Identification of a Bacteriophage Containing a Silent Staphylococcal Variant Enterotoxin Gene (sezA\(^+\))

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A variant enterotoxin gene, referred to as sezA\(^+\), has been identified. *Staphylococcus aureus* FR11106, a staphylococcal enterotoxin type D producer (sed\(^+\)), contained HindIII fragments of 3.8 and 9.4 kilobase pairs (kbp) that hybridized in Southern blot analysis to a probe containing only staphylococcal enterotoxin type A structural gene sequences. Presumably, probe A-624 hybridized to the 9.4-kbp HindIII fragment because of the sequence homology between sea\(^+\) and sed\(^+\). This 9.4-kbp HindIII fragment, which was part of a staphylococcal plasmid, was isolated and ligated into an *Escherichia coli* plasmid vector; Sed\(^+\) E. coli recombinant clones were isolated. The 3.8-kbp HindIII fragment was shown to be part of a viable lysogenic bacteriophage, and it contained sezA\(^+\). This sezA\(^+\)-containing fragment was cloned into *E. coli*, and its DNA sequence was determined. Examination of the nucleotide sequence revealed a 771-bp region that contained an open reading frame with 85 and 77% nucleotide and derived amino acid sequence identities with sea\(^+\) and staphylococcal enterotoxin type A, respectively. This open reading frame has 83 to 50% nucleotide sequence identities with the other types of staphylococcal enterotoxin genes. sezA\(^+\) was shown to be transcribed into stable mRNA. However, the sezA\(^+\) mRNA was not translated into an enterotoxin-like protein because it lacks an appropriate translation initiation codon.

Staphylococcal enterotoxins are emetic toxins that cause staphylococcal food-poisoning disease (4). In addition, enterotoxins are T-cell mitogens and potent inducers of gamma interferon (for a review, see reference 24), interleukin-1 (35), and tumor necrosis factor (16). They may be involved in some cases of toxic shock syndrome (13, 17, 39).

There are five major serological types of staphylococcal enterotoxins, A through E (SEA through SEE). The amino acid sequences have been determined for SEA, SEB, and SEC (22, 23, 40), and the nucleotide sequences have been determined for the genes of SEA, SEB, SEC, SEF, and SEE (sea\(^-\), seb\(^-\), sec\(^-\), sed\(^-\), and see\(^-\), respectively) (3, 7–9, 11, 12, 27). Among these genes, there is 49 to 85% nucleotide sequence identity, with sea\(^-\) and see\(^-\) being the most closely related (12). On the basis of nucleotide sequence comparison, the enterotoxins are related to the streptococcal pyrogenic exotoxins types A (26, 44) and C (20); of the enterotoxin genes, seb\(^+\) is the most closely related to streptococcal pyrogenic exotoxin type A gene (speA), with 61% nucleotide sequence identity.

A variety of genetic elements encode the enterotoxins. sea\(^+\) is encoded by a family of lysogenic bacteriophages in *Staphylococcus aureus* (6). In at least two strains, see\(^-\) is associated with UV-inducible DNA that appears to be defective phases (12). The SEC and SEF phases are associated with the same plasmid in one *S. aureus* strain (1), while in other strains, seb\(^+\) is part of a chromosomal element of at least 26.8 kilobase pairs (kbp) that is absent in some non-enterotoxin-producing *S. aureus* strains (25). sed\(^+\) is plasmid encoded (3).

We have identified a staphylococcal UV-inducible phage (designated phage FR11106-1) that encoded an enterotoxin-like gene (designated sezA\(^+\)). Phage FR11106-1 DNA efficiently hybridized to both sea\(^-\)-containing phage FR1337-1 and a probe that consisted of sea\(^-\)-specific DNA. An sezA\(^+\)-containing DNA fragment was cloned into an *Escherichia coli* plasmid vector. Nucleotide sequence analysis of sezA\(^+\) revealed a region of 771 bp that contained an open reading frame (ORF). This region has 85 and 77% nucleotide and derived amino acid sequence identities with sea\(^+\) and SEA, respectively. sezA\(^+\) was transcribed into stable mRNA; however, this mRNA apparently was not translated into an enterotoxin-like protein because it lacked a translation initiation codon. In a preliminary report of this work, sezA\(^+\) was referred to as entA\(^+\) (M. T. Soltis, J. J. Mekalanos, and M. J. Betley, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, P19, p. 277).

