Nucleotide Sequence of the Type A Staphylococcal Enterotoxin Gene

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We determined the nucleotide sequence of the gene encoding staphylococcal enterotoxin A (*entA*). The gene, composed of 771 base pairs, encodes an enterotoxin A precursor of 257 amino acid residues. A 24-residue N-terminal hydrophobic leader sequence is apparently processed, yielding the mature form of staphylococcal enterotoxin A (M_r , 27,100). Mature enterotoxin A has 82, 72, 74, and 34 amino acid residues in common with staphylococcal enterotoxins B and C₁, type A streptococcal exotoxin, and toxic shock syndrome toxin 1, respectively. This level of homology was determined to be significant based on the results of computer analysis and biological considerations. DNA sequence homology between the *entA* gene and genes encoding other types of staphylococcal enterotoxins was examined by DNA-DNA hybridization analysis with probes derived from the *entA* gene. A 624-base-pair DNA probe that represented an internal fragment of the *entA* gene hybridized well to DNA isolated from EntE⁺ strains and some EntA⁺ strains. In contrast, a 17-base oligonucleotide probe that encoded a peptide conserved among staphylococcal enterotoxins A, B, and C₁ hybridized well to DNA isolated from EntA⁺, EntB⁺, EntC₁⁺, and EntD⁺ strains. These hybridization results indicate that considerable sequence divergence has occurred within this family of exotoxins.

Staphylococcal enterotoxins are protein toxins that, when ingested, cause staphylococcal food poisoning syndrome (4). These proteins have other biological activities including pyrogenicity (10), T-cell mitogenicity (2, 20, 36, 55; B. G. Smith and D. L. Archer, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, p. 71), and induction of human immune interferon (20). Some investigators have speculated that the enterotoxins have a role in diseases other than food poisoning, because the biological effects of parenteral toxins can mirror symptoms observed in *Staphylococcus aureus* infections (20).

Characterized staphylococcal enterotoxins are classified into five serological groups: A, B, C, D, and E (referred to as SEA, SEB, SEC, SED, and SEE, respectively [4]). SEC is further subdivided into three groups (SEC₁, SEC₂, and SEC₃) based on differences in minor epitopes (4, 39).

Previously we reported the cloning of the gene of SEA (*entA*) and, using the cloned gene as a probe, showed that the *entA* gene is encoded by a staphylococcal phage (6, 7). Here we report the complete nucleotide sequence of the *entA* gene from *S. aureus* FRI337.

The deduced amino acid sequence of SEA was shown to contain regions of homology to the amino acid sequences of SEB (14, 18), SEC₁ (43), type A streptococcal exotoxin (SPEA) (16, 54), and toxic shock syndrome toxin 1 (TSST-1) (8). DNA probes derived from the cloned *entA* gene as well as a synthetic oligonucleotide probe (representing the coding sequences for a highly conserved peptide present in three of the enterotoxins) were shown to hybridize to DNA isolated from other types of staphylococcal enterotoxin producers. The hybridization results also demonstrate that a high amount of sequence divergence has occurred in this family of bacterial exotoxins.

MATERIALS AND METHODS

Plasmid, strains, phages, and media. pMJB9 and pMJB38 were the sources of *entA*-containing fragments used in DNA sequence analysis and for probes derived from the *entA* gene. pMJB9 is a recombinant clone of pBR322 that contains the *entA* gene from *S. aureus* FRI337 on a 2.5-kilobase (kb) *Hind*III fragment (6). pMJB38 is a recombinant pBR322 plasmid that contains a 624-base-pair *Hinc*II-*Hgi*AI fragment that was derived from the *entA* gene. The construction of pMJB38 is described below. First pMJB27 was constructed by attaching a *Bam*HI linker (22) to the sole *Hinc*II site of the *entA* gene. pMJB27 DNA was digested with *Hgi*AI, the *Hgi*AI 4-base protruding 3' ends were made blunt ended with T4 DNA polymerase, and then the DNA was digested with *Bam*HI. This blunt-ended-*Bam*HI fragment was ligated into the polylinker region of M13mp8 replicative form that had been linearized with *Bam*HI-*Hinc*II. The insert, which contained 624 base pairs from the *entA* gene, was reisolated as a *Bam*HI-*Hind*III fragment and cloned into the *Bam*HI and *Hind*III sites of pBR322.

