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Genetics of Staphylococcal Enterotoxin B in Methicillin-Resistant Isolates of *Staphylococcus aureus*†

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Biophysical and genetic analysis of staphylococcal enterotoxin B (SEB) synthesis in 16 methicillin-resistant (Mec^r) *Staphylococcus aureus* isolates demonstrated that the toxin gene (*entB*) can occupy either a plasmid or chromosomal locus. Biophysical analysis of the plasmid deoxyribonucleic acid content of these strains by agarose gel electrophoresis revealed the presence of a 1.15-megadalton plasmid in six isolates that appears to contain the *entB* gene. Genetic manipulation of SEB synthesis by transduction and elimination procedures demonstrated that this plasmid is critical for enterotoxigenesis. Nevertheless, the majority of the Mec^r SEB$^+$ isolates (62.5%) analyzed in this investigation were found to lack the 1.15-megadalton plasmid. In at least two of these strains (COL and 57-dk), transduction and elimination procedures showed that *entB* was chromosomal. Genetic studies involving strains harboring either a plasmid or chromosomal *entB* gene demonstrated that toxin synthesis was coeliminated with *mec*. However, analysis of the *entB* and *mec* loci by transformation or transduction showed that the genes are not closely linked. On the other hand, transduction of *entB*, regardless of the donor, was observed when both *mec* and the Tc^r plasmid were jointly cotransduced. This finding suggests that, during transduction, a transient association between *entB*, *mec*, and the Tc^r plasmid may exist.

Studies dealing with the genetics of staphylococcal enterotoxin B (SEB) biosynthesis indicate that the toxin gene (*entB*) may be either plasmid or chromosomal (3, 4, 17, 19). Dornbusch et al. (3, 4) studied the genetics of SEB synthesis and reported that, in the methicillin-resistant (Mec^r) isolate *Staphylococcus aureus* DU-4916, the *entB* and *mec* determinants were cotransduced and coeliminated at frequencies suggestive of linkage on a small plasmid. As a result, the *mec* gene became the subject of intense investigation, and the cumulative data have unambiguously shown that this determinant is chromosomal (6, 8, 19, 20), maps within *pyr-his-nov-pur-mec* linkage group (8), and exhibits properties of a pseudoplasmid (19, 20, 23).

The *entB* gene was not included in those investigations but, using the Mec^r strain of Dornbusch et al., Shalita and co-workers (19) reinvestigated the genetics of SEB synthesis. They provided biochemical and genetic data which strongly implicated the involvement of a small plasmid (pentB) in SEB production. Whether this plasmid contains the *entB* structural gene or some determinant critical for enterotoxigenesis was not determined, although the latter seems an unlikely alternative because of the complete correlation observed.

We (17) have been able to confirm the data of Shalita et al. (19) in the Mec^r strain DU-4916, but in five methicillin-sensitive strains we were unable to detect the presumptive SEB miniplasmid. Genetic analysis of the plasmid deoxyribonucleic acid (DNA) in these Mec^s strains failed to reveal a plasmid *entB* locus, and SEB synthesis was shown to be directed by chromosomal determinants.

This study of the *entB* gene was undertaken to further clarify the genetic status of SEB synthesis. Specifically, we have been concerned with the disposition of *entB* in *mec* isolates because of the reported genetic linkage of these two determinants (3, 4). Additionally, we have also been concerned with the frequency with which pentB is present among enterotoxigenic Mec^r isolates. The results presented in this communication demonstrate that the toxin determinant in Mec^r isolates can be either plasmid or chromosomally borne but, in either case, is not physically linked to the *mec* gene as previously suggested (3, 4). However, transduction results suggest a transient association of *entB* with the *mec* gene and a tetracycline resistance plasmid.

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MATERIALS AND METHODS

Organisms. The *S. aureus* strains employed in this investigation are listed in Tables 1 and 2.

Media. For enterotoxin analysis, the various strains were grown in 3% NP broth (5). Trypticase soy broth (TSB; BBL Microbiology Systems, Cockeysville, Md.) was used for routine culture media, phage propagation, and as the growth media in ethidium bromide curing experiments (17, 18). When agar plates were required, 1.5% agar (Difco Laboratories, Detroit, Mich.) was added to either TSB or NP broth.

Marker analysis. Cadmium nitrate [$Cd(NO_3)_2$] sensitivity was determined as previously described (17, 21). Antibiotic sensitivity was determined as described by Dornbusch et al. (3, 4), with the Mec^r phenotype scored at 30°C or at 37°C in the presence of 5% NaCl.

Enterotoxin B production and assay. Conditions for maximal enterotoxin synthesis have been described (5). Analysis of SEB production was performed by Laurell immunoelectrophoresis (5) or gel double diffusion (15). When necessary, supernatants were concentrated by pervaporation. Monospecific antiserum was prepared in New Zealand white albino rabbits using highly purified SEB (kindly provided by R. Bennett, U. S. Food and Drug Administration, Washington, D.C.). SEB production by individual colonies was analyzed by replica plating onto NP agar containing a 1:40 dilution of anti-SEB serum or by Elek plate (1) analysis. Normal rabbit serum controls were also run to distinguish nonspecific immunoprecipitation due to protein A interaction.

Genetic manipulations. (i) Transduction. All transduction experiments were performed with phage 29 of the International Typing Series that were plaque purified on the donor strain. Transducing lysates were prepared on the appropriate donor strains and sterilized as previously described (16, 17). The phage dilution buffer was used according to Novick (13). Transduction was carried out (3, 4, 17) with multiplicities of infection ranging from 0.1 to 1.0. The recipient strain for all transduction experiments was *S. aureus* 8325-4(ϕ11) or derivatives of this strain. After 2 h of preincubation at 37°C or 4 h at 30°C, transductants were selected by the antibiotic soft agar overlay procedure of Sjöström et al. (20). The concentrations of the selective agents were as follows: cadmium nitrate, 25 μg/ml; tetracycline (Tc), 5 μg/ml; methicillin (Mec), 5 μg/ml; novobiocin (Nov), 10 μg/ml; and penicillin G (Pc), 10 μg/ml. Further incubation was carried out at 37 or 30°C for 48 to 72 h before scoring for transductants. The transduced phenotype was confirmed by replica plating onto Trypticase soy agar (TSA) plates containing the appropriate selective agent. Uninfected and phage sterility controls were always included.

