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Chromosomal Locus for Staphylococcal Enterotoxin B†

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The genetic locus of staphylococcal enterotoxin B (SEB) was investigated in the *Staphylococcus aureus* food-poisoning isolates, strains S6 and 277. Direct neutral sucrose gradient centrifugation analysis of sodium dodecyl sulfate-sodium chloride-mediated cleared lysates demonstrated that strain S6 contained a single 37S plasmid. Transductional analysis revealed that the 37S plasmid in S6 encoded for cadmium resistance (Cad) but not SEB. Additionally, elimination of cadmium resistance in S6 provided a plasmid-negative derivative that produced SEB at the same level as the parent. Examination of strain 277 showed two plasmids, a 37S species encoding for penicillin resistance (Pen^r) and a 21S species containing the gene(s) responsible for tetracycline resistance (Tet^r). Elimination of the 37S, *pen*^{r} plasmid in 277 had no effect on SEB production, whereas introduction of the 21S *tet*^{r} plasmid via transformation into strain 8325 (SEB$^-$) did not confer enterotoxigenesis upon the transformants. The data obtained in this investigation suggest that the SEB gene(s) in these food-poisoning isolates of *S. aureus* is chromosomal.

The genetic determinants of the staphylococcal enterotoxins among food-poisoning strains of *Staphylococcus aureus* have not been clearly defined. The studies of Dornbusch et al. (1) and Shalita et al. (10) represent the only definitive approaches to this problem, but they focused on the status of staphylococcal enterotoxin B (SEB) in a methicillin-resistant (Mec^r) hospital isolate (*S. aureus* DU-4916) rather than known food-poisoning strains. Nevertheless, both groups reported that SEB exhibited properties of a plasmid-linked gene, and Shalita et al. (10) have physically and genetically identified a 14S plasmid determinant of SEB. However, because both groups also have observed varying degrees of linkage of SEB to methicillin resistance, which was shown by Sjöström et al. (11) to be chromosomal in nature, further examination is in order.

We have chosen to examine the SEB locus in *S. aureus* S6 because enterotoxin synthesis has been extensively studied in this strain and because it is ordinarily considered the type strain for SEB production. We present data to show that SEB is determined by a chromosomal gene(s) in strain S6 and 277 and that this genetic arrangement may be characteristic of many food-poisoning isolates of *S. aureus*.

MATERIALS AND METHODS

Several strains of *S. aureus* were employed in this investigation and are listed in Table 1.

Media and chemicals. For toxin analysis, the various strains were routinely cultured in NAK-PHP broth (2). However, CY broth (5), supplemented with 250 μg of 2-deoxyadenosine per ml, was used for labeling cellular deoxyribonucleic acid (DNA), and Trypticase soy broth (8) was used for phage production and as the growth medium in ethidium bromide curing experiments. When agar plates were required, 2% agar (Difco Laboratories, Detroit, Mich.) was added to either Trypticase soy broth or NAK-PHP broth. When necessary, anti-SEB serum was added to NAK-PHP agar at a dilution of 1:40. [2-*methyl*-^{3}H]thymidine, specific activity 50 Ci/mmol, was purchased from Moravek Biochemicals (Industrial City, Calif.).

Marker analysis. Cadmium nitrate sensitivity was determined as described by Smith and Novick (12). Antibiotic sensitivity was determined as described by Dornbusch et al. (1).

Transduction of plasmid DNA. Phage 29 of the International Typing Series was propagated on the donor strains in Trypticase soy broth supplemented with 400 μg of $CaCl_2$ per ml at 37°C as described by our laboratory (8). Routinely, titers of 10^9 to 10^{10} plaque-forming units were obtained, but the precise titer varied depending upon the host strain. Transduction was carried out as described by Dornbusch et al. (1). Cadmium-resistant (Cad^r) transductants were selected on Trypticase soy agar plates supplemented with 50 μg of $Cd(NO_3)_2$ per ml. Penicillin- and tetracycline-resistant (Pen^r and Tet^r) transductants were selected on Trypticase soy agar supplemented with either 50 μg of penicillin G per ml or 12.5 μg of tetracycline per ml. All plates were incubated at 37°C for 48 h before scoring for transductants. Uninfected lawns and phage sterility controls were similarly tested.

Transformation analysis. Transformation by plasmid DNA was performed as described by Stiffler et al. (13). Transforming DNA in 0.1× SSC (0.15 M NaCl-0.015 M sodium citrate, pH 7.0) was used at a

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final concentration of 1 μg/ml.