Escherichia coli AB259 (30) was the host strain for pBR322 recombinant plasmids. These strains were routinely grown in LB broth (28) with 100 µg of ampicillin per ml. Derivatives of phage M13 for sequencing were propagated with L broth and stored as previously described (26, 32, 51). *E. coli* JF626 [Δ (*lac pro*) *thi rpsL supE endA sbcB15 hsdR4* (F' *traD36 proA B lacI^a lacZ*ΔM15)] (gift of J. Felton) was used as the host strain for all M13 phages. Transfection with M13 derivatives was performed as described previously (45).

The following *S. aureus* strains were obtained from Merlin S. Bergdoll: DU4916 (SEB producer [EntB⁺]) (13), FRI137 (EntC₁⁺), FRI184 (Ent⁻), FRI361 (EntC₂⁺), FRI578 (EntE⁺), FRI776 (EntD⁺), FRI918 (EntE⁺), S6 (EntA⁺ EntB⁺), and S6R (an EntA⁺ EntB⁻ derivative of S6 [11]). *S. aureus* RN450 (Ent⁻) was from Richard P. Novick (33). *S. aureus* MJB265 is RN450 lysogenized with the *entA*-encoding phage FRI337-1 (7).

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S. aureus strains were routinely cultured on brain heart infusion agar plates (Difco Laboratories). For genomic DNA preparation, *S. aureus* strains were cultured in Trypticase soy broth (BBL Microbiology Systems). All *S. aureus* culture media were routinely supplemented with 5 µg of adenine, guanine, cytosine, and uracil and 20 µg of thymine per ml.

DNA preparations. Large-scale plasmid preparations were purified from lysates of chloramphenicol-amplified cultures by cesium chloride-ethidium bromide equilibrium density centrifugation (22). Restriction fragments were extracted from agarose gels by a "freeze-squeeze" technique (50).

Replicative-form phage DNA to be analyzed for insert DNA was isolated by a modified alkaline lysis method (23). Single-stranded DNA of recombinant M13 phages used for DNA sequence analysis was purified as described previously (26).

The procedure used to isolate *S. aureus* genomic DNA was based on a method for purifying *S. aureus* genomic DNA from a 100-ml staphylococcus culture (35). *S. aureus* genomic DNA used in DNA-DNA hybridization analysis was purified from 0.7 ml of an overnight Trypticase soy broth culture. The bacteria were collected by centrifugation and washed in 50 mM Tris–50 mM disodium EDTA (pH 7.5). The cell pellet was suspended in 0.6 ml of 50 mM Tris–50 mM EDTA that contained 0.5 mg of lysostaphin per ml and incubated at 37°C for 30 min, after which 1 µl of RNase A (300 µg/ml) and 3 µl of RNase T₁ (20 U/µl) were added. The mixture was incubated for 20 min at 37°C, and 18 µl of 20% sodium lauryl sulfate (SDS), 35 µl of pronase (10 mg/ml), and 5 µl of proteinase K (5 mg/ml) were then added. After 2 h of incubation at 37°C the DNA was extracted by inversion for 5 min with a 50% (vol/vol) solution of chloroform and phenol that had been saturated with 10 mM Tris–1mM EDTA, pH 8.0 (TE). The DNA was precipitated from the aqueous phase by adding 1/10 volume of 3 M sodium acetate (pH 7.0) and filling with 2 volumes of isopropanol followed by cooling at –70°C for 10 min. The DNA pellet was collected by centrifugation (10 min, 4°C), dried under a vacuum, suspended in 180 µl of TE, and stored at 4°C for at least 2 days before use.

The oligonucleotide 5' ACACCACCATACATACA3' was prepared with an Applied Biosystems DNA synthesizer and purified by passage through a Partisil 10SAX column (Whatman Ltd.) by high-pressure liquid chromatography (kindly done by Joseph Barbieri).

DNA sequence analysis. DNA sequencing was by the Sanger et al. dideoxy chain-termination procedure (41). Reagents for sequencing including [α -³²P]dATP were obtained from Amersham Corp. DNA data were analyzed by using Intelligenetics sequencing programs.

Labeling probes. Double-stranded DNA was labeled with [³²P]ATP (Amersham) by nick translation (22).