(ii) Transformation. Transforming chromosomal DNA in sterile 0.1× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) was prepared as described by Sjöström et al. (20) and used for *mec* transformation (8, 20). DNA concentrations were determined by ultraviolet absorbance at 260 nm. Controls consisted of individually plating the transforming DNA and untreated cells onto selective agar plates. Incubation of transforming DNA with deoxyribonuclease (20) resulted in the complete loss of transforming activity.

(iii) Marker elimination. Ethidium bromide curing experiments were performed as previously described (17, 18). Spontaneous elimination of a particular marker was determined by plating approximately 100 colony-forming units per plate (after sonication to disrupt clumps) onto TSA and then replica plating onto selective or antiserum agar.

The spontaneous elimination of either the Mec^r or SEB$^+$ phenotype was not observed in strains DU-4916, 592, COL, or 57-dk when even 500 colonies grown on TSA plates were replica plated to methicillin and antibody agar plates. Therefore, the isolation of the negative variants was due to the elimination procedures used.

Analysis of plasmid DNA. The *S. aureus* strains were propagated in 50 ml of TSB at 37°C for 24 h, and cleared lysates were produced (14, 17). The volume of the cleared lysates was doubled with chloroform-isoamyl alcohol (24:1), and multiple extractions were carried out by shaking at 37°C for 5 min followed by 30 min at 4°C. The aqueous phase was collected after centrifugation at 5,000 × *g* for 30 min at 4°C. A clear aqueous phase was generally present after two to three extractions. Generally, ribonuclease treatment (7, 12) of the lysates was not necessary. After the final extraction, two volumes of cold 95% ethanol were added and the cleared lysate was stored at −20°C for at least 4 h to precipitate plasmid DNA. The DNA precipitates were collected by centrifugation at 12,000 × *g* for 20 min at −10°C. The ethanol was carefully drained, and the pelleted material was gently redissolved in 0.2 ml of 0.03 M tris(hydroxymethyl)aminomethane–0.0025 M ethylenediaminetetraacetic acid–0.05 M NaCl (pH 8.0).

Plasmid DNA species from cleared lysates were analyzed by horizontal gel electrophoresis. For electrophoresis, 1% agarose gels (Seakem HGT Agarose, Marine Colloids) were run at 60 V and 28 mA. The electrophoretic run lasted 4.5 h or until the tracking dye (12) reached the end of the gel. The electrophoresis buffer consisted of 0.089 M tris(hydroxymethyl)aminomethane, 0.089 M boric acid, and 0.0025 M ethylenediaminetetraacetic acid. Generally, 10 to 50 μl of the plasmid DNA solutions was analyzed. After electrophoresis, the gels were stained with a 0.5-μg/ml solution of ethidium bromide for 30 min and photographed over a short-wave ultraviolet transilluminator using Polaroid type 55 P/N film. The electrophoretic position of each plasmid species in the agarose gels was checked with CsCl-ethidium bromide density gradient-purified plasmid DNA (12, 17). To purify individual plasmid species, preparative gel electrophoresis was carried out by placing an entire cleared lysate in a trough in the gel and subjecting it to electrophoresis under the same conditions as the analytical technique. The desired plasmid band was cut from the gel and electrophoretically eluted into dialysis sacs. The purified plasmid preparation was then dialyzed against electrophoresis running buffer for 24 h, precipitated with ethanol, redissolved in 0.03 M tris(hydroxymethyl)aminomethane–0.0025 M eth-

TABLE 1. *Designation, phenotype, and origin of S. aureus test strains*

Strain	Relevant phenotype[a]	Origin
DU-4916	Pc^r, Tc^r, Mec^r, $SEB^{+(50)b}$	S. Cohen
DU-4916S	Pc^s, Tc^r, Mec^s, SEB^-	S. Cohen
COL	Tc^r, Mec^r, $SEB^{+(12.5)b}$	B. Wilkinson
57-dk	Pc^r, Tc^r, Mec^r, $SEB^{+(30)b}$	B. Wilkinson
592	Pc^r, Tc^r, Mec^r, $SEB^{+(25)b}$	B. Wilkinson
8325-4 (ϕ11)	Mec^s, SEB^-	P. A. Pattee
ISP2	Nov^r, Mec^s, SEB^-	P. A. Pattee
RN 492	Pc^r, SEB^-	R. Novick
8325-4 (pC194)	Cm^r, SEB^-	E. Lederberg; PRC[c]
8325-4 (pT169)	Tc^r, SEB^-	E. Lederberg; PRC[c]

[a] Marker abbreviations are as follows: Pc^r (penicillin resistance), Tc^r (tetracycline resistance), Mec^r (methicillin resistance), SEB^+ (production of enterotoxin B), Nov^r (novobicin resistance), Cm^r (chloramphenicol resistance).

[b] Expressed as micrograms per milliliter of SEB produced by 36-h shake cultures as determined by Laurell immunoelectrophoresis (see text).

[c] PRC, Plasmid Reference Center, Stanford, Calif.

ylenediaminetetraacetic acid–0.05 M NaCl (pH 8.0), and stored at −20°C until used.