MATERIALS AND METHODS

Bacterial strains, plasmids, phages, and culture conditions. Bacterial strains, plasmids, and phages used in this study are described in Table 1. The cultural conditions for propagation of cells used for DNA isolation procedures, for propagation of staphylococcal and *E. coli* phages, and for UV induction of staphylococcal lysogenic phages have been described (6, 7, 12).

DNA manipulations. Whole-cell DNA was isolated from *S. aureus* protoplasts (7). Staphylococcal plasmid DNA was isolated from cleared lysates and purified by CsCl-ethidium bromide dye-buoyant density centrifugation (34). Staphylococcal phage DNA was isolated by phenol extraction (6). Procedures for purification of single-stranded M13 phage DNA have been described elsewhere (2). *E. coli* plasmid and replicative forms of M13 phage were purified by an alkaline lysis method (2).

DNA endonuclease restriction, alkaline phosphatase treatment, ligation, and transformation and transfection of competent *E. coli* cells were done by standard methods (2, 30). Restriction enzyme fragments to be used as probes in hybridization experiments or in construction of recombinant plasmids or phages were fractionated by agarose gel electro-
TABLE 1. Strains, plasmids, and phages used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Plasmids</th>
<th>Phages</th>
<th>Relevant genotype or phenotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td>ISP456 (PS 80CR 3)</td>
<td>FRI1106</td>
<td>Non-enterotoxin producer, restriction enzyme deficient</td>
<td>P. A. Pattee (41)</td>
</tr>
<tr>
<td></td>
<td>RN450</td>
<td>FRI1106</td>
<td>Non-enterotoxin producer</td>
<td>R. P. Novick (33)</td>
</tr>
<tr>
<td></td>
<td>FRI337</td>
<td></td>
<td>Sea* Sed*</td>
<td>M. S. Bergdoll</td>
</tr>
<tr>
<td></td>
<td>FRI1106</td>
<td></td>
<td>Sed* sezA*</td>
<td>M. S. Bergdoll</td>
</tr>
<tr>
<td></td>
<td>MJB265</td>
<td></td>
<td>RN450 lysogenized with phage FRI137-1, Sea*</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>MJB269</td>
<td></td>
<td>ISP456 lysogenized with phage FRI1106-1, sezA*</td>
<td>This work</td>
</tr>
<tr>
<td>E. coli</td>
<td>AB259</td>
<td></td>
<td>Amp*</td>
<td>S. Lofdahl (5)</td>
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<tr>
<td></td>
<td>JF626</td>
<td></td>
<td>Amp*</td>
<td>J. Felton (7)</td>
</tr>
<tr>
<td></td>
<td>MJB329</td>
<td></td>
<td>AB259 containing pMJB48</td>
<td>This work</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MJB333 Sed*, AB259 containing pMJB52</td>
<td>This work</td>
</tr>
<tr>
<td></td>
<td>pBR322</td>
<td></td>
<td>Contains bla</td>
<td>Promega</td>
</tr>
<tr>
<td></td>
<td>pGEM-7Zf(+)</td>
<td></td>
<td>Contains bla</td>
<td>This work</td>
</tr>
<tr>
<td>pFRI1106-1</td>
<td></td>
<td></td>
<td>pBR322 with a 2.5-kbp insert containing sea* of FRI337</td>
<td>5</td>
</tr>
<tr>
<td>pMJB9</td>
<td></td>
<td></td>
<td>pBR322 with a 2.6-kbp HinIIRsAl (designated A-D64) that has only sea* structural gene sequences</td>
<td>6</td>
</tr>
<tr>
<td>pMJB38</td>
<td></td>
<td></td>
<td>pgem-7Zf(+) with a 3.8-kbp HindIII fragment of phage FRI1106-1 that contains sezA*</td>
<td>This work</td>
</tr>
<tr>
<td>pMJB48</td>
<td></td>
<td></td>
<td>pBR322 with a 9.4-kbp HindIII fragment of phage pFRI1106 that contains sed*</td>
<td>This work</td>
</tr>
<tr>
<td>pMJB52</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FRI137-1</td>
<td>sea* containing phage isolated from FRI1137</td>
<td></td>
<td>6</td>
<td>This work</td>
</tr>
<tr>
<td>FRI1106-1</td>
<td>sezA* containing phage isolated from FRI1106</td>
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</tbody>
</table>