Oligonucleotides were ³²P labeled by using T4 polynucleotide kinase and [³²P]ATP by a published procedure (3). After the kinase reaction, the free [³²P]ATP was separated from the ³²P-labeled oligonucleotide with a Sepak-100 column (Waters and Associates) according to the manufacturer's instructions. Deionized water was the polar solvent used for washing the column and sample application. The ³²P-labeled oligonucleotide was eluted in 2 ml of 50% (vol/vol) methanol in water. The solution containing the ³²P-labeled oligonucleotide was evaporated to dryness in a vacuum centrifuge (8 h). The probe was dissolved in 200 µl of TE and stored at –20°C.

DNA-DNA hybridization analysis. Blot hybridization analysis was performed essentially by the method of Southern

(46), except that Zeta-probe (Bethesda Research Laboratories, Inc.) was used in place of nitrocellulose membranes. The Zeta-probe membranes were washed in 1.0% (wt/vol) SDS–0.1× SSC (20× SSC is 0.3 M sodium citrate–3.0 M sodium chloride, pH 7.0) for 30 min at 60°C before prehybridization.

³²P-labeled double-stranded DNA probes were hybridized to filters by using conditions of high or low stringency as described previously (24). The high-stringency buffer solution contained 50% (vol/vol) formamide, 5× SSC, 0.1% SDS, 1mM EDTA, 250 mg of heat-denatured salmon sperm DNA per ml, 0.05% bovine serum albumin, 0.05% polyvinylpyrrolidone, and 0.05% Ficoll. The low-stringency buffer solution was the same, except it contained 25% (vol/vol) formamide. Also, the filters were washed at 65 and 50°C for the high- and low-stringency blots, respectively. The high- and low-stringency conditions allow maximum mismatches of about 20 and 33%, respectively (29).

The ³²P-labeled oligonucleotide probe was hybridized to membrane-bound DNA as described previously (3). This procedure differed from that used to hybridized double-stranded DNA in that the hybridization buffer contained 0.9 M NaCl, 6 mM EDTA, 90 mM Tris (pH 8.0), 0.25% bovine serum albumin, 0.25% polyvinylpyrrolidone, 0.25% Ficoll, 0.1% SDS, and 250 mg of yeast tRNA per ml. Also the filters were washed in 0.9 M NaCl–6 mM EDTA–90 mM Tris (pH 8.0) that contained 0.3% (wt/vol) SDS at 25°C. Probes were removed from membranes by washing for 3 h in 0.025 M NaOH and rinsing the filters in 2× SSC.

RESULTS AND DISCUSSION

Nucleotide sequence of the *entA* gene. The cloning and expression of the *entA* gene on a 2.5-kb *Hind*III fragment from *S. aureus* FRI337 has been reported (6). A 1.3-kb *Rsa*I–*Hind*III fragment, which is a subsection of this 2.5-kb *Hind*III fragment (data not shown), was sequenced. The sequencing strategy, which allowed both strands to be sequenced independently, is presented in Fig. 1.

The complete nucleotide and predicted amino acid sequence of the *entA* structural gene is given in Fig. 2. The coding sequence for SEA is contained in an open reading frame of 774 base pairs which contains 258 codons, including the translation initiation and termination codons (Fig. 2). The sequence just upstream and including the initiation codon of *entA* is 5'GCTTTAGAGGTGAGCAAATG3'. This sequence agrees well with other ribosome-binding sites

FIG. 1. Partial restriction endonuclease site map and sequencing strategy of the *entA* gene. The arrows indicate the direction and extent of sequences derived from the 5' end of the restriction endonuclease sites. The nucleotides of the *entA* gene are numbered below the map.

FIG. 2. *entA* nucleotide sequence and derived amino acid sequence of SEA. The nucleotides are numbered (above the DNA sequence) starting with the initiation codon. The amino acid residues are numbered (below the protein sequence). An arrow indicates the presumptive processing site between amino acid residues -1 and 1.

(40, 44), (i) there is a purine-rich segment about 9 base pairs upstream of the ATG (40), (ii) A and U are the preferred codons on both sides of the purine-rich segment (40), and (iii) the sequence is highly complementary to the 3' end of *Bacillus subtilis* 16S rRNA (homologous bases are underlined above).