Marker elimination. Strains were propagated overnight in Trypticase soy broth diluted 1,000× in fresh media containing 9×10^{-6} M ethidium bromide and incubated in the dark for 24 h at 37°C. After sonic treatment to disrupt clumps, about 100 colony-forming units were plated onto Trypticase soy agar or NAK-PHP and incubated for 24 h at 37°C. The colonies were then replica plated onto NAK-PHP agar containing anti-SEB serum or plated on Trypticase soy agar containing penicillin, tetracycline, or cadmium nitrate. Further incubation was carried out at 37°C for 48 h, and then colonies were scored for the appropriate markers.

Enterotoxin B production and assay. *S. aureus* strains were grown in 10-ml shake cultures at 37°C for 36 h. After incubation, the cells were pelleted by centrifugation, and the spent medium was analyzed for the presence of SEB by Laurell immunoelectrophoresis (2) or Ouchterlony double diffusion (7). These assays were also performed on culture supernatants after 10× concentration by pervaporation. Purified SEB was kindly provided by R. Bennett (Food and Drug Administration, Washington, D.C.) and used to prepare antitoxin in white albino rabbits.

Preparation of sodium dodecyl sulfate-salt lysates. Exponentially growing cultures in CY broth at 37°C were labeled with [^{3}H]thymidine (4 μCi/ml) for at least 3 generations. The cells were centrifuged from the growth medium and washed two times in TES (0.03 M tris(hydroxymethyl)aminomethane–0.005 M ethylenediaminetetraacetic acid–0.05 M NaCl, pH 8.0) and finally resuspended in 1 ml of TES–2.5 M NaCl, pH 8. After the addition of 200 μg of lysostaphin, protoplasting was carried out by incubating the cell suspensions at 37°C for 15 min. After enzyme treatment, 1.5 volumes of 1.67% sodium dodecyl sulfate was added to lyse the protoplasts, and the mixture was immediately placed in ice. The lysed cells were stored at 4°C overnight, and then the suspension was centrifuged at 27,000 × *g* for 30 min (9) to pellet the chromosomal DNA. The supernatant solution was removed and dialyzed overnight at 4°C against sterile TES buffer. After dialysis, the cleared-lysate solution was concentrated approximately 10-fold against 30% polyethylene glycol (20 M).

Centrifugal analysis of plasmid DNA. Cesium chloride-ethidium bromide buoyant density gradient centrifugation of the dialyzed and concentrated cleared lysate was carried out in an SW50.1 rotor at 42,000 rpm for 36 h at 20°C. The gradients were prepared by adding CsCl to DNA in TES buffer to a final density of 1.54 g/cm^2; ethidium bromide was added to a final concentration of 100 μg/ml. After centrifugation, 5-drop fractions were collected on glass-fiber filters. The filters were trichloroacetic acid treated, ethanol washed, dried, and counted as previously described (4).

Direct neutral sucrose gradient centrifugation of the dialyzed, concentrated cleared lysates was carried out in 5 to 20% sucrose in 1× SSC–0.05 M tris(hydroxymethyl)aminomethane (pH 7.9) in an SW50.1 rotor at 45,000 rpm for 180 min at 4°C. Ten-drop fractions were collected on filters, processed, dried, and counted. Reference DNAs consisted of either the [^{3}H]thymidine-labeled 21S component I of polyoma DNA (provided by R. A. Consigli) or the [^{3}H]thymidine-labeled pI$_{524}$ plasmid from *S. aureus* RN492.

RESULTS

To ascertain if the strains employed in this investigation contained covalently closed circular DNA, cells were propagated in CY broth containing [^{3}H]thymidine. Bulk cellular DNA was labeled for approximately three generations, after which cleared lysates were produced. The dialyzed, concentrated cleared lysates were then centrifuged to equilibrium in cesium chloride-ethidium bromide density gradients (6, 10, 13–15). All of the strains employed were analyzed in this manner by using strain 8325 as the plasmid-negative control. Figure 1 shows the equilibrium density gradient profile of strain S6 and is representative of all the data. A satellite peak of radioactivity, indicative of covalently closed circular DNA, is present (fraction 30). Chromosomal and/or possibly open circular plasmid DNA are located at fraction 36.

