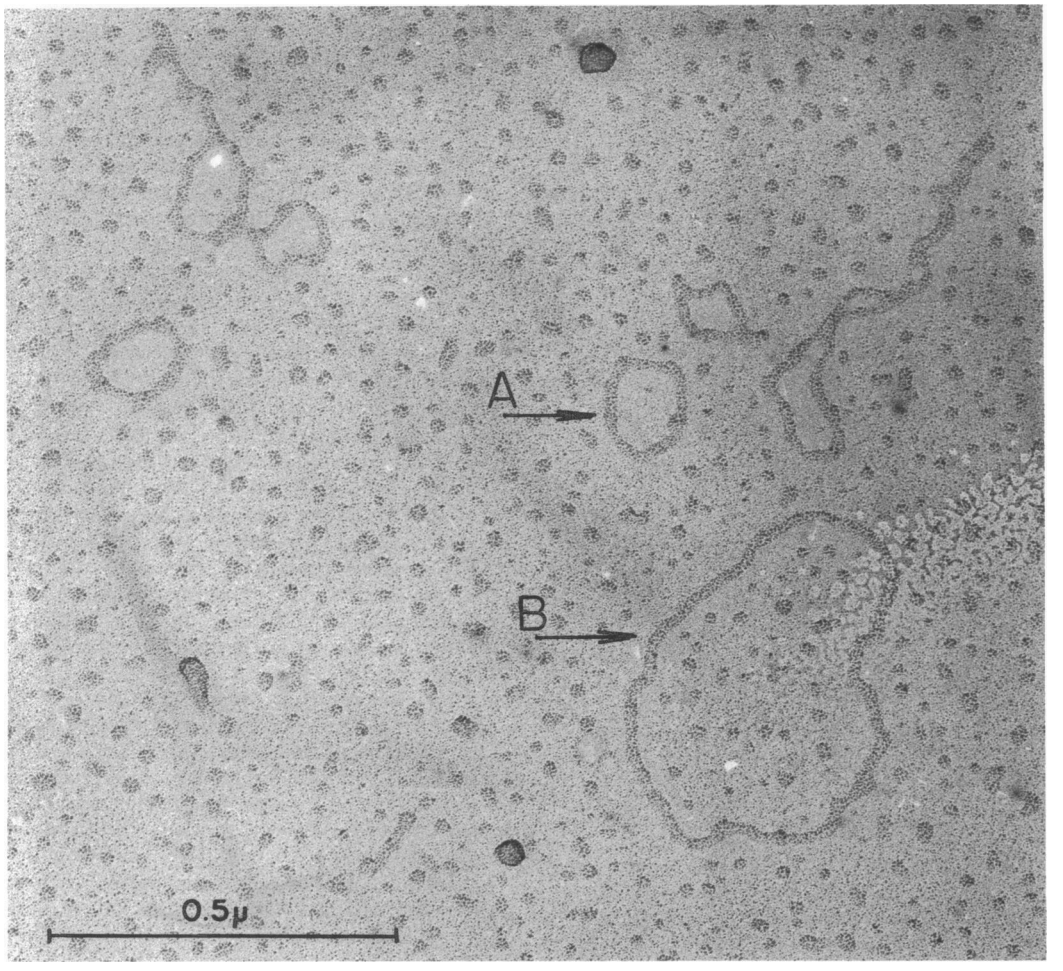

FIG. 4. *Electron micrographs of plasmid DNA. Fractions 13–15 described in the legend to Fig. 1 and peak fractions of a parallel gradient of strain SN109 were prepared by the spreading method of Kleinschmidt (9). (A) Open circles of the small plasmid; (B) open circles of the large plasmid.*

TABLE 4. *Plasmid contour length*

Origin of plasmid DNA	Designation of molecules	OC molecules measured[a]/total screened[b]	Mean contour length (μm)	Mol wt[c] (×10⁻⁶)
SN161 (*pen*^{r} *mec*^{r} *tet*^{r} *entB*$^+$)	Large form	5/68	1.43 ± 0.05	2.72
	Small form	8/63	0.36 ± 0.05	0.69
SN109 (*pen*^{s} *mec*^{r} *tet*^{r} *entB*$^+$)	Large form	8/63	1.55 ± 0.05	2.95
	Small form	13/47	0.42 ± 0.05	0.80

[a] The negatives of electron micrographs were projected, and molecular contours were drawn on paper sheets. OC molecules were measured by a map-measuring device.
[b] Number of both CCC and OC molecules screened.
[c] Tentative molecular weights were calculated from mass to length ratio of 1.91 × 10⁶ daltons/μm (12).

TABLE 5. *Transformation with CCC DNA from strain SN109*

Recipient	CFU/ml[a]	DNA (μg/ml)	No. of *tet*^{r} transformants/ml	No. of cotransformants/ml	
				entB$^+$	*mec*^{r} + *entB*$^+$
SN6	1.6 × 10⁸	1	110	0	0
SN6(φ11)	1.9 × 10⁹	1	420	0	8 (1.9%)
8325-4(φ11)	1.3 × 10⁸	1	120	0	0
	1.3 × 10⁹	10	2,500	10 (0.4%)[b]	0

[a] CFU, Colony-forming units.
[b] Unstable character that was lost during reisolation.