Analysis of the nucleotide sequence of *entA* predicts that SEA is synthesized as a protein precursor of 257 amino acid residues (29,700 M_r) which includes a signal sequence of 24 amino acid residues (Fig. 2). The hydropathicity curve of the predicted N-terminal amino acids was determined by using the parameters of Kyte and Doolittle (19) (data not shown). The degree of polarity of the N terminus of SEA is consistent with the structure of known signal sequences (27); that is, after the initiation Met residue there are two positively charged amino acids which are followed by a stretch of uncharged nonpolar residues (Fig. 2). The N-terminal peptide of mature SEA is Ser-Glu-Lys-Ser-Glu-Glu (M. S. Bergdoll and I. Y. Huang, personal communication). Therefore, the precursor of SEA is probably processed between Gly-24 and Ser-25, giving a mature protein of 233 residues (27,100 M_r).

The predicted amino acid sequence of *entA* gene agrees well with other data on partial amino acid sequence derived

from mature SEA. Huang et al. (15) reported the partial sequence of two peptides that were thought not to be contiguous and that actually correspond to amino acid residues 85 to 103 and 104 to 119 in the mature form of SEA (Fig. 2). Our data agree with their sequence data, except that we predict an extra Tyr residue at position 88 and we were able to order residues 99 through 103. The C-terminal residue is predicted to be a Ser residue, which agrees with protein analysis (42).

Amino acid sequence homology between SEA and other bacterial exotoxins. The mature amino acid sequence of SEA was compared with those of SEB, SEC₁, SPEA, and TSST-1. Of the two slightly different peptide sequences reported for SEB, we elected to use the one of Jones and Kahn (18) because it was derived from the nucleotide sequence rather than the protein sequence (14). For our comparisons we used the SPEA sequence reported by Weeks and Ferritti (54), because in the region corresponding to SEA amino acid residues 145 to 156 this SPEA sequence had 10 of 12 residues in common with three enterotoxins, whereas the SPEA sequence in this region reported by Johnson et al. (16) had little or no homology with the enterotoxins. The two reported SPEA-derived amino acid sequences differ in the region encoding amino acid residues 165 to 178 of the

precursor form of SPEA (54) (which corresponds to mature SEA amino acid residues 145 to 156), because one of the *speA* nucleotide sequences has three additional nonconsecutive adenine residues (54) compared with the other sequence (16).

Optimal alignments were determined by using the computer program FASTP developed by Lipman and Pearson (21). Based on these alignments, mature SEA has 83, 72, and 75 amino acid residues homologous with SEB, SEC₁, and SPEA, respectively (Fig. 3). Optimized homology scores (ktup = 1) for each comparison are given in Table 1. These were obtained by FASTP analysis, which not only computes a similarity score, allowing for insertions and deletions, but

also takes into account evolutionarily conserved amino acid substitutions (21). The statistical significance of each optimized homology score was evaluated using the RFD program of FASTP (Table 2).

Homology scores and *z* values can be used to assess the relative relatedness with respect to amino acid similarities among a group of proteins (21). Thus, SEA is related to SEB, SEC₁, and SPEA to about the same degree (Tables 1 and 2). SPEA is more closely related to SEB and SEC₁ than it is to SEA. SEB and SEC₁ are the most similar pair among the four toxins.

Because the enterotoxins are closely related proteins, it is reasonable to expect that the critical amino acid residues

FIG. 3. Comparison of amino acid sequences of SEA, SEB, SEC₁, SPEA, and TSST-1. The residues that a given protein has in common with SEA are indicated with blank spaces. Only the residues that a given protein has that differ from those of SEA are shown. The numbers given above the line correspond to the amino acid residues of the mature form of SEA. Dashes indicate gaps of one amino acid residue. An asterisk is placed before and after the N- and C-terminal residue, respectively, of each protein. The sequence of the mature SEB is taken from Jones and Khan (18); the sequence of mature SEC₁ is taken from Schmidt and Spero (43); the sequence of the mature SPEA is taken from Weeks and Ferretti (54); and the sequence of mature TSST-1 is taken from Bloomster-Hautmaa et al. (8). The alignments of SEB, SEC₁, and SPEA relative to SEA were determined by the algorithm used in the computer program FASTP (21). The alignment of TSST-1 relative to SEA was derived from the nucleotide sequence alignment between the *entA* and *tst* genes that was obtained with the computer program GAP (Genetics Computer Group, Madison, Wis.) (12). The alignments between SEA and TSST-1 as determined by the FASTP program (data not shown) compared with the alignment derived by the nucleotide sequence comparison was the same for peptides corresponding to SEA residues 91 through 138 and 142 through 174; according the FASTP program analysis, the only other two residues SEA and TSST-1 had in common were SEA residues 119 and 177, which correspond to TSST-1 residues 94 and 151.

