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Penicillinase Plasmid-Linked Genetic Determinants for Enterotoxins B and C_1 Production in *Staphylococcus aureus*

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The genes encoding for β-lactamase (*bla*$^+$) and resistance to metallic ions (cadmium, mercury, lead, arsenate, and arsenite) were located in a 56.2-kilobase plasmid, pZA10, isolated from a clinical strain, *Staphylococcus aureus* 6344. This strain produced enterotoxin B and enterotoxin C_1. Elimination of pZA10 by either sodium dodecyl sulfate or heat treatment (43°C) resulted in the loss of the capability of the bacteria to produce both enterotoxin B and enterotoxin C_1. A physical map of pZA10 was constructed with *Bam*HI, *Sal*I and *Bgl*II restriction endonucleases. Penicillin-resistant, enterotoxin B- and C_1-producing cotransformants were isolated by transformation with pZA10 DNA with either *S. aureus* RN450 or cured *S. aureus* 6344 as recipients. The transferred plasmids exhibited genetic instability shown by changes in restriction pattern and molecular size, loss of plasmid DNA, and addition of chromosomal DNA. Enterotoxin B production was related to a 18.1-kilobase pZA10 fragment carried by such a rearranged plasmid. Chromosomal cointegration of *bla*$^+$ with genetic determinants for metallic ion resistance and enterotoxin B and C_1 production were detected in heat-treated *S. aureus* 6344. Transformation employing chromosomal DNA containing the integrated plasmid resulted in excision and reestablishment of pZA10-related plasmids in the transformants. pZA10-linked resistance to cadmium, which was lost upon the integration of pZA10 into the host chromosome, reappeared in transformants carrying the excised plasmid.

Enterotoxinogenic strains of *Staphylococcus aureus* produce six known immunologically distinct enterotoxins which cause food poisoning (1). Each strain may produce a single type of enterotoxin or several types simultaneously. The genetic determinant for enterotoxin A (SEA) production (*entA*) was found in several strains to be located on the bacterial chromosome between the *purB110* (purine requirement) and *ilv-129* (isoleucine-valine requirement) marker, close to the *hla*$^+$ gene (α hemolysin production [8, 16]). Although published evidence indicates that the structural gene for enterotoxin B (SEB) production is chromosomal, its precise locus is still controversial (4, 6, 16, 20, 22). Results of plasmid elimination and transduction experiments indicate that SEB production in the strain DU4916 is associated with the presence of a 1.3-kilobase (kb) plasmid pSN2 (22). In vitro transcription of pSN2 DNA in cell extracts from *S. aureus* (6) and *Escherichia coli* (4, 6) and in *Bacillus subtilis* minicells (4) results in the production of two polypeptides (20 and 12 kilodaltons) which do not react with anti-SEB immunoglobulins. Similar results have been obtained in translation of pSN2 mRNA in either reticulocyte lysate or in wheat germ systems (E. Israeli, E. Kaufmann, and A. Shafferman, Isr. J. Med. Sci. **16**:470, 1979). Transformation of pSN2 to heterologous nontoxigenic recipients does not result in SEB-producing transformants (4, 6). Thus, although Dyer and Iandolo (4) concluded that pSN2 may provide regulatory functions essential for SEB synthesis, the results of Khan and Novick (6) indicate that the plasmid is not in any way involved in the production of SEB. They proposed that *entB* is part of a special genetic system, such as a hitchhiking transposon. The chromosomal location of determinants for enterotoxin C_1(SEC_1) production was suggested by Betley and Bergdoll (M. J. Betley and M. S. Bergdoll Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, D38, p. 49).

We have used transformation techniques to determine the involvement in SEB and SEC_1 synthesis of a 56.2-kb penicillinase plasmid (pZA10) which also carries genetic determinants for resistance to penicillin, cadmium, mercury, lead, arsenate, and arsenite. Upon the basis of successful transformation of *S. aureus* RN450 recipients to SEB and SEC_1 production, we propose that pZA10 carries either structural genes for SEB and SEC_1, or essential regulatory genes which switch on cryptic structural genes in the recipients.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are described in Table 1. The wild-type *S. aureus* 6344 was isolated from a clinical specimen by M. Komarov from The National Center for Staphylococcal Enterotoxins in Israel. This strain is sensitive to bacteriophages 29 and 96, and it does not produce protein A, a protein which interferes in immunoassays.

Culture media. Trypticase soy broth (BBL Microbiology Systems) and the solid medium, tryptic soy agar (Trypticase soy broth supplemented with 1.5% agar; Difco Laboratories), were the standard nutrient media used in all the genetic manipulations. CH medium (22) was used to grow bacteria for determination of enterotoxin.

Identification and quantitation of enterotoxins. *S. aureus* strains were grown in CH medium for 24 h at 37°C. The cultures were centrifuged, and the supernatant was analyzed for the presence of SEB and SEC_1 by one of the following methods: the Oudin method for quantitative determination (26), the optimal sensitivity plate method (19), the microslide gel double diffusion test (3), or radioimmunoassay. Radioimmunoassay was carried out by the following procedure. The reaction mix contained 10 μl of anti-enterotoxin serum (diluted to the concentration binding 20 to 30% of iodinated enterotoxin), 10 μl of iodinated enterotoxin (5×10^4 cpm),

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TABLE 1. *S. aureus* strains