RESULTS

The *entB* gene is a chromosomal determinant in all of the Mec^s SEB^+ isolates of *S. aureus* we have so far examined (17). On the other hand, the Mec^r SEB^+ isolate, DU-4916, has been shown by Shalita et al. (19) and verified by us (17) to possess a small plasmid that apparently contains the *entB* gene. Since enterotoxigenicity is characteristic of most Mec^r strains (10), we wished to determine if the plasmid *entB* genotype was characteristic of Mec^r SEB^+ strains. Cleared lysates of 16 such strains were produced and subjected to agarose gel electrophoresis. In Fig. 1, an agarose gel profile of the plasmid DNA from four test Mec^r SEB^+ isolates (592, COL, DU-4916, and 57-dk) is presented. The banding patterns of the pentB plasmid (1.15 megadaltons [Mdal]), isolated and purified from strain DU-4916 by preparative gel electrophoresis, and of chromosomal DNA from the plasmid-negative strain 8325-4(ϕ11) are also presented. Strains DU-4916 and 592 were found to contain a plasmid species migrating at 1.15 Mdal (Fig. 1). These two strains were also found to contain a 17.5- and a 3.0-Mdal plasmid responsible for Pc^r and Tc^r, respectively (Fig. 1). Molecular weights of the plasmid species were determined by the linear migration of reference staphylococcal plasmids (Fig. 2). Strain COL contains only a single 3.0-Mdal plasmid which was shown to encode for Tc^r, whereas strain 57-dk harbors a 3.0-Mdal Tc^r plasmid and a 17.5-Mdal Pc^r plasmid. To ascertain the frequency at which the pentB plasmid is present among such isolates, we extended this analysis to examine the plasmid DNA profile of 12 additional strains. The plasmid DNA content of all 16 Mec^r SEB^+ isolates is presented in Table 2. Of 16 isolates examined, only 37.5% (6/16) contained the pentB plasmid. Each of these six also possessed a 3.0-Mdal Tc^r plasmid and a 17.5- to 22.4-Mdal Pc^r plasmid. The remaining strains possessed a varying array of similar plasmid species.

Since agarose gel electrophoresis of cleared lysate DNA resolves not only covalently closed circular DNA, but also the open circular and linear plasmid forms as well as contaminating chromosomal DNA (12), it was necessary to purify bulk covalently closed circular DNA to verify the band positions of the individual plasmids. This was accomplished by cesium chloride-ethidium bromide equilibrium density gradient centrifugation of [^{3}H]thymidine-labeled cleared lysates (14, 17). The covalently closed circular DNA peak was collected and electrophoresed in 1% agarose gels. The results (not presented) were identical to those already discussed.

The limited distribution of pentB among these Mec^r cultures led us to question the genetic configuration of SEB in toxin-positive strains

TABLE 2. *Plasmid DNA profile of Mec^r SEB^+ strains*[a]

Strain	Mol wt of plasmids[b]	SEB synthesis[c]
DU-4916	17.5 (Pc^r), 3 (Tc^r), 1.15 (SEB^+)	50
592	17.5 (Pc^r), 3 (Tc^r), 1.15 (SEB^+)	25
Kasanjian	22.4, 3.1	30
57-dk	17.5 (Pc^r), 3.0 (Tc^r)	23
5814R[d]	22.4 (Pc^r), 1.8 (Cm^r)	1.5
Meuse	17.5, 3.1	22
Japan	20, 3	23
Dumas	20, 3, 1.15	21.6
5106R	20, 3	16.7
7074R	20, 3, 1.15	11.9
5619	20, 3, 1.15	14.3
69129	20, 3, 1.15	15.5
COL	3 (Tc^r)	12.5
456-33	20	18
5205-R	20	3.4
639-45I	20	10.9

[a] All strains except for DU-4916 were supplied by B. Wilkinson.

[b] Expressed in megadaltons as determined by agarose gel electrophoresis.

[c] Expressed as micrograms per milliliter as determined by Laurell immunoelectrophoresis.

[d] A Mec^s SEB^- derivative (5814S), obtained from B. Wilkinson, was found to contain an identical plasmid profile.

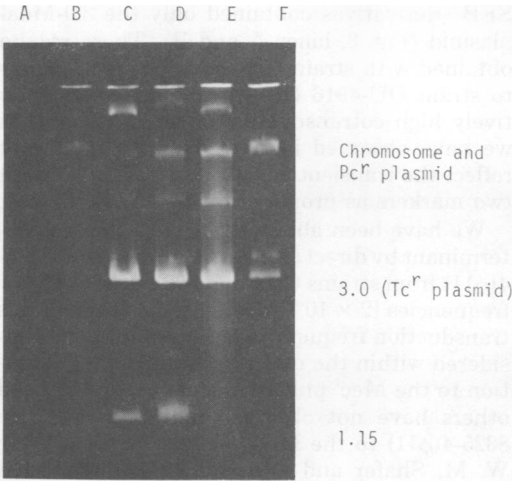

FIG. 1. *Agarose gel electrophoresis of ethanol-precipitated cleared lysate DNA from SEB⁺ isolates, strain 8325-4(φ11), and purified SEB plasmid. (A) Electrophoretically purified SEB plasmid; (B) chromosomal DNA from S. aureus 8325-4(φ11); (C) S. aureus DU-4916; (D) S. aureus 592; (E) S. aureus 57-dk; and (F) S. aureus COL. Bands migrating behind the chromosomal and Pc^r plasmid DNA represent the open circular form of the Pc^r plasmid, whereas bands migrating between the Pc^r and Tc^r plasmids represent the open circular form of the Tc^r plasmid. The position of the covalently closed circular form of each plasmid was confirmed by electrophoresis of cesium chloride-ethidium bromide-purified covalently closed circular DNA.*

lacking pentB. Therefore, we analyzed strains 592 and COL. Strain 592 was chosen as a plasmid control because it contained pentB, whereas COL was selected because it did not contain this small plasmid but only a single 3.0-Mdal Tc^r plasmid. Further, analysis of 592 would provide genetic evidence of pentB in a second strain in addition to DU-4916 (17, 19).