To resolve the individual plasmid species, the cleared lysates were centrifuged in a 5 to 20% neutral sucrose gradient after dialysis and poly-

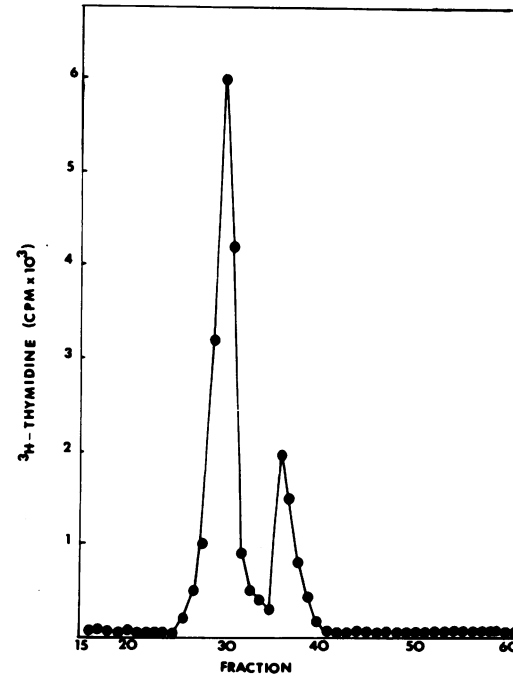

FIG. 1. *Cesium chloride-ethidium bromide equilibrium density gradient profile of the cleared-lysate fraction of S. aureus S6. Cleared lysates in TES buffer were made up to contain CsCl at a final density of 1.54 g/cm^2 and 100 μg of ethidium bromide per ml. Centrifugation was carried out in an SW50.1 rotor for 36 h at 42,000 rpm.*

ethylene glycol concentration. We elected to use this procedure instead of centrifuging cesium-purified material so that plasmid DNA existing in the open circular form would be resolved.

The neutral sucrose gradient profile of strain S6 is shown in Fig. 2B. This strain was found to contain only a single-plasmid species of 37S that corresponds to a molecular weight of 17.5×10^6. We additionally examined the plasmid DNA of strains 277 and 279 (Fig. 2C). The plasmid profiles of both strains were identical, and only 277 is presented. Both of these strains are known food-poisoning isolates and produce both staphylococcal enterotoxin A and SEB, as does strain S6. They both were found to contain a 37S and a 21S plasmid. A summary of these data is shown in Table 1. It is important to point out that none of the three SEB positive (SEB^+) known food-poisoning strains we have examined contain the 14S plasmid species previously reported by Shalita et al. (10) to code for SEB. However strain S6 as well as the other two are multiple-enterotoxin producers, whereas the strain used by Shalita et al. (DU-4916) produced only SEB. We therefore questioned whether the plasmid profile of single-enterotoxin producers would exhibit the 14S plasmid peak they reported. Therefore, we also analyzed the extrachromosomal DNA profile of two strains that produce only SEB (strains 315 and 838, provided by R. Bennett) and have been implicated in food-poisoning incidents. The data showed that strain 838 possessed only a 37S plasmid, which tentatively encodes for penicillin resistance (data not shown, but the profile is identical to S6, Fig. 2B), whereas strain 315 did not contain any plasmid DNA.

We do not, however, feel that these data in any way contradict the findings of Shalita et al. (10) because when strain DU-4916 was analyzed (Fig. 2A), three distinct plasmids sedimenting at 37S, 21S, and 14S were resolved. The putative SEB plasmid (14S) was clearly evident, and from the position of sedimentation, a molecular weight of 1.15×10^6 was determined. Strain DU-4916S, which is Pen^s Mec^s Tet^r SEB^-, is also presented in Fig. 2A with the parent DU-4916. This strain was found to contain only the 21S