Strain	Plasmid profile	Relevant phenotype	Derivation or description
6344	pZA10, pZA1	Pc^r Tc^r SEB^+ SEC_1^+	Wild-type strain
AZ1110	pZA10	Pc^r Tc^s SEB^+ SEC_1^+	Spontaneous elimination from 6344
AZ1016	pZA10	Pc^r Tc^s SEB^+ SEC_1^+	Et Br-treated 6344
AZ1116	pZA10	Pc^r Tc^s SEB^+ SEC_1^+	SDS-treated 6344
AZ1025	pZA10	Pc^r Tc^s SEB^+ SEC_1^+	Heat-treated 6344
AZ1024	None	Pc^r Tc^s SEB^+ SEC_1^+	Heat-treated 6344
AZ1022	None	Pc^s Tc^s SEB^- SEC_1^-	Heat-treated 6344
AZ1112	None	Pc^s Tc^s SEB^- SEC_1^-	SDS-treated 6344
AZ1002	None	Pc^s Tc^s SEB^- SEC_1^-	EtBr-treated 6344
AZ1072	pZA1	Pc^s Tc^r SEB^- SEC_1^-	AZ1002 × pZA1
AZ1160	pZA10	Pc^r Tc^s SEB^+ SEC_1^+	AZ1002 × pZA10
AZ1708	pZA1708	Pc^r SEB^+ SEC_1^+	RN450 × pZA10
AZ1194	pZA1194	Pc^r SEB^+ SEC_1^+	RN451 × pZA10
AZ1147	pZA1147	Pc^r SEB^+ SEC_1^-	AZ1112 × pZA10
AZ1144	pZA1144	Pc^r SEB^+ SEC_1^-	AZ1112 × pZA10
AZ3311	None	Pc^r SEB^- SEC_1^-	SDS-treated AZ1144
AZ1502	None	Pc^r SEB^+ SEC_1^+	ISP125 × AZ1024
AZ1361	pZA1361	Pc^r Cad^r SEB^+ SEC_1^+	RN450 × AZ1024
AZ1369	None	Pc^r Cad^s SEB^+ SEC_1^+	RN450 × AZ1024
RN450	None	Pc^s Tc^s SEB^- SEC_1^-	NCTC 8325-4[b]
RN451	None	Pc^s Tc^s SEB^- SEC_1^-	NCTC 8325 (Φ11)[b]
ISP125	None	Pc^s SEB^- SEC_1^- Thy^- Pur^- Pig^- Tmm^r	NCTC 8325[c]

[a] Phenotype designations: Tc^r, tetracycline resistant; Tc^s, tetracycline sensitive; Pc^r, penicillin resistant; Pc^s, penicillin sensitive; Cad^r, cadmium resistant; Cad^s, cadmium sensitive; SEB^+, SEB producer; SEB^-, SEB nonproducer; SEC_1^+, SEC_1 producer; SEC_1^-, SEC_1 nonproducer: Pur^-, purine requirement; Thy^-, thymine requirement; Pig^-, inability to elaborate yellow pigment; Tmn^r, tetracycline and minocycline resistant.

[b] Strains RN450 and RN451 were obtained from R. P. Novick, Department of Plasmid Biology, The Public Health Research Institute of the City of New York, Inc., New York, N.Y.

[c] Strain ISP125 was obtained from P. A. Pattee, Department of Bacteriology, Iowa State University, Ames, Iowa.

and either cold standard solutions of enterotoxin (0.5 to 250 ng) or up to 300 μl of test sample, in a total volume of 500 μl of a buffer containing (wt/vol): 1.5% barbitol (pH 8.8), 0.9% NaCl, 0.27% EDTA, 0.5% Triton X-100, 1% bovine serum albumin 0.01% sodium dodecyl sulfate (SDS), and 0.01% sodium azide. The mixture was incubated for 16 h at room temperature, and 20 μl of a chloroform-treated *S. aureus* Cowan I (NCTC 8530) cell suspension was added. After incubation for 30 min at room temperature, the cells were pelleted and washed once with reaction buffer containing 0.2% SDS. The radioactive immunological complex adsorbed to the cells was measured in a γ counter. Direct identification of enterotoxin-producing colonies was performed by plating a diluted suspension of bacteria (ca. 50 colonies) on agar-serum plates. The agar-serum plates were prepared from CH medium containing 1% agar, 0.005% trypan blue, and a proper dilution of antienterotoxin serum. An enterotoxin-producing colony was identified by a precipitating halo. Monovalent anti-SEB and anti-SEC_1 sera were prepared by the method of Silverman (23).

Plasmid elimination. For plasmid elimination experiments, 10^2 cells from exponential growth phase were inoculated into 25 ml of Trypticase soy broth containing various concentrations of ethidium bromide (EtBr) (6×10^{-6} to 2×10^{-5} M) or SDS (0.05 to 0.09%). The cultures were grown for 24 h at 37°C. In high-temperature elimination experiments, the cells were grown for 48 h at 43°C. After incubation, the bacteria were plated onto nonselective tryptic soy agar plates. The resulting colonies were replica plated to selective plates containing either 5 μg (7.5 U) of penicillin per ml or 5 μg of tetracycline per ml, and the colonies formed were scored for unselected genetic markers.

Preparation of transforming DNA and transformation. Plasmid and chromosomal DNA were prepared by procedures described by Novick et al. (14) and Lindberg et al. (7), respectively. Cultures were grown to a level that results in a maximum induced competence (17) and transformed with 1 μg of plasmid DNA or 20 μg of chromosomal DNA per 1×10^9 cells. Transformants were selected on tryptic soy agar plates containing 0.05 μg (0.075 U) of penicillin per ml. The colonies were transferred twice on selective plates and subsequently scored for cotransformation phenotypes for resistance to metal ions by the disc test (15) and for SEB and SEC_1 production after cultivation in CH medium.

Enzymes and reactions. Restriction endonucleases *Bam*HI, *Bgl*II, and *Eco*RI were obtained from Bethesda Research Laboratories. *Sal*I and *Hin*dIII were obtained from Boehringer Mannheim Biochemicals. Digestion conditions were as suggested by the manufacturer. Usually, the reaction was performed in a total volume of 25 μl, containing 1 μg of DNA, the appropriate buffer, and a twofold excess of enzyme units to give complete cleavage in 1 h. For double and triple digests, the reaction was done by incubating for 1 h with the enzyme requiring a buffer of lower ionic strength, adjusting the buffer for the second enzyme, and incubating the reaction for another hour. In all cases, the reactions were stopped by incubating for 10 min at 65°C and adding 5 μl of dye solution containing 33% glycerol, 7% SDS, and 0.07% bromophenol blue (10).