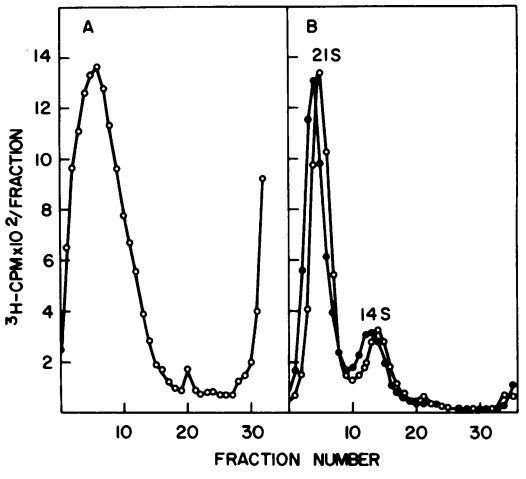

FIG. 5. *Zonal rate sedimentation analysis of plasmid DNA of entB*$^+$ *and entB*$^-$ *transformants. Cleared lysates were prepared and sedimentation in 5 to 20% neutral sucrose gradients, performed as described in the legend to Fig. 2. The counts were normalized for label incorporation by cultures. (A) Strain SN513* (○); *(B) strain SN518* (○) *and strain SN521 (superimposed)* (●).

joint cotransformation of *mec*^{r} and *entB*$^+$ (1.9%) with *tet*^{r} selection was observed (Table 5). A *mec*^{r} *tet*^{r} *entB*$^+$ transformant was unstable and segregated methicillin-sensitive progeny. A single stable *mec*^{s} *tet*^{r} *entB*$^+$ clone (SN518) was isolated. Cotransformation of *entB*$^+$ with *tet*^{r} selection was observed in 8325-4(φ11). However, these transformants were unstable and lost the *entB*$^+$ character.

Analysis of transformants for circular duplex DNA. Cleared lysates of one representative strain for each stable transformant type were subjected to zonal sedimentation in neutral sucrose gradients (Fig. 5). Transformant SN513 (*mec*^{s} *tet*^{r} *entB*$^-$) exhibited one distinct 21*S* peak corresponding to the tetracycline plasmid of the SN109 donor. Transformant SN521 (*mec*^{r} *tet*^{r} *entB*$^+$) exhibited two distinct peaks with sedimentation coefficients of 21*S* and 14*S*. Segregant SN518, which lost methicillin resistance, exhibited a sedimentation profile indistinguishable from that of transformant SN521.

The results of transformation analysis indicated that the 14*S* plasmid, having a molecular weight of 0.75 × 10⁶, carries a genetic determinant for SEB. No distinct plasmid carrying determinants for methicillin resistance could be identified.

DISCUSSION

The present work was undertaken to detect plasmid localization of the genetic determinant(s) for SEB production in *S. aureus*. Based on results of coelimination and cotransduction, Dornbusch (7) suggested that SEB, methicillin resistance, and β-haemolysin in *S. aureus* DU4916 may be plasmid linked. Results of our coelimination experiments suggest association of the SEB gene(s) with both methicillin and tetracycline resistance. Zonal rate centrifugation of plasmid DNA from a DU4916 isolate showed three different size CCC DNA entities with sedimentation coefficients of 37*S*, 21*S*, and 14*S*, respectively. Sedimentation profiles of

plasmid DNA from cured derivatives, transformants, and transductants identified the 37*S* and 21*S* entities as penicillinase and tetracycline plasmids, respectively. Cotransformation to *mec*^{r}-*tet*^{r}-*entB*$^+$ with SN109 (*pen*^{s} *mec*^{r} *tet*^{r} *entB*$^+$) plasmid DNA resulted in the appearance of 21*S* and 14*S* plasmids. Isolation of a *mec*^{r} *tet*^{r} *entB*$^+$ transformant which segregated the *mec*^{r} character enabled us to show that methicillin resistance is not stably associated with the 21*S* or 14*S* plasmids.

No direct chemical evidence for CCC or OC DNA carrying genes for SEB production has so far been published. No attention was given by other authors to the 14*S* plasmid present in DU4916 (17), but according to our results it may carry a genetic determinant for enterotoxin production. A minimal estimate of 16 to 18 plasmid copies per chromosome was calculated (12) for each the tetracycline and the suggested SEB plasmid. The 14*S* plasmid, having a molecular weight of 0.75×10^6, can accommodate only one or two genes (12). There is no evidence available at present indicating whether it carries the structural gene for the SEB polypeptide or some other gene essential for production of this toxin. Recent experiments in our laboratory (unpublished) suggest that the 14*S* plasmid is transcribed into mRNA in the late-stationary phase of growth, as shown by ribonucleic acid-DNA hybridization. Our finding that the disappearance of 2.8×10^6-dalton plasmid DNA resulted in the loss of enterotoxin production may indicate that the tetracycline plasmid is essential for efficient maintenance of the 14*S* SEB plasmid. Since *entB*$^+$ cannot be used as selective marker in transduction or transformation, positive evidence for this assumption is not available at present.

The nature of the genetic element carrying the determinant for methicillin resistance in *S. aureus* is still in dispute (6, 10, 17). In this work methicillin resistance could not be ascribed to one of the CCC DNA components resolved in strain DU4916. Similar results were obtained by other authors (17, 20). Our results are indicative of a transient association of the *mec*^{r} gene(s) with the tetracycline plasmid in *S. aureus* DU4916, and therefore the genetic element carrying determinants for methicillin resistance may be defined as a "pseudoplasmid" according to criteria suggested by Novick et al. (13).

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