phoresis and then isolated by using Gene Clean (Bio 101, Inc.).

By using single-stranded M13 phages (32) as templates, nucleotide sequences were determined by the dideoxy-chain termination method (38) as outlined in the sequencing kits (Amersham Corp. and U.S. Biochemical). Nucleotide sequence comparisons were done with the GAP program set to default parameters (University of Wisconsin Genetics Computer Group [14]). Comparison of amino acid sequences derived from nucleotide sequence data was done with the FASTP program (stringency K-tuple = 1) of Lipman and Pearson (29).

DNA-DNA hybridization. For staphylococcal plaque hybridizations, PFUs were transferred to nitrocellulose filters (Schleicher & Schuell, Inc.) by a technique described previously for E. coli phage λ (30). For Southern blot analysis, restriction enzyme-digested DNA was electrophoresed on a 1% agarose gel and passively transferred to nitrocellulose filters (30).

The same prehybridization and hybridization conditions were used for plaque and Southern hybridization experiments. 32P-labeled (30) double-stranded DNA probes were hybridized to filters under low-stringency conditions (25% [vol/vol] formamide) (7).

Extractions of RNA and Northern blot. The methods described by Sandler and Weisblum (37) were used for RNA preparation, transfer of RNA from gels to Nytran filters (Schleicher & Schuell), and hybridization, except that the gels consisted of 1.0% agarose-2.2 M formaldehyde and the gel running buffer was 0.2 M morpholinepropanesulfonic acid (pH 7.0), 50 mM sodium acetate, and 5 mM EDTA (pH 8.0). The size of an mRNA species was estimated by comparison of its gel position (visualized on the autoradiogram) with the gel positions of RNA molecular weight standards (Bethesda Research Laboratories, Inc.) that were visualized on the filter with a methylene blue stain (30).

Serological assays. Polyclonal antibodies to SEA, SED, and SEE, prepared in rabbits, were provided by Merlin S. Bergdoll (University of Wisconsin-Madison). The gel double-diffusion assay has been described previously (36). Western (immunoblot) analysis was done by using ProtoBlot (Promega Corp.) as described by the manufacturer. When necessary, culture supernatant fluids were concentrated 20-fold by dialysis against a 20% (w/vol) solution of polyethylene glycol (20 M; Union Carbide Corp.) in 0.008 M sodium phosphate buffer (pH 6.8). Samples were electrophoresed on a 12.5% polyacrylamide-sodium dodecyl sulfate gel and electrophoretically transferred to nitrocellulose (2).

RESULTS

Identification of sezA*. SED was the only known enterotoxin produced by S. aureus FRI1106. We expected an sea*-derived probe to hybridize to the sed*-containing fragment of FRI1106 under low-stringency hybridization conditions because sea* and sed* have 60% nucleotide sequence identity (3, 7). However, FRI1106 contained two HindIII fragments (3.8 and 9.4 kbp) that hybridized to the sea*-specific probe, A-624, in Southern blot experiments (Fig. 1). A first step towards establishing the identity of the FRI1106 DNA which hybridized to the A-624 probe was to construct strains that contained only one of these DNA fragments.

Analysis of recombinant clones demonstrated that sed* of FRI1106 was encoded by the 9.4-kbp HindIII fragment that hybridized to probe A-624. This 9.4-kbp HindIII fragment was isolated from plasmid DNA of FRI1106 (purified by CsCl-ethidium bromide ultracentrifugation) and ligated into
gene has 85, 53, 50, 60, and 83% nucleotide sequence identities with the sea' (7), seq' (27), sec' (8, 9, 11), sed' (3), and see' (12) genes, respectively, as determined by the 
GAP program.