Restriction analysis of plasmid DNA. Digests of plasmid DNA with restriction enzymes were analyzed by electrophoresis in various concentrations of agarose (0.7 to 1%; Sigma Chemical Co.) in Tris borate buffer (10). Electrophoresis was carried out in a horizontal slab gel apparatus at 4 V/cm until the bromophenol blue tracing dye reached the end of the gel. The gel was stained in a solution on EtBr for 30 min, illuminated with UV transillumination, and photographed with Polaroid 667 film. The standard reference digests used were λ C1857 S7 (New England BioLabs) cleaved with *Eco*RI, *Bgl*II, *Bam*HI, or *Hin*dIII (25).

TABLE 2. Influence of plasmid elimination on the production of SEB and SEC_1 by *S. aureus* 6344

Representative strain	Plasmid profile	Plasmid curing method[a]	Frequency of curing (%)	No. of cured isolates tested	Enterotoxin production	
					SEB	SEC_1
6344	pZA1, pZA10				+	+
AZ1110	pZA10	Spontaneous	1.5	2	+	+
AZ1116	pZA10	SDS	73.0	160	+	+
AZ1016	pZA10	EtBr	1.78	4	+	+
AZ1025	pZA10	43°C	1.85	13	+	+
AZ1112	None	SDS	0.45	3	−	−
AZ1002	None	EtBr	0.2	1	−	−
AZ1022	None	43°C	0.14	1	−	−

[a] Elimination experiments were performed as described in the text.

Preparation and hybridization of DNA blots. Plasmid DNA (1 μg) was cleaved with restriction enzymes, subjected to electrophoresis on 0.8% agarose in TAE buffer (21), and blotted to nitrocellulose membrane (Schleicher & Schuell, Inc.) by the method of Southern (24). The DNA was dried under vacuum for 2 h at 80°C and hybridized with plasmid DNA that had been labeled with [α-^{32}P]dCTP to a specific activity of 5×10^8 cpm per μg of DNA by the nick translation procedure (9). Hybridization was carried out at 68°C for 24 h with 10^6 cpm in a total volume of 5 ml of hybridization solution consisting of 6× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate), 0.02% Ficoll, 0.02% polyvinylpyrollidon, 0.02% bovine serum albumin, and 100 μg of sonicated and denatured sperm DNA per ml. The filters were washed with 0.1× SSC–0.1% SDS at 68°C. Agfa Curix RP2 X-ray film was exposed to the filter for various times.

RESULTS

Plasmid-linked enterotoxinogeny. *S. aureus* 6344 is an enterotoxinogenic strain, producing both SEB and SEC_1 at concentrations of 100 and 5 μg per ml, respectively, in overnight cultures. This strain contains two plasmids: a tetracycline resistance plasmid (4.3 kb, pZA1) and a penicillinase plasmid (56.2 kb, pZA10). Our previous work (22) and the work of others (4, 20) indicate correlation between the presence of plasmids and production of SEB in *S. aureus*. Therefore, at the first stage of this work, a detailed analysis was undertaken to clarify the involvement of plasmids in SEB and SEC_1 production in the clinical strain *S. aureus* 6344. Plasmid elimination experiments were performed, and the cured mutants were checked for production of SEB and SEC_1. Elimination of the plasmids was achieved by growing the strain either at high temperature (43°C) or in the presence of EtBr or SDS (Table 2; see above for details). Cured mutants were selected for sensitivity to tetracycline and penicillin and were checked for production of SEB and SEC_1. The results presented in Table 2 indicate that the elimination of pZA1 had no effect on the production of SEB and SEC_1, whereas coelimination of pZA10 with pZA1 resulted in loss of the ability to produce both enterotoxins. Curing of pZA10 alone was not observed. The results of the curing experiments indicated that pZA10 contains, in addition to determinants for *bla*$^+$ and enterotoxin (SEB and SEC_1) production genes which confer resistance to the metal ions cadmium, mercury arsenate, arsenite, and lead.

Transformation with pZA10 DNA. To show that enterotoxinogenic determinants are located on pZA10, we transformed nontoxinogenic recipients with plasmid DNA. The recipients were either cured penicillin-sensitive nontoxinogenic *S. aureus* 6344 or the heterologous strains RN450 and RN451. Transformants were selected for resistance to penicillin and were scored for the production of SEB and SEC_1. The results of the transformation experiments are summarized in Table 3. Cotransformation of resistance to penicillin with SEB and SEC_1 production was observed. About 20% of all penicillin-resistant transformants produced either one or both enterotoxins.

The linkage between the enterotoxinogenic determinants and the metal ion resistance genes was analyzed in the enterotoxinogenic transformants. The results (Table 4) indicate linkage between those genes. Although the isogenic pZA10 transformants AZ1144 and AZ1147 lost different pZA10-linked markers, the heterologous transformant AZ1708 retained all pZA10-linked genes (Table 5). The loss of the SEC_1 determinants was observed in AZ1144 and AZ1147. SEB$^-$ SEC_1^+ transformants were not detected, and this asymmetry in the loss of determinants for enterotoxinogeny was confirmed in transformation experiments with chromosomal DNA (see Table 7).