required for the structural integrity and the biological activities should be among the residues that are conserved among the mature forms of SEA, SEB, and SEC₁. Data from amino acid modification studies have suggested that Met and His residues are involved in the formation of the emetic active sites of SEB and SEA, respectively (9, 47). There is only one Met residue and one His residue conserved among the mature forms of SEA, SEB, and SEC (Fig. 3, SEA residues 107 and 114, respectively). These two amino acids may constitute critical residues in the active sites (or receptor-binding sites) of these molecules because they are (i) close to each other, (ii) in a highly conserved region (Cys-Met-Tyr-Gly-Gly, the sequence corresponding to residues 106 to 110, is conserved among all three enterotoxins), and (iii) adjacent to a disulfide bridge, which is present in all enterotoxins (4).

TSST-1 probably belongs in the staphylococcal enterotoxin-SPEA group of proteins based on several considerations. The amino acid sequence alignment between SEA and TSST-1 was determined by using two different algorithms. TSST-1 and SEA have 34 homologous amino acid residues as determined by the analysis that used the nucleotide sequence alignment between *entA* and *tst* genes (GAP program; Genetics Computer Group, Madison, Wis. [12]) as the basis for the amino acid sequence alignment (Fig. 3). SEA and TSST-1 had only 18 homologous amino acid residues based on the alignment as determined by the algorithm of Lipman and Pearson (21) (Computer Program FASTP) (locations of homologous residues are described in the legend to Fig. 3). The amino acid sequence similarities between SEA and TSST-1, as determined by using the program FASTP, is classified as possibly significant by the criteria suggested by Lipman and Pearson for evaluation *z* values (21) (Tables 1 and 2). Additional evidence for including TSST-1 in the enterotoxin-SPEA protein family is that amino acid sequence similarity between TSST-1 and SPEA is judged to be probably significant on the basis of Lipman and Pearson's algorithms (Tables 1 and 2). The amino acid sequence of SPEA is unquestionably related to that of the enterotoxins (Tables 1 and 2) (16, 54). TSST-1 and SEA are both produced by *S. aureus* and have similar biological properties, which are shared by the other staphylococcal enterotoxins as well as SPEA, such as T-cell mitogenicity, pyrogenicity, and induction of enhanced susceptibility to endotoxin shock (1, 2, 20, 36, 37, 52; Smith and Archer, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977). The question of relatedness will be resolved once the amino acid residues critical for these functions and the precise mode of action at the molecular level are understood for each of these toxins.

Staphylococcal enterotoxins, TSST-1, and SPEA are members of a group of related toxins that are found in wild-type strains of different genera. Several other highly

TABLE 2. *z* values for optimized homology scores for comparison between mature amino acid sequences^a

Bacterial toxin	<i>z</i> value of shuffled sequence				
	SEA	SEB	SEC ₁	SPEA	TSST-1
SEA	120	27.4	29.6	35.8	5.23
SEB	27.9	96.7	72.4	49.4	4.26
SEC ₁	26.7	72.6	118.8	53.4	3.84
SPEA	35.8	56.0	60.4	112.4	7.26
TSST-1	5.47	4.00	3.24	7.00	101.8

^a The *z* values were calculated by using the RDF program, which is part of the software FASTP (21). The *z* value is the number of standard deviation units the aligned optimized score (calculated from comparison of the query sequence to the unshuffled sequence, given in Table 1) is above the mean of the aligned optimized score for 500 permuted versions of the shuffled sequence (*ktup* = 1). The guidelines of Lipman and Pearson (21) for interpretation of *z* values are that the homology is possibly significant if *z* is >3, probably significant if *z* is >6, and significant if *z* is >10.

related toxins show a distribution in multiple genera that may be related to the fact that these toxins are encoded by accessory genetic elements (phages, plasmids, and transposons). For example, Shigalike toxin, a phage-encoded toxin in *E. coli*, occurs in two other genera, *Shigella* (31) and *Vibrio* (34). Some *Yersinia* and *Klebsiella* strains possess genes for heat-stable toxins that have substantial DNA or amino acid homology to the transposon-encoded heat-stable toxin gene of *E. coli* (48, 49). Cholera toxin of *Vibrio cholerae* and heat-labile toxin of *E. coli* are highly homologous and are each encoded by an accessory genetic element (25). The evolutionary events that have resulted in the distribution of genes for related bacterial toxins in various genera are not fully understood. Both the *speA* (17, 53) and *entA* (7) genes are located on phage, and perhaps these phages can occasionally cross species boundaries. Alternatively, these toxin genes may be on transposons and either the transposons or phage carrying these transposons move between genera.