A 771-bp fragment that encoded an ORF of 257 derived amino acid residues was identified in seqA'. This derived amino acid sequence had 77, 34, 28, 49, and 79% amino acid sequence identities with the amino acid sequences derived from sea', seq', sec', sed', and see', respectively (comparison with sea' is shown in Fig. 2). Compared with sea' and see', seqA' had a deletion of one nucleotide (from the regions that contain the putative ribosome-binding sites through the translation termination codons of sea' and see' ) (see Fig. 2 for a comparison of seqA' with sea'). The location of this seqA' deletion corresponded to the locations of the translation initiation codons of both the sea' and see' genes and resulted in an asparagine codon, which is not among the known initiation signals (18) (Fig. 2).

Consistent with this apparent lack of a translation initiation signal, we were unable to demonstrate production of enterotoxinlike protein by strain MJB269. Thirty-five milliliters of MJB269 culture supernatant, administered via gastric tube to each of three rhesus monkeys (body weight, 2 to 3 kg), did not cause emesis. No signals were detected in Western analysis of supernatants of MJB269 concentrated 20-fold and reacted against polyclonal antibodies produced against SEA and SEE (data not shown).

seqA' appeared to have a functional promoter, because seqA' was transcribed into stable mRNA that was about the same size as sea' mRNA. Transcription of seqA' was demonstrated by Northern (RNA) analysis of RNA isolated from MJB269 and ISP456 by using as probe a 375-bp Dral-NsiI fragment (corresponding to nucleotides 230 through 605, respectively, in Fig. 2) isolated from seqA' (Fig. 3).

**DISCUSSION**

*S. aureus* FR11106, an Sed' strain, contained two HindIII fragments (3.8 and 9.4 kbp) that, under low-stringency hybridization conditions, hybridized to A-624, a probe derived from sea'. seqA' was encoded by a 9.4-kbp HindIII fragment, as shown by analysis of the recombinant *E. coli* strain, MJB333. Hybridization between probe A-624 and the 9.4-kbp HindIII fragment was most likely due to the homology between sea' and sed'. To date, all Sed' strains examined (including FR11106) contain identical EcoRI restriction fragment patterns (3; data not shown for plasmid FR11106-1). This is in contrast to the restriction-fragment-length polymorphisms that occur among sea'-containing phages (6).

The 3.8-kbp HindIII fragment of FR11106, which hybridized to A-624, contained a gene designated seqA'. Of the sequenced enterotoxin genes, seqA' was most closely related to sea' and see', with 85 and 83% nucleotide sequence identities, respectively.

We have no evidence that the seqA' mRNA was translated into an enterotoxinlike protein. No enterotoxinlike protein produced by strain MJB269 was detected by the Western and monkey-feeding assays. It is possible (i) that these assays were not sensitive enough to detect low concentrations of an enterotoxinlike protein, (ii) that this protein is not emetic, or (iii) that it does not serologically cross-react with SEA or SEE. The derived amino acid sequence of the 771-bp ORF of seqA' had 77 and 79% sequence identities with SEA and SEE, respectively. It is reasonable to expect
FIG. 3. Northern hybridization analysis of RNA isolated from S. aureus strains probed with 32P-labeled A-624 (lanes 1 through 4) or a 32P-labeled 375-bp Dral-NsiI fragment that contains only seaA+ DNA sequences (lanes 5 and 6). The location of a 1.4-kb fragment is indicated.

such closely related proteins to serologically cross-react, because mucous forms of SEA and SEE, with 81% amino acid sequence identity, cross-react (15, 28, 43). The most likely reason that we were unable to demonstrate an enterotoxin-like seaA+ product is that the 771-bp ORF was not translated. Visual examination of DNA sequences 20 bp upstream of ATG and GTG codons (two of the most frequently used translation initiation codons) that were in frame with the 771-bp ORF did not reveal any likely ribosome-binding sites (note that in ribosome-binding sites, the purine-rich regions [GGAGG] are usually 6 to 13 bp from the translation initiation codon [18]).