Plasmid analysis. The size of pZA10 was determined by restriction enzyme analysis. The plasmid was cleaved with the enzymes *Bam*HI, *Bgl*II, and *Eco*RI. The results indicate that the total length of pZA10 is 56.2 kb (Fig. 1). *Sal*I cleaved

TABLE 3. Transformation of *S. aureus* strains with pZA10 DNA

Experiment no.	Recipient[a]	No. of Pc^r transformants	Frequency of transformation	No. of isolates tested for enterotoxin production	No. of Pc^r Ent^+ cotransformants		
					SEB$^+$ SEC_1^+	SEB$^+$ SEC_1^-	SEB$^-$ SEC_1^+
1	AZ1002	42	3×10^{-10}	42	3	0	0
2	AZ1112	14	4.6×10^{-9}	14	0	9	0
3	RN450	18	5×10^{-10}	18	2	0	0
4	RN451	7	4×10^{-10}	7	3	0	0

[a] For bacterial strains, see Table 1.

TABLE 4. Linkage between enterotoxin production and metal ion resistance in transformants of *S. aureus* with pZA10 DNA

Recipient	No. of Pc^r SEB$^+$ transformants scored:	No. of transformants of the following phenotype[a]:				
		SEC_1^+	Asa^r	Mer^r	Cad^r	Lea^r
AZ1002	2	2	2	2	2	2
AZ1112	8	0	8	8	5	4
RN450	2	2	2	2	2	2
RN451	2	2	2	2	2	2
Total (%)	14	6 (45.7)	14 (100)	14 (100)	11 (78.6)	10 (71.4)

[a] Asa, Arsenate; Mer, mercury; and Lea, lead. For other phenotype abbreviations, see footnote *a* of Table 1.

pZA10 into three fragments of 34.5, 18.1, and 3.6 kb (Fig. 1, lane 1). The addition of *Bam*HI resulted in cleavage of the large *Sal*I fragment alone (into 27.1- and 7.4-kb fragments; Fig. 1, lane 2). *Bgl*II cleaved pZA10 into five fragments of 25.0, 15.8, 8.6, 6.0, and 0.8 kb (Fig. 1, lane 5). The addition of *Bam*HI resulted in cleavage of the 15.8-kb *Bgl*II fragment (into 11.0- and 4.8-kb fragments; Fig. 1, lane 4). These results established a single *Bam*HI cleavage site as a reference point to construct a pZA10 restriction cleavage map (Fig. 2). Accordingly, the alignment of *Bgl*II cleavage sites within the *Sal*I fragments was established.

Restriction digest analysis of plasmid DNA in transformants (Table 6) showed that pZA1708 is identical to pZA10. pZA1147 contains an additional DNA of 30.2 kb, resulting in a total length of 86.4 kb. pZA1144 has a deletion of 15.3 kb, resulting in a length of 40.9 kb. Cross-hybridization experiments were performed between these plasmids (Fig. 3a and b). Although results of hybridization experiments between pZA10 (Fig. 3b, lanes 1 and 4) and pZA1147 (Fig. 3b, lanes 2 and 5) showed homology between the two plasmids, a low hybridization intensity with pZA10 *Bgl*II fragments of 8.6 and 6.0 kb may indicate deletions in these pZA10 DNA fragments. This observation was substantiated by additional blot hybridization experiments (Fig. 4) in which pZA10 *Bam*HI-*Sal*I-*Bgl*II digests were hybridized in parallel to labeled pZA10 and pZA1147. Since AZ1147 does not produce SEC_1, the 8.6- and 6.0-kb *Bgl*II fragments may contain the determinants for SEC_1 production. pZA1147 shows a ladder of nine small fragments which hybridize with pZA10. This pattern was observed at a lower intensity in pZA10 and may result from partial digestion of a repeated sequence of ca. 0.2 to 0.5 kb. Homology was found between the 18.1-kb fragment of pZA10 (Fig. 3b, lanes 1 and 7) and a single fragment of pZA1144 exceeding the size of 18.1 kb (Fig. 3b, lanes 3 and 9). Fragments of pZA1144 and pZA1147 which were not of pZA10 origin were homologous (Fig. 3b, lanes 5, 6, 8, and 9). These fragments were of chromosomal origin since they hybridized to *Bgl*II digest of chromosomal DNA from the recipient strain AZ1112 (Fig. 5B, lane 4). Cloning and expression of the pZA10 *bla*$^+$ fragment showed that the *bla*$^+$ gene is located on the 6.0-kb *Bgl*II fragment. Hybridization experiments between nick-translated ^{32}P-labeled pZA10 *Bgl*II 6.0-kb fragments and plasmids pZA10, pZA1147, and pZA1144 (Fig. 6) indicated that plasmids pZA10 and pZA1147 (Fig. 6b, lanes 1 and 2) contain the *bla*$^+$ gene. In transformant AZ1144, which was selected for penicillin resistance, the *bla*$^+$ fragment was detected in the chromosome by hybridization (data not presented).

Plasmid-curing experiments with SDS were performed in strain AZ1144 to determine whether the plasmid-borne 18.1-kb fragment is involved in SEB production. Of ca. 1,000 colonies screened on agar-serum plates, 3 did not produce SEB. These isolates were plasmid free and did not contain the 18.1-kb fragment in the chromosome as tested by hybridization with nick-translated ^{32}P-labeled pZA1144 (data not presented). These results indicated that determinants for SEB production are located on pZA10 *Bgl*II-*Sal*I fragment of 18.1 kb.

TABLE 5. Phenotypes of *S. aureus* strains 6344, AZ1112, and RN450 and some pZA10 transformants

Strain[a]	pZA10-linked markers[b]							
	Pc	SEB	SEC_1	Asa	Asi	Mer	Cad	Lea
S. aureus 6344	r	+	+	r	r	r	r	r
AZ1112	s	−	−	s	s	s	s	s
RN450	s	−	−	s	s	s	s	s
AZ1708	r	+	+	r	r	r	r	r
AZ1147	r	+	−	r	r	r	r	r
AZ1144	r	+	−	r	r	r	s	s

[a] For bacterial strain, see Table 1.

[b] r, Resistant; s, sensitive; +, production of indicated enterotoxin; −, no production of indicated interotoxin; and Asi, arsenite resistant or sensitive as indicated. For other phenotype abbreviations see footnote *a* of Table 1 and footnote *a* of Table 4.