DNA homology between *entA* and DNA from other Ent⁺ strains. Currently five separate assays are used to detect the five major serological types of staphylococcal enterotoxins (5). It has been suggested that a DNA-DNA hybridization assay with a probe derived from an enterotoxin gene may be used to design a single assay capable of identifying all types of enterotoxin-producing staphylococci. Therefore, DNA purified from EntB⁺, EntC₁⁺, EntC₂⁺, EntD⁺, and EntE⁺ strains of *S. aureus* was examined for homology to *entA* sequences by the method of Southern (46). Two probes were used. The first was a 624-base-pair *HincII*-*HgiAI* fragment (designated A-624) containing only *entA* structural gene

TABLE 1. Optimized homology scores for comparisons between mature amino acid sequences^a

Toxin	Amino acid homology scores with the following toxins:				
	SEA	SEB	SEC ₁	SPEA	TSST-1
SEA	1,160	326	294	344	81
SEB		1,211	825	577	74
SEC ₁			1,161	555	— ^b
SPEA				1,110	96
TSST-1					936

^a Optimized homology scores were calculated by using the algorithm of Lipman and Pearson (21) with the FASTP program, which is part of the software FASTP (*ktup* = 1).

^b No significant amino acid homology.

FIG. 4. DNA probes were derived from the indicated regions of the *entA* structural gene. A-624, A-252, and A-322 are double-stranded restriction fragments (restriction enzyme sites are indicated) containing 624, 252, and 322 base pairs of DNA originating from the *entA* structural gene, respectively. Probe A-17 is a 17-base oligonucleotide, 5'-ACACCACCATACATACA-3', that corresponds to the negative strain of the strain FRI337 *entA* gene.

FIG. 5. Southern hybridization analysis of genomic DNA from *S. aureus* strains. Genomic DNA was digested with *Hind*III, fractionated by agarose gel electrophoresis, transferred to a Zeta probe membrane, and probed with 32 P-labeled A-17 (even lanes). Then the probe was eluted with 0.025 N NaOH, and the filters were probed with 32 P-labeled A-624 (odd lanes). The strains are indicated above the blots. The type of enterotoxin(s) a strain produced is given below the blots. Dashes indicate that the strains make no known enterotoxins. The numbers next to the arrows indicate the sizes of the fragments in kilobase pairs.

sequences (Fig. 4). The other probe was a 17-base oligonucleotide, 5'ACACCACCATACATACA3' (designated A-17; Fig. 4). This probe corresponds to a coding sequence for the longest conserved polypeptide among SEA, SEB, and SEC₁ (Fig. 4). Probe A-17 is derived from the nucleotide sequence of *entA* that represents the negative strand that encodes Cys-Met-Tyr-Gly plus the first two nucleotides that encode Val (residues 106 through 110 in Fig. 3). The sequence of this oligonucleotide is identical to a portion of the *entB* gene (18). Probe A-17 was expected to have at least partial homology with a portion of the *entC1* gene because the corresponding peptide of SEC₁ is the same as that of SEA, except SEC₁ has an Ile residue in place of the C-terminal Val residue (Fig. 3).

No simple explanation accounts for all of the hybridization patterns observed. The most intense autoradiographic signals were observed between A-624 and DNA isolated from EntA⁺ strain MJB265 and EntE⁺ strains (FRI578 and FRI918) (Fig. 5, lane 3; Fig. 6; Table 3) and between A-17 and DNA from EntA⁺ (MJB265), EntB⁺ (DU4916), and EntA⁺ EntB⁺ (S6) strains (Fig. 5, lanes 4, 8, and 10; Table 3). Less intense autoradiographic signals were observed between either probe A-624 or A-17 and DNA from EntA⁺ (S6R), EntD⁺ (FRI776), and EntC₁⁺ (FRI137) strains (Fig. 5, lanes 5, 6, 11, 12, 15, and 16; Table 3). DNA from an EntC₂⁺ strain (FRI361) did not have detectable homology to either A-624 or A-17 (Fig. 5, lanes 13 and 14; Table 3).