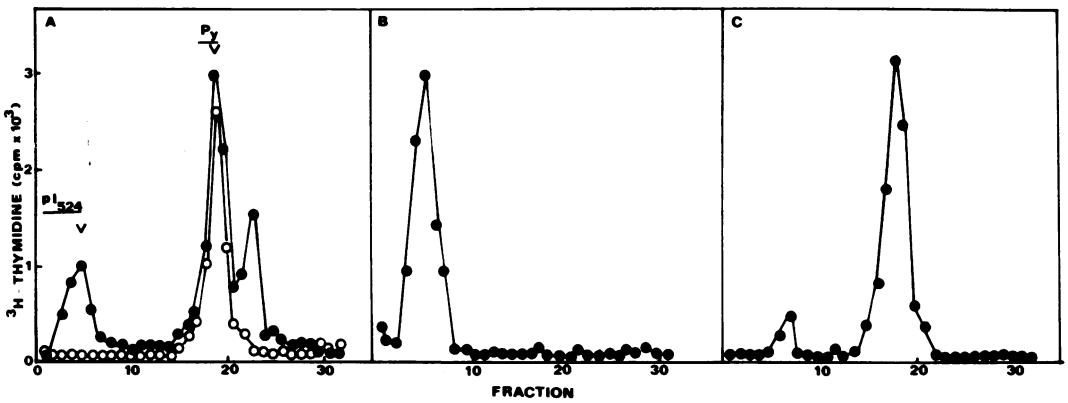

FIG. 2. *Direct neutral sucrose gradients of cleared lysates of S. aureus. (A) Closed circles, strain DU-4916; open circles, strain DU-4916S; (B) strain S6; and (C) strain 277. In (A) the position of the marker DNAs (pI_{524} plasmid fraction 4 and polyoma virus fraction 19) is indicated. Cleared lysates were centrifuged through 5 to 20% neutral sucrose in 1× SSC–0.05 M tris(hydroxymethyl)aminomethane, pH 7.9 in an SW50.1 rotor for 180 min at 45,000 rpm.*

TABLE 1. *Marker analysis of test strains*

Strain	Plasmid profile	Cad^r	Pen^r	Tet^r	SEB[a]	Source
DU-4916	37S, 21S, 14S	+	+	+	50	S. Cohen
DU-4916S	21S	−	−	+	0	S. Cohen
S6	37S	+	−	−	375	M. S. Bergdoll
277	37S, 21S	+	+	+	65	M. S. Bergdoll
279	37S, 21S	+	+	+	125	M. S. Bergdoll
8325	None	−	−	−	0	R. P. Novick
S6 (cad^s)	None	−	−	−	375	This laboratory
8325 (cad^r)	37S	+	−	−	0	This laboratory Transductant of Cad^r from S6

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

tet^r plasmid. In addition, we have successfully transduced the 37S plasmid from DU-4916 into strain 8325 and have found that the 8325 *pen*^{r} derivatives are also non-enterotoxigenic. Although we do not provide primary data to implicate the 14S plasmid in SEB synthesis, it is apparent that in an SEB$^-$ derivative the 14S plasmid is no longer present and that the establishment of either the 37S or 21S plasmid does not alone confer enterotoxigenesis. Therefore, as stated by Shalita et al. (10), the 14S plasmid either codes for SEB or for a gene essential for toxigenesis.

The lack of the 14S plasmid in the strains that we investigated prompted a more detailed evaluation of the locus of the SEB gene(s).

Because SEB production does not provide a growth advantage to positive cells, both antibiotic and heavy-metal resistances were used as selective pressures in transduction experiments. Penicillin, tetracycline, and cadmium resistance were found to be suitable markers for plasmid analysis in the strains used. A summary of these data is presented in Table 1.

To determine if the SEB and cadium resistance (*cad*^{r}) markers were located on the 37S plasmid in strain S6, phage 29-mediated transduction was carried out with phage grown on S6 and infected onto strain 8325. $Cd(NO_3)_2$ was used to select transductants, and anti-SEB serum in agar was used to screen for toxin-positive colonies. Transduction of *cad*^{r} occurred at a frequency of 6×10^{-7} but none of the transductants were SEB positive. Several single 8325 *cad*^{r} clones were individually propagated in labeled CY broth, and the concentrated, dialyzed cleared lysates were centrifuged in neutral sucrose gradients. A cleared lysate from wild-type 8325 was similarly analyzed. The presence of a 37S plasmid was clearly apparent in the transductant (Fig. 3). As a control to determine if the transductants produced low levels of SEB that were not detected in the initial screen, 10 strain 8325 *cad*^{r} transductants were propagated in NAK-PHP broth at 37°C for 36 h. The culture supernatants were then concentrated 10-fold and assayed for SEB. SEB was assayed by agar diffusion and by immunoelectrophoresis. Although as little as 0.1 μg/ml was detectable in the concentrated supernatants, all clones selected remained negative.

To confirm these data, the *cad*^{r} gene was eliminated from an 8325 *cad*^{r} transductant clone by culturing in ethidium bromide. The curing frequency in this experiment was 5% (5 out of 100 colonies tested). Subsequent gradient analysis of the cured cells showed that the 37S plasmid was no longer present in a *cad*^{s} derivative.