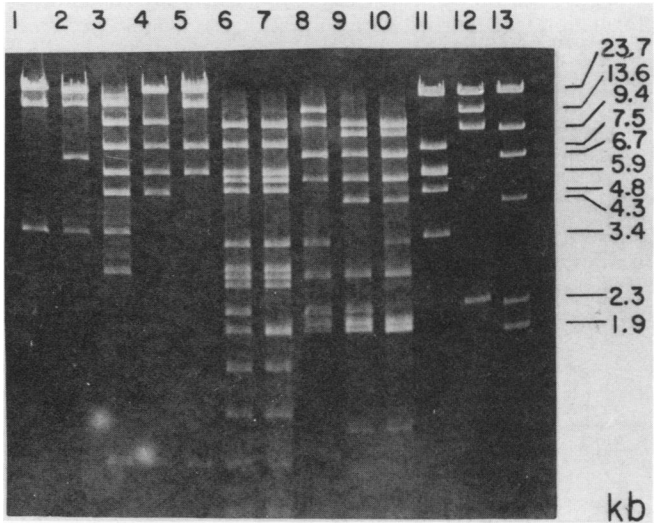

FIG. 1. Agarose gel electrophoresis of restriction fragments produced by digestion of pZA10 with the following restriction endonuclease enzymes: *Sal*I (lane 1); *Sal*I and *Bam*HI (lane 2); *Sal*I, *Bam*HI, and *Bgl*II (lane 3); *Bam*HI and *Bgl*II (lane 4); *Bgl*II (lane 5); *Bgl*II and *Eco*RI (lane 6); *Bgl*II, *Bam*HI, and *Eco*RI (lane 7); *Eco*RI (lane 8); *Eco*RI and *Sal*I (lane 9); and *Eco*RI, *Sal*I, and *Bam*HI (lane 10). For estimation of molecular weights of the endonuclease fragments, electrophoretic mobilities were compared with the molecular weights of coliphage λ digested with *Eco*RI (lane 11), *Bgl*II (lane 12), and *Hin*dIII (lane 13).

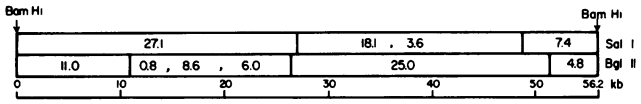

FIG. 2. Linear restriction cleavage map of pZA10. Numbers indicate fragment sizes in kilobase pairs. Placement of more than one fragment in a box indicates that their relative positions are not yet determined.

Chromosomal integration and excision of pZA10. *S. aureus* 6344 grown at 43°C was plated on nonselective plates; the resulting colonies were replica plated on tetracycline, penicillin, and cadmium (10^{-3} M) plates. A Pc^r Tc^s colony exhibiting resistance to all metallic ions except cadmium was isolated, and the strain was designated AZ1024. AZ1024 produced SEB and SEC_1 at the same level as did *S. aureus* 6344. Plasmid analysis showed absence of extrachromosomal DNA. The integration of pZA10 into the chromosome of strain AZ1024 was confirmed in hybridization experiments between restriction endonuclease digest of chromosomal DNA from AZ1024 and nick-translated ^{32}P-labeled pZA10 (Fig. 7). The results of these hybridization experiments indicated that the chromosome of strain ZA1024 contains DNA fragments which are homologous to pZA10. Homology to pZA10 could not be detected in strain RN450 (Fig. 7, lane 1), and in pZA10-cured strains, as shown for chromosomal DNA of AZ1112 (the cured strain), which did not hybridize to pZA10 DNA sequences in pZA1144, which is a related plasmid (Fig. 5B, lane 4 as compared with lane 1).

To examine whether the integrated *bla*$^+$ gene in AZ1024 is linked with the enterotoxinogenic determinants, we transformed penicillin-sensitive nontoxinogenic recipients, either pZA10-cured 6344 or RN450 and RN451, with ZA1024 chromosomal DNA. Transformants were selected for Pc^r and tested for production of enterotoxins (Table 7). Of 34 Pc^r transformants tested, 20 (58.8%) produced both SEB and SEC_1 or SEB alone. Cotransformation of metal ion resistance genes with enterotoxinogeny was scored. The cotransformation frequencies were 66.6% for Lea^r, 66.6% for Asa^r, and 33.3% for Mer^r. These results confirm the linkage between enterotoxinogeny, *bla*$^+$, and metal ion resistance genes in the plasmid-integrated state.

Three of the *S. aureus* RN450 enterotoxinogenic transformants (Table 7) showed resistance to cadmium, which was not expressed in the parental strain AZ1024 carrying the integrated plasmid. Plasmid analysis of Cad^r transformants showed the presence of extrachromosomal DNA, whereas Cad^s transformants were plasmid free. The plasmid locations of genetic determinants for SEB and SEC_1 in these Cad^r transformants were tested by curing the plasmids with SDS. Four Cad^s mutants were isolated, all of which were also SEB^- SEC_1^- Pc^s Hg^s Lea^s Asa^s Asi^s and did not carry plasmid DNA.