Based on hybridization data alone it is not possible to prove that either A-624 or A-17 hybridizes to DNA of another type of enterotoxin gene. However, based on other considerations, A-17 must be hybridizing to the *entB*-encoding DNA of S6. Specifically, analysis of data from cloning experiments demonstrated that the *entB* gene of S6 is on a 6-kb *Hind*III fragment (38), and the nucleotide sequence of a portion of the *entB* gene (18) is homologous to A-17. Therefore, the observed homology between A-17 and the

6-kb *Hind*III fragment of S6 was expected (Fig. 5, lane 8). Additional evidence that A-17 hybridizes to the *entB* gene is that probe A-17 does not hybridize to a 6-kb *Hind*III fragment of S6R (EntA⁺ EntB⁻). S6R is a derivative of S6 (EntA⁺ EntB⁺) that lacks the *entB* gene (11, 38) (Fig. 5, lanes 6 and 8, Table 3).

FIG. 6. Southern hybridization analysis of genomic DNA from *S. aureus* strains producing SEE was done by using four different *entA*-derived probes. *Hind*III-digested genomic DNA from the strains was fractionated by agarose gel electrophoresis and transferred to a Zeta probe membrane. The membrane was cut into four pieces, each piece containing DNA from both strains, and then each piece was hybridized to the probe indicated at the top of the lane. The numbers next to the arrows indicate the sizes of the fragments in kilobase pairs.

TABLE 3. DNA-DNA hybridization data between genomic DNA from various *S. aureus* strains and probes derived from the *entA* genes^a

Strain	Toxin(s) produced	Hybridization (size of fragment in kb) to probe	
		A-624	A-17
RN450	None	—	—
MJB265	SEA	+++ (2.5)	+++ (2.5), + (4.6)
S6R	SEA	++ (3.7)	+ (3.7), + (4.6)
S6	SEA, SEB	++ (3.7), + (6.0)	+ (3.7), +++ (6.0)
DU4916	SEB	+ (6.0)	+++ (6.), + (11.), + (12.5)
FRI137	SEC ₁	+ (5.1)	++ (5.1), + (4.4)
FRI361	SEC ₂	—	—
FRI776	SED	++ (11)	++ (11)
FRI578	SEE	+++ (14)	++ (8.6)
FRI918	SEE	+++ (5.1)	—

^a —, No fragment hybridized to the probe; +, weak hybridization signal on autoradiogram; ++, fair hybridization signal on autoradiogram; +++, strong hybridization signal on the autoradiogram. Fragments with sizes in parentheses are those to which the probe hybridized.

Curiously, probe A-17 does not hybridize to the same size fragment that probe A-624 hybridized to in either FRI578 or FRI918. Instead, A-17 hybridizes to an 8.6-kb *Hind*III fragment of FRI578 and no detectable fragment of FRI918 (Fig. 6). In FRI578 the 14- and 8.6-kb *Hind*III fragments that hybridize to probes A-624 and A-17, respectively, are not contiguous pieces of DNA that each encode part of a putative *entE* gene and share a *Hind*III site. This is indicated by the fact that two probes, A-252 and A-322, which flank opposite regions of the DNA corresponding to probe A-17 (Fig. 4), both hybridize to only a 14-kb *Hind*III fragment of FRI578 and a 5.1-kb *Hind*III fragment of FRI918 (Fig. 6).

The significance of the homology between the A-17 probe and the 8.6-kb *Hind*III fragment of FRI578 is not clear. However, the fact that A-17 hybridized not to the putative *entE* gene (i.e., the 14-kb *Hind*III fragment of FRI578 that has substantial homology to A-624) but to some other DNA of FRI578 illustrates a potential problem in using an oligonucleotide probe for detection of all types of enterotoxin-encoding DNA. Stringency conditions that allow hybridization between the probe and each type of enterotoxin gene (some are not homologous to the probe) may allow hybridization of the probe to non-enterotoxin-related DNA. This problem may be overcome by using more stringent hybridization conditions and a mixture of DNA probes that are derived from various types of enterotoxin genes. This mixture of probes need not consist of a probe for each type of enterotoxin gene, because A-624 hybridized well to DNA from EntA⁺ and EntE⁺ strains; probe A-624 potentially could be used for the detection of EntA⁺ and EntE⁺ *S. aureus* strains.

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