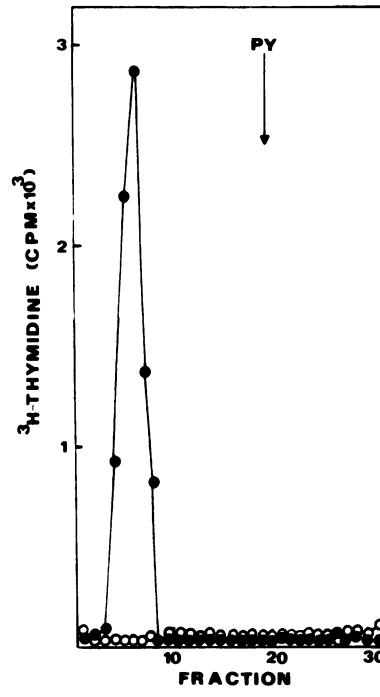

FIG. 3. *Direct neutral sucrose gradients of S. aureus transductants. Closed circles, strain 8325 cad*^{r}*; open circles, cured S6. The position of the polyoma marker is indicated.*

As additional confirmation, elimination of *cad*^{r} in S6 by growth in ethidium bromide was also tested. S6 was cured of *cad*^{r} at a frequency of 2% (4 out of 200 colonies tested), and neutral sucrose gradient analysis showed that the S6 *cad*^{s} derivatives were devoid of plasmid DNA (Fig. 3). To test whether the SEB marker had been eliminated along with *cad*^{r}, the sensitive clones were propagated in broth. Examination of the culture supernatants for the presence of SEB showed that the *cad*^{s} derivatives produced equivalent amounts of SEB as compared to the parent S6 (Table 1).

Attempts to directly cure S6 of SEB production were similarly performed. Strain S6 was grown for 24 h in NAK-PHP containing 9×10^{-6} M ethidium bromide. The culture was then diluted to give approximately 100 colony-forming units per ml and plated onto antiserum agar. After 48 h of incubation at 37°C, 200 colonies were examined, and all remained SEB positive. Further attempts at elimination were carried out. A 24-h culture was diluted to 10^3 colony-forming units per ml in fresh medium containing ethidium bromide and was reincubated for 24 h at 37°C. This procedure was performed every 24 h for 7 days, and 3,500 clones were tested, all of which remained SEB positive. Thus, SEB pro-

duction in strain S6 is not only unassociated with the 37S plasmid, but most importantly, gives evidence of being a stable chromosomal gene in a plasmid-negative derivative. We can conclude, therefore, that the genetic determinants for SEB are chromosomal in this organism.

A second *S. aureus* strain, 277, was also genetically analyzed. Strain 277 was cultured in ethidium bromide broth, and colonies sensitive to penicillin and tetracycline were individually selected. Elimination of the *pen*^{r} marker occurred at a frequency of 9% (9 of 100 colonies tested). Neutral sucrose gradient analysis of a *pen*^{s} derivative showed that the 37S plasmid had been eliminated (data not presented). When the 277 *pen*^{s} derivatives were propagated in NAK-PHP broth, SEB production was found to be at the same level as the present strain. A similar approach to the analysis of the 21S *tet*^{r} plasmid was unsuccessful. With either the wild-type 277 or the *pen*^{s} derivatives, tetracycline-sensitive clones were not obtained even after repeated subculturing in ethidium bromide, or after culturing at 43°C, or in the presence of 0.008% sodium dodecyl sulfate or 25 μg of acriflavine per ml. To analyze the possible involvement of the 21S plasmid with SEB production, transformation of *tet*^{r} into 8325 by plasmid DNA prepared from the 277 *pen*^{s} derivative was carried out. Tetracycline-resistant clones of strain 8325 were analyzed in neutral sucrose gradients and were found to contain a plasmid species sedimenting at 21S (data not shown). Subsequent analysis of 36-h concentrated-culture supernatants from 10 selected 8325 *tet*^{r} transformants showed that SEB was not synthesized. Therefore, analysis of the two plasmid species in strain 277 failed to show an extrachromosomal involvement with SEB synthesis.

An Ouchterlony gel diffusion profile of the toxins from the various strains and derivatives is presented in Fig. 4. Culture supernatants from strain DU-4916, S6, 277, S6 *cad*^{s}, and 277 *pen*^{s} all show a single precipitin band. Strain 8325 and the 8325 *tet*^{r} transformant did not produce any precipitin bands. These data additionally show the SEB produced from DU-4916, S6, and 277 are immunologically identical and point out that the genetic differences are not due to an aberrant SEB molecule.