TABLE 6. Sizes of restriction endonuclease fragments (kb) of plasmids isolated from three pZA10 transformants

Restriction enzyme	Plasmid	Restriction fragment size (kb)	Plasmid size (kb)
*Bgl*II	pZA10	25.0, 15.8, 8.6, 6.0, 0.8	56.2
	pZA1708	25.0, 15.8, 8.6, 6.0, 0.8,	56.2
	pZA1147	30.0, 30.0, 14.7, 4.2, 4.2, 2.5, 0.8	86.4
	pZA1144	30.0, 4.2, 4.2, 2.5	40.9
*Eco*RI	pZA10	13.5, 8.8, 7.4, 7.4, 5.7, 3.5, 2.8, 2.3, 2.2, 2.1, 0.5	56.2
	pZA1708	13.5, 8.8, 7.4, 7.4, 5.7, 3.5, 2.8, 2.3, 2.2, 2.1, 0.5	56.2
	pZA1147	23.8, 12.6, 10.2, 7.8, 5.8, 5.2, 4.2, 3.8, 3.4, 3.0, 2.5, 2.0, 1.0, 0.8, 0.3	86.4
	pZA1144	23.8, 7.8, 4.2, 3.8, 1.0, 0.3	40.9

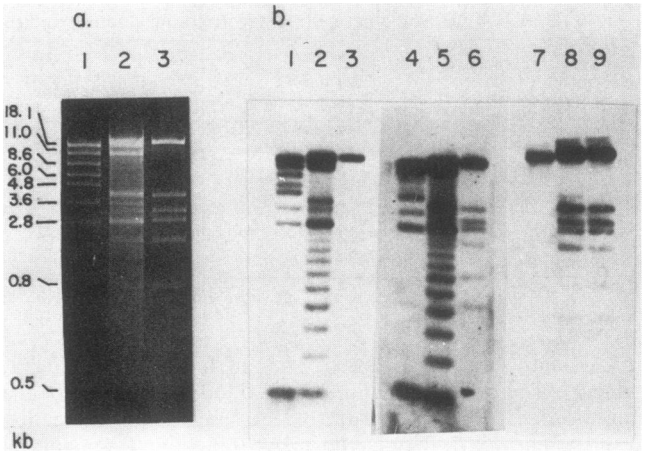

FIG. 3. Cross-hybridization between various plasmids. (a) Agarose gel electrophoresis of restriction fragments produced by the enzymes *Bam*HI, *Sal*I, and *Bgl*II cleaving the plasmids pZA10 (lane 1), pZA1147 (lane 2), and pZA1144 (lane 3). (b) After gel electrophoresis, the fragments were transferred to nitrocellulose membranes where they were hybridized to nick-translated ^{32}P-labeled pZA10 (lanes 1 through 3); ^{32}P-labeled pZA1147 (lanes 4 through 6), and ^{32}P-labeled pZA1144 (lanes 7 through 9).

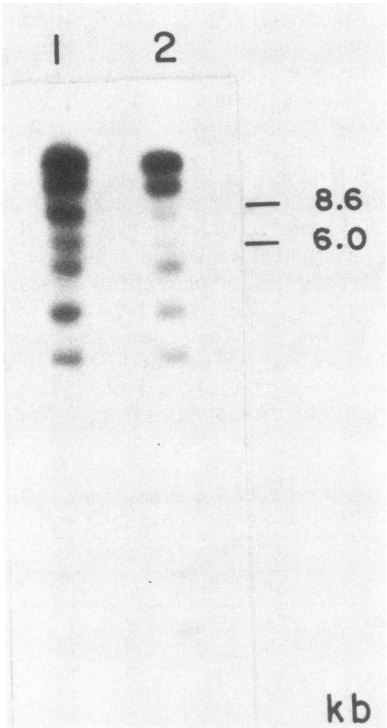

FIG. 4. Identification of pZA10 DNA sequences in pZA1147. pZA10 was cleaved by the restriction enzymes *Bam*HI + *Sal*I + *Bgl*II. After electrophoresis in agarose gel, the DNA fragments were transferred to nitrocellulose membrane and hybridized to nick-translated ^{32}P-labeled pZA10 (lane 1) and ^{32}P-labeled pZA1147 (lane 2).

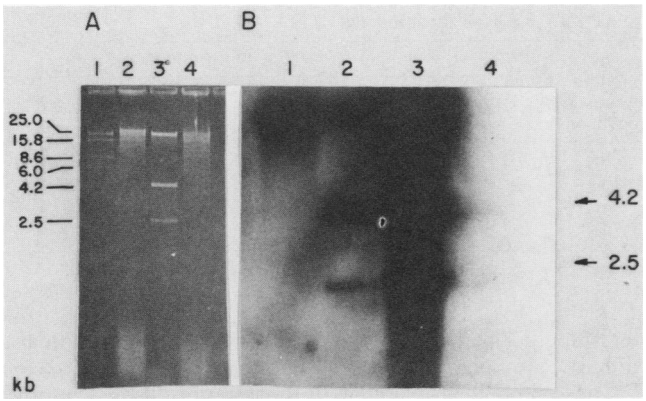

FIG. 5. Identification of host chromosomal DNA fragments in plasmid pZA1144. (A) Restriction endonuclease *Bgl*II cleavage of pZA10 (lane 1), total DNA from strain AZ1144 (lane 2), pZA1144 (lane 3), and total DNA from strain AZ1112 (lane 4). (B) After gel electrophoresis, the DNA fragments were transferred to nitrocellulose membrane and hybridized to nick-translated ^{32}P-labeled pZA1144.

The plasmid carried by the Pc^r Cad^r transformant (AZ1361) was analyzed by *Eco*RI endonuclease cleavage (Fig. 8a, lane 2) and hybridization to either nick-translated ^{32}P-labeled pZA10 (Fig. 8b) or to ^{32}P-labeled pZ1361 (Fig. 8c). The analysis of the transformant showed (i) homology with pZA10, (ii) change in restriction pattern, and (iii) increase in size. Comparison of the *Eco*RI cleavage fragments of pZA1361 to those of pZA10 revealed the absence of pZA10 *Eco*RI doublet fragments of 7.4 kb and the addition of four new fragments of 21.8, 8.0, 5.2, and 4.4 kb. The first two new fragments did not hybridize to ^{32}P-labeled pZA10 (Fig. 8b, lane 2) and were lost in later plasmid isolations without any effect on SEB and SEC_1 production. *Eco*RI digest pattern of the stable derivative of pZA1361 is shown in Fig. 8d, lane 1. However, in a reciprocal experiment, the labeled pZA1361 hybridized to all pZA10 *Eco*RI fragments (Fig. 8c, lane 1). This finding suggested DNA rearrangement of pZA10 sequences in pZA1361.The hybridization pattern of pZA10 to *Eco*RI digest of AZ1024 chromosomal DNA (from which pZA1361 was excised) (Fig. 7b, lane 2) indicated that DNA rearrangement had already occurred during integration of pZA10. Thus, pZA10 can integrate into and excise from the host chromosome. Plasmid-linked determinants for SEB and SEC_1 production and for resistance to penicillin and metal ions (except cadmium) were expressed in both the integrated and the excised form. These results may indicate that integration by a recombination event occurs within or near the *cad* gene and involves DNA rearrangement.