Previous genetic manipulation of SEB⁺ strains has demonstrated that the *entB* gene is not physically linked to plasmids encoding for resistance to cadmium, penicillin, or tetracycline (3, 4, 9, 17, 19). However, cotransduction of *entB* with the Tc^r plasmid and the *mec* gene from strain DU-4916 has been observed (3, 4, 19). Therefore, as a strategy in genetic evaluation of *entB* linkages, we employed transduction of the plasmid Tc^r marker and analyzed for cotransduction of *mec* and *entB*. Phage 29 was propagated on strains COL and 592, and the sterile lysates were used to transduce strain 8325-4(φ11) for resistance to tetracycline. The transduction frequency of the Tc^r marker from strain COL was 4.6×10^{-6} and that from strain 592 was 2.4×10^{-6}. As a control to rule out linkage of *entB* with the Tc^r plasmid, 10 randomly selected 8325-4(φ11) Tc^r Mec^s transductants from each donor were propagated in NP broth for 36 h and the spent medium was analyzed for SEB. Additionally, analysis of 25× concentrated supernatants from the Tc^r Mec^s transductants was performed. The data demonstrated that none of the transductants synthesized SEB whereas the donor strains COL and 592 produced 12.5 and 25 μg/ml, respectively. Plasmid DNA analysis of the Tc^r Mec^s transductants by agarose gel electrophoresis of cleared lysate preparations from either donor showed that the selected clones contained only the 3.0-Mdal plasmid (Fig. 3, lanes B and C). These results demonstrate that the *entB* gene is not linked to the 3.0-Mdal Tc^r plasmid in either strain. These data are consistent with our earlier findings (17) in various Mec^s SEB⁺ isolates. Moreover, since the 3.0-Mdal Tc^r plasmid is the only extrachromosomal DNA species harbored by strain COL, the *entB* gene is chromosomal in this particular isolate.

To determine if *entB* from strains 592 and COL, as in strain DU-4916 (3, 4, 19), could be

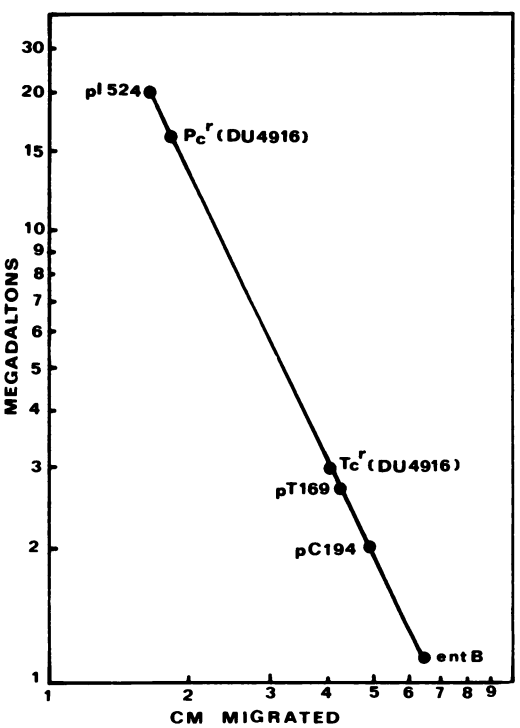

FIG. 2. *Relative migration in 1% agarose gels of plasmid DNA from S. aureus DU-4916 to reference plasmids. The molecular weights of the reference plasmids (pI524, pT169, pC194) have been described elsewhere (11, 14, 17). The molecular weights of the DU-4916 plasmids were derived from the linear migration of the reference plasmids.*

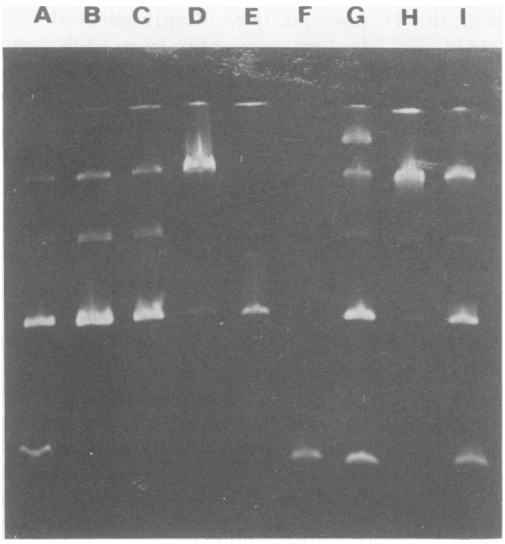

FIG. 3. *Agarose gel electrophoresis of ethanol-precipitated cleared lysate DNA from SEB⁺ donors (S. aureus strains 592, COL, and DU-4916) and transductants. (A) 8325-4(φ11) Tc^r Mec^r SEB⁺; (B) 8325-4(φ11) Tc^r Mec^s SEB⁻; (C) 8325-4(φ11) Tc^r Mec^s SEB⁻; (D) 8325-4(φ11) Tc^r Mec^r SEB⁺; (E) electrophoretically purified 3-Mdal Tc^r plasmid from S. aureus COL; (F) electrophoretically purified 1.15-Mdal SEB plasmid from S. aureus DU-4916; (G) S. aureus 592; (H) S. aureus COL; (I) S. aureus DU-4916. The plasmid DNA profiles of the transductants in lanes A and B were obtained from transducing lysates obtained on S. aureus 592 whereas lanes C and D were obtained from phage propagated on S. aureus COL.*

cotransduced with *mec* and *tc*, we selected for tetracycline-resistant clones of 8325-4(φ11) and scored the transductants for the Mec^r phenotype (Table 3). Clones exhibiting both phenotypes, as determined by replica plating on agar medium containing both drugs, were analyzed for toxin production and plasmid DNA. When strain COL served as the donor, *mec* was contransduced with *tc* at a frequency of 6% (12/200). All of the double transductants were SEB⁺ (5 to 12.5 μg/ml). Agarose gel electrophoresis of cleared lysates from representative clones demonstrated only the presence of the 3.0-Mdal Tc^r plasmid. The plasmid profiles of the Tc^r Mec^r SEB⁺ and the Tc^r Mec^s SEB⁻ derivatives were therefore identical (Fig. 3, lanes C and D). When strain 592 served as the donor for *tc* (Table 3), the Mec^r phenotype was cotransduced at a frequency of 2% (4/200). In agreement with the results obtained with COL, all of the *mec* cotransductants were also SEB⁺. However, all of the Tc^r Mec^r SEB⁺ clones contained both the 3.0- and 1.15-Mdal plasmids, whereas Tc^r Mec^r SEB⁻ derivatives contained only the 3.0-Mdal plasmid (Fig. 3, lanes A and B). These results obtained with strain 592 show that it is similar to strain DU-4916 (19). Additionally, the relatively high cotransduction of *mec* with *tc* that we have observed in both COL and 592 may reflect the transient genetic association of these two markers as proposed by Shalita et al. (19).