seaA+ may have evolved from a functional enterotoxin gene that is distinct from the characterized enterotoxin genes: this gene lost the ability to be translated because of a one-nucleotide deletion. Alternatively, seaA+ or sea+ may have incurred a single-nucleotide deletion that destroyed the translation initiation codon, and subsequent missense mutation may have resulted in the divergence observed between seaA+ and the sea- and see+ genes. Both explanations are consistent with the observations that seaA+ was transcribed into stable mRNA and that compared with either sea+ or see+, seaA+ had a deletion of one nucleotide which corresponded to the locations of the translation initiation codons for both sea+ and see+ (Fig. 2). Also, seaA+ had a ribosome-binding-like sequence which corresponded to the putative ribosome-binding sites of sea+ and see+ (7, 12). The purine-rich region that is characteristic of ribosome-binding sites and homologous to the 3' end of Bacillus subtilis 16S rRNA is indicated in Fig. 2 (31). The presence of TAA between the

FIG. 2. Comparison of seaA+ and its derived amino acid sequence for the 771-bp ORF with sea+ (7) and its derived amino acid residues in SEA that are different from those derived from the 771-bp ORF of seaA+. , Purine-rich regions that resemble other known ribosome-binding sites (18) and are complementary to the 3' end of B. subtilis 16S rRNA (31); , initiation codon for sea+; , translation termination codon; , gap between the nucleotide sequence alignments.
ribose-binding-like sequence of \( sezA^+ \) and the 771-bp ORF would not prevent translation of this ORF. It has been observed that the region 20 bp upstream of initiation codons is high in TAA in most positions (19) and that there are several bacterial proteins that have TAA next to their translation initiation codons on the 5' side (21).

\( sezA^+ \) may represent a class of silent enterotoxin genes that are used as a DNA reservoir, similar to that observed for the pil of Neisseria gonorrhoeae, for the generation of new enterotoxin genes. \( N. gonorrhoeae \) contains a complete pilin gene as well as a repertoire of partial pilin genes. The gonococcal pili can undergo antigenic variation as well as phase variation (pilin-positive to pilin-negative, or vice versa). Phenomena responsible for the variations in pilin expression include recombination between the complete and partial pilin genes, as well as single-base-pair additions or substitutions that result in a premature termination codon (such pilin-negative strains do revert to pilin positive) (for a review, see reference 42). Recombination between \( sezA^+ \) and either the \( sea^+ \) or the \( sea^- \) gene may result in production of a new antigenic type of enterotoxin. This scenario of producing new enterotoxin genes by recombination between existing genes is very plausible, because information from nucleotide sequence comparisons is consistent with the suggestion that \( sec^+ \) of MN Don strain resulted from recombination between an \( sec^- \) strain FR11230 and \( seb^- \) (11).

The existence of a silent, variant enterotoxin gene does complicate identification of enterotoxin producers by DNA-DNA hybridization assay, because in a DNA-DNA hybridization assay, an \( sezA^- \)-containing strain may give a true-positive reaction. Indeed, FR11106 appears not to be unique in containing a silent enterotoxin gene, because recently, another group reported that DNA from two \( S. aureus \) strains hybridized efficiently to an \( sea^- \)-derived oligonucleotide probe in a colony hybridization assay, but these two strains did not produce SEA, as determined by a Western blot assay (G. Fanning, R. Neill, F. DeLatloz, R. Wolff, and P. Gemski, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, 230, p. 120). Previously, we have described a family of phages that includes \( sea^- \)-containing phages (6). These phages share substantial DNA homology but differ by restriction-fragment-length polymorphisms; the family contains phages with functional \( sea^+ \) as well as phages that have no detectable homology with \( sea^- \)-specific probes (6). This report expands this phage family to include phage FR11106-1, which has DNA with 85% nucleotide sequence identity with \( sea^+ \) but is apparently not translated into an enterotoxinlike protein.

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LITERATURE CITED