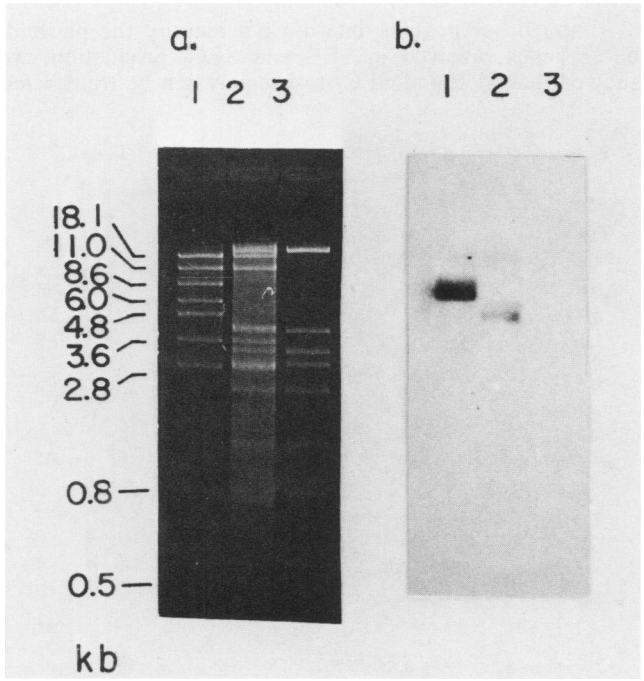

FIG. 6. Hybridization between pZA10 *bla*$^+$ gene and various plasmids. (a) Agarose gel electrophoresis of restriction fragments from plasmids pZA10 (lane 1), pZA1147 (lane 2), and pZA1144 (lane 3), digested by the enzymes *Bam*HI, *Sal*I, and *Bgl*II. (b) After gel electrophoresis, the DNA fragments were transferred to nitrocellulose membrane and hybridized to nick-translated ^{32}P-labeled pZA10 *Bgl*II, a 6.0-kb fragment which contains the *bla*$^+$ genes.

DISCUSSION

This work was undertaken to extend previous investigations (22) on possible localization of genetic determinants of staphylococcal enterotoxins. The identification of genes involved in enterotoxin production has been recently the subject of several studies (4, 6, 8, 16, 20). The results of these studies led to the conclusion that the genetic determinants of staphylococcal enterotoxins are chromosomal. How-

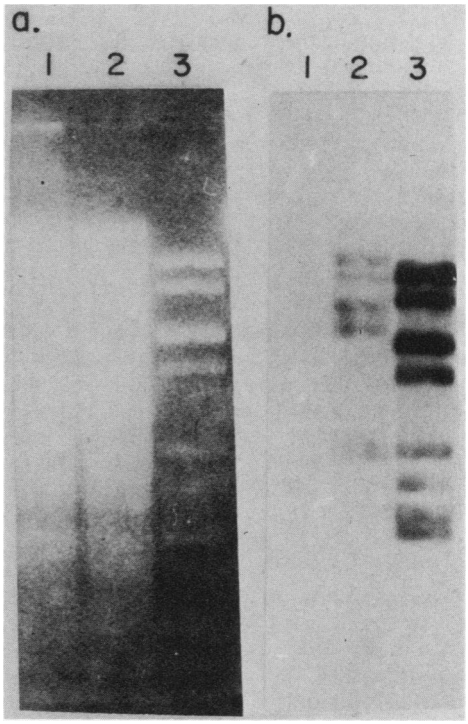

FIG. 7. Identification of pZA10 sequences in a strain containing the integrated plasmid. Restriction endonuclease cleavage pattern of *Eco*RI digestion of pZA10 and chromosomal DNA from strains AZ1024 and RN450. After electrophoresis in agarose gel (a), the DNA fragments were transferred to nitrocellulose membrane by the Southern blotting procedure and hybridized to nick-translated ^{32}P-labeled pZA10 (b). Lanes: 1, RN450 *Eco*RI; 2, AZ1024 *Eco*RI; and 3, pZA10 *Eco*RI.

TABLE 7. Transformation of *S. aureus* strains with AZ1024 chromosomal DNA

Experiment no.	Recipient[a]	No. of Pc^r transformants	Frequency of transformation	No. of isolates tested for enterotoxin production	No. of Pc^r Ent$^+$ cotransformants		
					SEB$^+$ SEC$_1$$^+$	SEB$^+$ SEC$_1$$^-$	SEB$^-$SEC$_1$$^+$
1	AZ1002	6	4.0×10^{-10}	6	3	0	0
2	RN450	470	3.0×10^{-8}	10	4	4	0
3	ISP125	4	2.2×10^{-10}	4	2	0	0
4	RN451	14	2.3×10^{-9}	14	3	4	0

[a] For bacterial strains, see Table 1. The enterotoxinogenic transformants of strain ISP125 were confirmed for Pur$^-$ and Tmn^r phenotype.

ever, only for SEA has the gene been identified and has its chromosomal site been determined (8, 16). The genetic determinants of SEB and SEC$_1$ remained unidentified. Khan and Novick concluded that although the determinants of SEB are chromosomal, it is possible that they are a part of a hitchhiking transposon (6).