DISCUSSION

The genetic analysis of the staphylococcal enterotoxins has been a subject of recent investigation (1, 10) but is as yet unclear. Early studies by Dornbusch et al. (1) with strain DU-4916 suggested a plasmid locus for this gene based on cotransduction and co-elimination data with methicillin resistance as the selective marker. However, the genetic status of SEB became quite uncertain with the reports of Stiffler and Cohen (13), in which they were unable to provide evidence for the presence of a *mec*^{r} plasmid in DU-4916, and Sjöström et al. (11) when they identified a chromosomal locus for *mec*^{r} by transformational analysis. Neither of the latter two groups employed SEB synthesis as a secondary marker, and the relationship of the *mec*^{r} gene to the SEB locus could not be determined. Recently, Shalita et al. (10) potentially resolved the question of SEB synthesis in strain DU-4916. They identified a 14S (0.75×10^6-dalton)

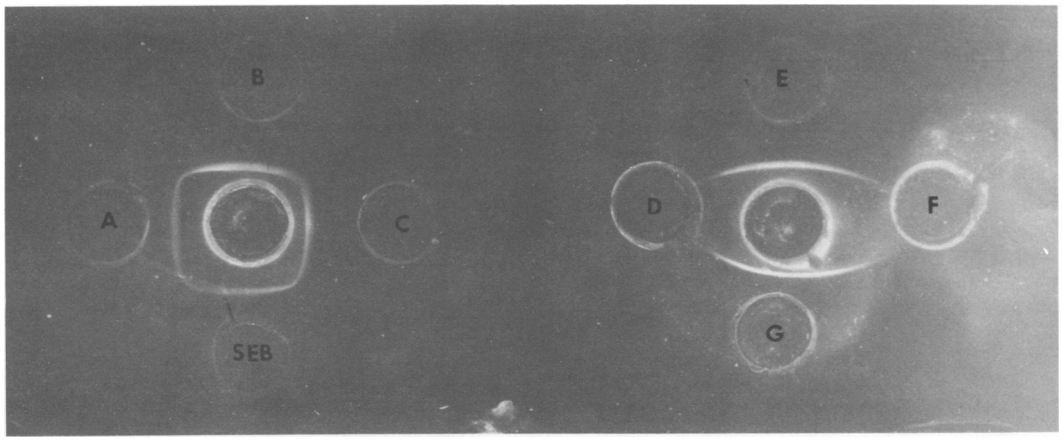

FIG. 4. *Ouchterlony double-diffusion analysis of toxin production by the various strains used in this study. Center wells contained anti-SEB serum. Culture supernatants from 36-h shake cultures were added to the outer wells. (A) DU-4916; (B) S6; (C) 277; (D) 8325; (E) S6 cad*^{s}*; (F) 8325 tet*^{r}*; and (G) 277 pen*^{s}*.*

plasmid species present in 12 to 16 copies per cell, which according to transductional and transformational data carries the gene for SEB. However, they also observed the segregation of *mec*^{r} and SEB on transduction. They report that this plasmid is somewhat unstable, and its autonomous maintenance appears to rely upon the presence of a 21S plasmid that confers tetracycline resistance on the host. We have confirmed the presence of a 14S plasmid in DU-4916 but find that by sedimentation analysis the molecular weight corresponds to 1.15×10^6, a value similar to the molecular weight calculated from the gradient data they present.

The studies presented in this communication add yet another measure of controversy to the genetic status of SEB. We have shown that among the food-poisoning isolates of *S. aureus* tested, strains S6 and 277 clearly contain chromosomal genetic determinants of SEB synthesis. The gene is stable and cannot be eliminated by conventional procedures, does not depend on the presence of a tetracycline plasmid for replication, is not influenced by the genes for other enterotoxin serotypes, and occurs in *mec*^{s} strains.

The etiology of the *S. aureus* cultures, especially regarding the *mec*^{r} determinant, may have a bearing on the data presented in this study. Strain DU-4916 is a hospital isolate and has no history of association with documented food-poisoning episodes, whereas the strains used in this study are all confirmed isolates of such investigations. Moreover, there have not been any *mec*^{r} food-poisoning isolates of staphylococci reported in this country (R. Bennett, personal communication). It may be then that the presence of the *mec*^{r} gene can confer different gene-processing capabilities on SEB$^+$ cells because, based on the immunological identity of SEB in strain DU-4916 and the others tested in this study, it seems clear that we are dealing with the same genetic determinant. More detailed genetic analysis is necessary to determine whether this possibility is viable and to determine if transfer of *mec*^{r} into strain S6 can exert similar aberrant behavior of the SEB$^+$ genotype in chromosomally determined strains.

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