We have been able to transduce the *mec* determinant by direct selection (Table 3) into 8325-4(φ11) from strains COL and 592 but at very low frequencies (2×10^{-9} to 5×10^{-9}). Although this transduction frequency would generally be considered within the expected frequency of mutation to the Mec^r phenotype, our laboratory and others have not observed mutation of strain 8325-4(φ11) to the Mec^r phenotype (3, 6, 8, 20; W. M. Shafer and J. J. Iandolo, unpublished data). When the *mec* determinant was transduced into 8325-4(φ11) from either 592 or COL, a 100% cotransduction frequency with *tc* and *entB* was observed. The Mec^r Tc^r SEB⁺ transductants obtained from phage propagated on COL and 592, as expected, contained the plasmid profile of the parental strains.

Transduction of methicillin resistance was shown by Cohen and Sweeney (2) to occur at high frequencies when the recipient contained the penicillinase plasmid pI524 and was lysogenized by the prophage φ11. The functions provided by these two elements in facilitating *mec* transduction remain unknown. We wished to determine if the low level of *mec* transduction into strain 8325-4(φ11) could be increased by the presence of pI524. Therefore, we constructed strain 8325-4(φ11)(pI524) by transducing pI524 from its natural host, RN492, into 8325-4(φ11). When strain 8325-4(φ11)(pI524) was employed as the recipient, the frequency of *mec* transduction from strains COL, 592, and Du-4916 increased approximately 100-fold (Table 3). The Mec^r transductants were scored for Tc^r and SEB synthesis. Neither the Tc^r plasmid nor *entB* was cotransduced with *mec* when the chromosomal SEB strain COL served as the donor; each marker individually and jointly segregated with *mec* at low frequencies when transducing lysates from the plasmid SEB strains, DU-4916 and 592, were employed. Cotransduction analysis demonstrated that the *entB* gene was only transduced when the clones were resistant to both tetracycline and methicillin. All of the Mec^r Tc^s clones analyzed were SEB⁻.

Regardless of the donor strain employed, analysis of the plasmid DNA profile of the 8325-4(φ11) (pI524) *mec* clones demonstrated only the presence of the pI524 plasmid (data not presented). The 8325-4(φ11)(pI524) *mec tc entB*

TABLE 3. *Cotransduction of SEB with Mec^r and Tc^r selection*

Donor strain	Recipient	Selected phenotype[a] (no. clones/frequency)	Cotransduction
COL	8325-4(φ11)	Tc^r 4600/4.6 × 10^{-6}	Mec^r (6%), SEB (6%) Mec^r and SEB (100)[b]
	8325-4(φ11)	Mec^r 4/2 × 10^{-9}	Tc^r (100%), SEB (100%) Tc^r and SEB (100%)[b]
	8325-4(φ11)(pI524)	Tc^r 1300/1.3 × 10^{-6}	Mec^r (0.923%), SEB (0.78%) Mec^r and SEB (85%)
	8325-4(φ11)(pI524)	Mec^r 260/2.6 × 10^{-7}	Tc^r (10%), SEB (0%)
592	8325-4(φ11)	Tc^r 2400/2.4 × 10^{-6}	Mec^r (2%), SEB (2%) Mec^r and SEB (100%)[b]
	8325-4(φ11)	Mec^r 6/5 × 10^{-9}	Tc^r (100%), SEB (100%) Tc^r and SEB (100%)[b]
	8325-4(φ11)(pI524)	Tc^r 1100/1.1 × 10^{-6}	Mec^r (3.8%), SEB (2%) Mec^r and SEB (53%)[b]
	8325-4(φ11)(pI524)	Mec^r 560/5.6 × 10^{-7}	Tc^r (6%), SEB (2.4%) Tc^r and SEB (40%)
DU-4916	8325-4(φ11)(pI524)	Tc^r 4500/4.5 × 10^{-6}	Mec^r (0.76%), SEB (0.3%) Mec^r and SEB (40.7%)[b]
	8325-4(φ11)(pI524)	Mec^r 650/6.5 × 10^{-7}	Tc^r (5%), SEB (3.8%) Tc^r and SEB (76%)[b]

[a] Frequencies are expressed as the number of transductants per plaque-forming unit.
[b] Expressed as the percentage of Mec^r Tc^r cotransductants that are SEB$^+$.

derivatives obtained from the 592 and DU-4916 transducing lysates were found to contain pI524, the 3.0-Mdal Tc^r plasmid, and pentB (data not presented).

To determine if the Tc^r plasmid was involved in the successful transduction of *mec* and *entB*, we also employed strain 8325-4(φ11)(pI524) as the recipient in *tc* transduction. The donor strains consisted of COL, 592, and DU-4916. Transduction of *tc* from strain COL occurred at a frequency of 1.3 × 10^{-6}, and the Mec^r phenotype was cotransduced at a frequency of 0.92%. Several Tc^r Mec^r and Tc^r Mec^s transductants were propagated in 3% NP, and the spent media were analyzed for toxin. All of the Tc^r Mec^s derivatives were found to be SEB$^-$, whereas six of seven Tc^r Mec^r cotransductants were SEB$^+$. Plasmid DNA analysis of these clones revealed only the presence of pI524 and Tc^r plasmid (data not presented). When strains DU-4916 and 592 were the donors of *tc*, both *mec* and *entB* were cotransducible. The frequency of *mec* cotransduction using phage propagated on DU-4916 was 0.76 and 3.8% when 592 was the donor strain. SEB analysis of the Tc^r Mec^r and Tc^r Mec^s transductants obtained from both DU-4916 and 592 demonstrated that tetracycline selection facilitated the joint cotransduction of *mec* and *entB*.