In the present work, *S. aureus* 6344, a penicillin- and tetracycline-resistant clinical isolate that produced both SEB and SEC$_1$, was analyzed for plasmid-linked enterotoxinogeny. Coelimination experiments indicated the association of SEB and SEC$_1$ determinants with a 56.2-kb penicillinase plasmid, pZA10, carried by the isolate. Transformation of standard nontoxinogenic recipients (NCTC 8325, RN450) with pZA10 DNA produced transformants which synthetized both SEB and SEC$_1$, expressed β-lactamase synthesis and resistance to metallic ions, and harbored the transferred plasmid. Thus, the present findings differ from those obtained with another plasmid, pSN2 (1.3 kb) isolated in our laboratory (22). Although the capability to produce SEB was coeliminated with pSN2, introduction of the plasmid into standard nontoxinogenic recipients did not yield transformants which produced SEB (4, 6). Our finding that pZA10 transforms standard nontoxinogenic strains may indicate that it either encodes for the two enterotoxins or that its gene products switch on cryptic chromosomal genes in the standard recipient.

Characterization of pZA10 DNA carried by transformants showed that genetic exchanges occur during transformation. Some pZA10-derived plasmids isolated from transformants carried deletions and insertions and had changed restriction cleavage patterns. Phenotypically, these changes correlated with the loss of various plasmid-encoded markers, including the capability to produce SEC$_1$ or SEC$_1$ and SEB but not SEB alone. The ability to produce SEB could be correlated with the presence of a plasmid-borne pZA10 18.1-kb fragment (in strain AZ1144). The genetic information for SEC$_1$ synthesis could be identified on a pZA10 segment which is deleted from the adjacent *Bgl*II fragments of 8.6 and 6.0 kb (in strain AZ1147). During transformation with pZA10, genetic exchanges with the host chromosome occurred. The established extrachromosomal DNA contained chromosomal DNA sequences (pZA1144, pZA1147) attached to pZA10 fragments.

By growth of the wild-type strain *S. aureus* 6344 at 43°C, pZA10 was integrated into the host chromosome (strain AZ1024). Transformation with this chromosomal DNA to RN450 recipients resulted in excision of the plasmid from the chromosome in some recipients. The reestablished plasmid (pZA1361) carried genetic determinants for the synthesis of SEB and SEC$_1$ and had a changed restriction cleavage pattern. The integration into the host chromosome caused the inactivation of the cadmium resistance gene alone; all the other plasmid markers were expressed. Reestablishment of the plasmid in the recipient restored cadmium resistance. Thus, the *cad* locus is implicated as the integrational recombination site.

These findings are similar to those reported by a number of authors on interaction of *S. aureus* plasmids with each other (11, 13) or with the host chromosome (5, 18). The penicillinase plasmid pI258 was found by Novick (12) and Pattee et al. (18) to integrate into the bacterial chromosome near the attachment site for bacteriophage Φ11.

pZA10 and its derivative exhibited genetic and physical stability in the plasmid state. When newly introduced into recipient cells by transformation or when forced to integrate into the chromosome, these plasmids exhibited DNA rearrangements. The integrated plasmid as well as its excised derivative carried pZA10 DNA sequences in new arrangements. Moreover, plasmids derived through pZA10 transformation carried chromosomal DNA attached to pZA10 fragments. We assume that DNA rearrangements of pZA10 are triggered through integration with the chromosome and that this event may precede plasmid establishment in transformants. Similar integrational events occurring during plasmid transformation were suggested in other microbial systems (2).

Although our present data did not identify the plasmid-borne genes involved in SEB and SEC$_1$ production, we showed that SEB and SEC$_1$ toxinogeny can be transferred

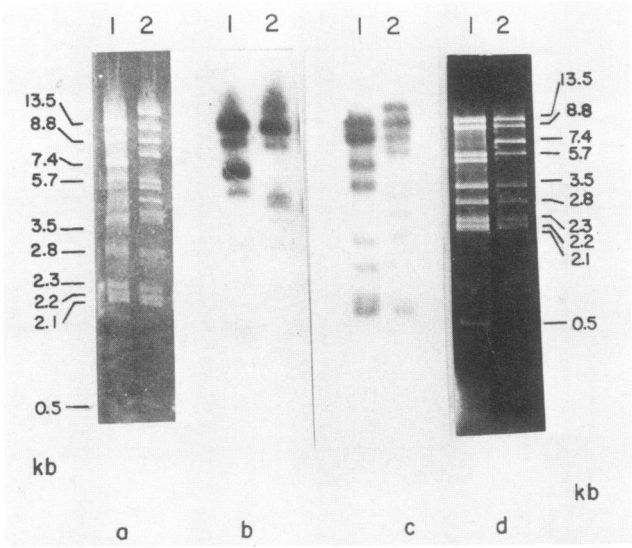

FIG. 8. Comparison between *Eco*RI digests of pZA10 and pZA1361. (a) Agarose gel electrophoresis of the *Eco*RI-generated fragments of pZA10 (lane 1) and pZA1361 (lane 2). (b and c) Autoradiography of hybridization experiments between pZA10 (lane 1) and pZA1361 (lane 2) with nick-translated ^{32}P-labeled pZA10 (b) and ^{32}P-labeled pZA1361 (c). (d) Agarose gel electrophoresis of a stable form of pZA1361 (lane 1) and pZA10 (lane 2).

with pZA10 DNA to standard nontoxinogenic strains. Two possible explanations may be offered for the latter observation: (i) pZA10 harbors SEB and SEC_1 structural genes, or (ii) pZA10 encodes for gene products which switch on cryptic chromosomal structural genes of SEB and SEC_1. The nature of the plasmid-borne genetic determinants involved in SEB and SEC_1 production is currently being studied by cloning of pZA10 restriction fragments and by the use of oligonucleotide probes specific for SEB and SEC_1 structural genes.

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