In summary, the cumulative transduction results obtained demonstrate that *entB* is not physically linked to the Tc^r plasmid or closely linked to the *mec* gene in any of the three strains analyzed. However, SEB$^+$ transductants were apparent when the recipients were jointly cotransduced for both *mec* and *tc* but neither marker alone. Plasmid DNA analysis of SEB$^+$ transductants also revealed that, in strain COL, the *entB* gene is chromosomal, whereas the determinant is linked to the pentB plasmid in strains 592 and DU-4916. A summary of the transduction results obtained in this study is provided in Table 3.

The involvement of the tetracycline plasmid in these manipulations led us to investigate whether a resident Tc^r plasmid was necessary for *entB* transduction. Specifically we wished to evaluate whether a resident Tc^r plasmid performs a role in establishing *entB* or *mec* into the chromosome. We transduced the Tc^r plasmid from DU-4916S (17) into 8325-4(φ11) and 8325-4(φ11)(pI524). Using these transductants as recipients, we transduced for Mec^r and screened for SEB production. Transduction of *mec* from COL was not observed when 8325-4(φ11) *tc* was the recipient but did occur when the pI524 derivative was used (frequency of 2.3 × 10^{-7}). Twenty randomly picked 8325-4(φ11)(pI524) *tc mec* derivatives were propagated in broth and analyzed for SEB. All were SEB$^-$. These data indicate that a resident Tc^r plasmid does not enhance either *mec* or *entB* establishment. Rather, the data shown in Table 3 indicate that transmission of the Tc^r plasmid plays a critical

role in establishment of the Mec^r SEB$^+$ phenotype. The *entB* genetic variability (i.e., plasmid versus chromosomal loci) and the fact that *mec* also behaves as a transposition element (20) suggests that the Tc^r plasmid may be involved as an intermediate vector of either or both genes.

In the foregoing experiments, we have been unable to separate *entB* from *mec*. That is, we have never isolated an SEB$^+$ transductant that was not also Mec^r. However, the converse is possible; the majority of the Mec^r clones that have been isolated are SEB$^-$ (this study; 9, 19). These findings prompted us to examine the degree and manner of linkage of the *mec* and *entB* genes.

Based on a high frequency of coelimination, Dornbusch et al. (3, 4) suggested that *mec* and *entB* genes were linked. Because our transduction data were radically different unless associated with a Tc^r plasmid, we examined this question using two different elimination strategies. We employed culturing in ethidium bromide and a recent procedure for *mec* elimination outlined by Kuhl et al. (8) involving transduction of novobiocin resistance (*nov*) into *mec* strains.

Curing experiments were carried out by culturing strain COL in TSB containing 9×10^{-6} M ethidium bromide and 592 in TSB containing 4.5×10^{-6} M ethidium bromide. These concentrations were the minimal inhibitory concentrations for each isolate. After incubation at 37°C for 36 h, 500 colonies from each isolate were analyzed for antibiotic resistance and SEB synthesis by replica plating onto TSA containing the desired antibiotic and NP antibody agar. When strain 592 was analyzed by this procedure, two classes of derivatives were obtained; Pc^s Tc^s Mec^r SEB$^+$ (5% elimination frequency) and Pc^r Tc^s Mec^s SEB$^-$ (0.80%) clones (Table 4). The former lacked only the 17.5-Mdal plasmid but synthesized SEB at the same level as the parental (25 μg/ml). Conversely, the latter lacked both the 3.0- and the 1.15-Mdal plasmids and did not produce SEB even when 25× concentrated culture supernatants were analyzed. When strain COL was propagated in ethidium bromide broth, the Tc^r and Mec^r phenotypes were not eliminated (500 surviving colonies were analyzed [Table 4]). We therefore analyzed the stability of the *entB* determinant by replica plating all colonies onto NP antibody agar plates. This procedure did not reveal any SEB$^-$ derivatives. As reported by others (3, 19), conventional elimination procedures strongly suggest linkage of these two genes among plasmid SEB producers. The chromosomal strains were remarkably stable and, to assess linkage in this group, we employed the following approach.

Kuhl et al. (8) recently demonstrated that the *mec* determinant is site specific and maps in the *pyr-his-nov-pur-mec* linkage group. Transduction revealed that, when Mec^r Nov^s recipients

TABLE 4. *Plasmid DNA profile of antibiotic-sensitive and toxin-negative derivatives*

Strain	Phenotype	Mol wt of plasmids[a]	Source
592	Pc^r, Tc^r, Mec^r, SEB$^+$	17.5, 3.0, 1.15	Wild type
592 A	Pc^s, Tc^r, Mec^r, SEB$^+$ (5.0%, 24/500)[b]	3.0, 1.15	Ethidium bromide curing
592 B	Pc^r Tc^s, Mec^s, SEB$^-$ (0.8%, 4/500)	17.5	Ethidium bromide curing
592 *nov*	Pc^r, Tc^r, Mec^r, SEB$^+$ (100%, 500/500)	17.5, 3.0, 1.15	Nov^r transductant from ISP2
COL	Tc^r, Mec^r, SEB$^+$	3.0	Wild type
COL *nov*	Tc^r, Mec^r, SEB$^+$ (96%, 288/300)	3.0	Nov^r transductant from ISP2
COL *nov*	Tc^r, Mec^s, SEB$^-$ (4%, 11/300)	3.0	Nov^r transductant from ISP2
COL *nov*	Tc^s, Mec^s, SEB$^-$ (0.3%, 1/300)	None	Nov^r transductant from ISP2
57-dk	Pc^r, Tc^r, Mec^r, SEB$^+$	17.5, 3.0	Wild type
57-dk A	Pc^s, Tc^r, Mec^r, SEB$^+$	3.0	Ethidium bromide curing
57-dk-*nov*	Pc^r, Tc^r, Mec^r, SEB$^+$ (88%, 176/200)	17.5, 3.0	Nov^r transductant from ISP2
57-dk-*nov*	Pc^r, Tc^r, Mec^r, SEB$^+$ (3.5%, 7/200)	17.5, 3.0	Nov^r transductant from ISP2
57-dk-*nov*	Pc^r, Tc^r, Mec^s, SEB$^+$ (1.5%, 3/200)	17.5, 3.0	Nov^r transductant from ISP2
57-dk-*nov*	Pc^r, Tc^r, Mec^s, SEB$^-$ (7%, 14/200)	17.5, 3.0	Nov^r transductant from ISP2

[a] Expressed in megadaltons as determined by agarose gel electrophoresis.
[b] Reported as the frequency of isolation.

were rendered Nov^r by transduction, the Mec^r phenotype was lost at low frequencies (1 to 5%). To test whether methicillin resistance and SEB synthesis could be eliminated by this procedure, phage 29 was propagated on strain ISP2 and used to infect strains 592 and COL. Using strain COL as the recipient, *nov* was transduced at a frequency of 2×10^{-7} (Table 4). The Nov^r transductants were subsequently replica plated onto TSA, TSA-methicillin, and NP antibody agar. This procedure demonstrated a 4% (12/300) frequency of *mec* elimination. Of the 12 Mec^s clones, all were SEB$^-$ and one derivative also lost the Tc^r phenotype. These derivatives were analyzed for plasmid DNA by agarose gel electrophoresis, and the Tc^r Mec^s SEB$^-$ clones contained the 3.0-Mdal Tc^r plasmid whereas a single Tc^s Mec^s SEB$^-$ clone lacked this 3.0-Mdal species. Therefore, except for one derivative, SEB$^-$ clones of strain COL contain the same plasmid DNA profile as the parental. When strain 592 was used as the recipient, the *nov^r* gene was transduced at a frequency of 9×10^{-8}. However, all of the Nov^r transductants retained both the Mec^r and SEB$^+$ phenotypes. A similar approach was employed with another chromosomal *entB* isolate, strain 57-dk. Approximately 12.0% of the Nov^r transductants were found to be Mec^s or SEB$^-$, and in about half (5%) the *mec* and *entB* genes were independently eliminated (Table 4).

Both approaches at elimination of genes (chemical curing and *nov* transduction) have resulted in an inconclusive general model of chromosomal linkage for *entB* and *mec*. Chemical curing of the *entB* gene was only effective with plasmid SEB$^+$ strains and indeed suggests linkage with *mec*. The use of *nov* transduction proved to be an adequate strategy for *entB* elimination in chromosomal strains, but different results were obtained with each strain. In strain COL the genes for methicillin resistance and SEB behave as though they are closely linked because they coeliminate totally. On the other hand, in strain 57-dk linkage is much less pronounced, with each gene being independently eliminated.

To examine this region of the chromosome in more detail, we have analyzed the *nov-pur-mec* linkage group as defined by Kuhl et al. (8) for the presence of the toxin gene. The strains employed in this investigation were Nov^s and Pur$^+$. We transduced the *nov* gene via phage 29 from ISP2 to the SEB$^+$ strains DU-4916, 592, COL, and 57-dk. The Nov^r transductants were subsequently scored for the Mec^r and SEB$^+$ phenotypes to insure that these phenotypes had not been eliminated. Selected clones exhibiting the Nov^r Mec^r SEB$^+$ phenotypes were infected with phage 29, and the resulting sterile lysates were employed for *nov* transduction. Using strain 8325-4(ϕ11) as the recipient, the frequency of *nov* transduction ranged from 3×10^{-8} to 5×10^{-7}, depending on the donor strain (Table 5). Cotransduction of *mec* occurred at low frequencies (2 to 8%). All of the 8325-4(ϕ11) *nov* transductants, regardless of the donor strain, however, remained SEB$^-$. Therefore, *entB* does not belong to the *pyr-his-nov-pur-mec* linkage group and reinforces our contention that these two genes are not closely linked.

To further confirm the results of these transduction experiments, we have employed Mec^r selection in transformation experiments with chromosomal DNA prepared as described by Sjöström et al. (20). Using this DNA preparation, Sjöström et al. were able to obtain high frequencies of *mec* transformation but were unable to obtain Mec^r transformants when the plasmid-containing fraction was employed. In our hands, using *S. aureus* strain 8325-4(ϕ11) as the recipient and donor DNA prepared from strains DU-4916, 592, COL, and 57-dk, the frequency of *mec* transformation ranged from 2×10^{-7} to 9×10^{-7}. Mec^r clones were subsequently replicated to NP antibody agar to score for the toxin phenotype. All Mec^r clones, regardless of the source of the donor DNA, did not produce SEB and were devoid of plasmids as determined by agarose gel electrophoresis (Fig. 4). Regardless of the source of the donor DNA, the only ethidium bromide-staining material evident from the 8325-4(ϕ11) *mec* transformants was chromosomal DNA. Therefore, by this criterion it also appears that *mec* and *entB* are not closely linked genes.

DISCUSSION

A previous report from this laboratory (17) demonstrated that the gene responsible for SEB synthesis is capable of existing in either the plasmid or the chromosomal state. However, a degree of uncertainty still remains regarding the plasmid locus. The problem revolves around the unselectability of the *entB* gene. Analysis has been carried out by cotransfer of this relevant genotype with the determinant specifying meth-

TABLE 5. *Transduction of nov from SEB-producing strains*

Donor	Recipient	Transduction Frequency		
		Nov$^{r a}$	Mec$^{r b}$	SEB^b
COL-*nov*	8325-4(ϕ11)	4.57×10^{-7}	8%	0
57-dk-*nov*	8325-4(ϕ11)	3.62×10^{-7}	3%	0
592-*nov*	8325-4(ϕ11)	3×10^{-8}	2%	0
DU-4916-*nov*	8325-4(ϕ11)	5×10^{-7}	4%	0

[a] Expressed as number per plaque-forming unit.
[b] Expressed as cotransduction frequency.

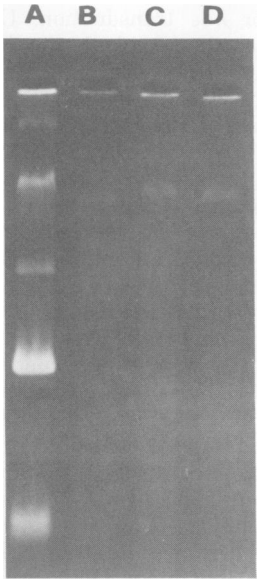

FIG. 4. *Agarose gel electrophoresis of cleared lysate DNA extracted from mec transformants, S. aureus strains DU-4916, and 8325-4(φ11). (A) S. aureus DU-4916; (B) S. aureus 8325-4(φ11); (C) S. aureus 8325-4(φ11) mec; (D) S. aureus 8325-4(φ11) mec. The mec transformant in panel C was obtained from donor DNA of S. aureus DU-4916, whereas the mec transformant in panel D was obtained from donor DNA of S. aureus COL.*

icillin resistance. Apparent genetic linkage has been reported between these two markers (3, 4), and although not 100% it is greater than one would predict for the random association of genes. Therefore, the SEB$^+$ phenotype has always been determined in either plasmid (3, 4, 19) or chromosomal strains (this study) by screening among a background of methicillin-resistant isolates. Although this situation might suggest that the plasmid *entB* locus in particular is an artifact of the assay system, the complete (i.e., 100%) correlation of the plasmid with the SEB$^+$ phenotype among such strains argues strongly for this configuration.

The purpose of this investigation was twofold. Firstly, we have been concerned with the physical disposition of the *entB* gene and have wished to confirm the existence of a plasmid responsible for SEB synthesis in a strain other than DU-4916. Secondly, we have been interested in detailing the linkage arrangement of *entB* with the tetracycline and methicillin resistance genes.

A survey of the extrachromosomal DNA profile of 15 Mec^r isolates demonstrated that only a minority of strains (37.5%) contain the 1.15-Mdal plasmid presently implicated in SEB synthesis. One of these isolates, *S. aureus* 592, was chosen for genetic examination, and the results obtained were consistent with previous work in strain DU-4916 (19). Specifically, the loss of SEB synthesis due to elimination by culturing in the presence of ethidium bromide resulted in the concomitant loss of the 1.15-Mdal plasmid. Additionally, transduction of *entB*, via selection for methicillin and tetracycline resistance, into strain 8325-4(φ11) or the pI524-containing derivative demonstrated that all SEB$^+$ clones harbored the 1.15-Mdal plasmid. Although we do not presently have direct biochemical evidence to demonstrate that the structural gene is present on this plasmid, it is clear from both our results and Shalita et al. (19) that this plasmid is critically involved in the establishment of enterotoxigenesis.

Our data have also demonstrated that, in other Mec^r isolates, the *entB* gene can occupy a chromosomal locus. This was shown when the single plasmid harbored by strain COL failed to confer the toxin phenotype when transferred. Further evidence showing that the *entB* gene is maintained differently in strains COL and 592 was reflected by the phenotypic stability of SEB synthesis. As is characteristic of plasmid and chromosomal genes, *entB* was eliminated in strain 592 but not COL when cultured in the presence of ethidium bromide.

Since the initial examination of *mec* and *entB* in *S. aureus* DU-4916 by Dornbusch and Hallander (3), numerous studies have attempted to elucidate the genetic status of *mec*. The cumulative data have unambiguously shown that *mec* is chromosomal in most if not all isolates (6, 8, 20, 22). Transformation of *mec* has been shown by Sjöström et al. (20) to be independent of *recA* function(s) and has therefore been implicated as a potential translocatable element. Kuhl et al. (8) have established the chromosomal map site for *mec* as belonging in linkage group II on the staphylococcal chromosome and have suggested that, if *mec* is a translocatable element, insertion is site specific.

The rigorous examination of *mec* by other investigators (6, 8, 20, 22) has ignored the question of linkage with *entB*. Therefore, the original proposal of linkage made by Dornbusch et al. (4) has not been satisfactorily verified. Although SEB synthesis is characteristic of most Mec^r isolates (10), the results of this investigation demonstrate that *mec* and *entB* do not belong to the same linkage group when analyzed by transformation and transduction. However, when analyzed by transduction, both genes can be transduced at low frequencies with the Tc^r plasmid. The surprisingly high frequency (ca. 5×10^{-9} to 2×10^{-7}) with which this event occurs argues against a chance packaging of these physically unlinked genes by a transducing particle.

In fact, cotransduction of the *entB* gene, regardless of the plasmid or chromosomal nature of the determinant, was only obtained when the recipient was rendered resistant to both methicillin and tetracycline. Transduction of either resistance marker alone did not promote cotransfer of *entB*. Furthermore, this role does not appear to be unique to the Tc^r plasmid carried by any single strain, since identical results have been obtained with at least three different strains. This occurrence reinforces the contention of Shalita et al. (19) that all three genes may be transiently associated during transduction. The basis for this anomaly is not presently understood, although our observations suggest that, during transduction, the Tc^r plasmid performs an obligatory step in the cotransfer of the *mec* and *entB* genes. This seems likely because prior residence of the Tc^r plasmid in the recipient does not result in productive transduction of both *mec* and *entB*. In a dynamic sense such transient associations would initially involve *mec* and the Tc^r plasmid forming a genetic unit which may or may not include *entB*, and that, once established, the genes are positioned in the correct configuration. This latter event is underscored by the lack of any single recipient clone containing a single plasmid element possessing all three genes.

The results obtained in this and our previous investigation (17) of the genetic determinant responsible for SEB clearly demonstrate the complexity of analysis of the *entB* gene. Our findings of chromosomal and plasmid loci involved in toxin synthesis and the interrelationship with other genetic elements suggest that the *entB* gene may be capable of transposition activity. Work with recombination-deficient mutants is in progress and should resolve this question. Moreover, additional study of the association of *mec* and *entB* and the role the Tc^r plasmid may play in the establishment of these genes is also necessary to understand the system.

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