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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* FRI1106, a staphylococcal enterotoxin type D producer (Sed$^+$), contained *Hin*dIII 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 *Hin*dIII fragment because of the sequence homology between *sea*$^+$ and *sed*$^+$. This 9.4-kbp *Hin*dIII 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 *Hin*dIII 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 enterotoxinlike protein because it lacks an appropriate translation initiation codon.

Staphylococcal enterotoxins are emetic toxins that cause staphylococcal food-poisoning syndrome (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, SED, 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 phages (12). The SEB and SEC phenotypes 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 FRI1106-1) that encoded an enterotoxin-like gene (designated *sezA*$^+$). Phage FRI1106-1 DNA efficiently hybridized to both *sea*$^+$-containing phage FRI337-1 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 enterotoxinlike 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-

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TABLE 1. Strains, plasmids, and phages used in this study

Strain, plasmid, or phage	Relevant genotype or phenotype	Source or reference
Strains		
S. aureus		
ISP456 (PS 80CR 3)	Non-enterotoxin producer, restriction enzyme deficient	P. A. Pattee (41)
RN450	Non-enterotoxin producer	R. P. Novick (33)
FRI337	Sea$^+$ Sed$^+$	M. S. Bergdoll
FRI1106	Sed$^+$ *sezA*$^+$	M. S. Bergdoll
MJB265	RN450 lysogenized with phage FRI337-1, Sea$^+$	6
MJB269	ISP456 lysogenized with phage FRI1106-1, *sezA*$^+$	This work
E. coli		
AB259	Amp^s	S. Lofdahl (5)
JF626	Amp^s	J. Felton (7)
MJB329	AB259 containing pMJB48	This work
MJB333	Sed$^+$, AB259 containing pMJB52	This work
Plasmids		
pBR322	Contains *bla*	10
pGEM-7Zf(+)	Contains *bla*	Promega
pFRI1106-1	*sed*$^+$-containing plasmid of FRI1106	This work
pMJB9	pBR322 with a 2.5-kbp insert containing *sea*$^+$ of FRI337	5
pMJB38	pBR322 with a 624-bp *Hin*cII/*Rsa*I (designated A-624) that has only *sea*$^+$ structural gene sequences	6
pMJB48	pGEM-7Zf(+) with a 3.8-kbp *Hin*dIII fragment of phage FRI1106-1 that contains *sezA*$^+$	This work
pMJB52	pBR322 with a 9.4-kbp *Hin*dIII fragment of phage pFRI1106 that contains *sed*$^+$	This work
Phages		
FRI337-1	*sea*$^+$-containing phage isolated from FRI337	6
FRI1106-1	*sezA*$^+$-containing phage isolated from FRI1106	This work

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. ^{32}P-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% (wt/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 *Hin*dIII 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 *Hin*dIII fragment that hybridized to probe A-624. This 9.4-kbp *Hin*dIII fragment was isolated from plasmid DNA of FRI1106 (purified by CsCl-ethidium bromide ultracentrifugation) and ligated into

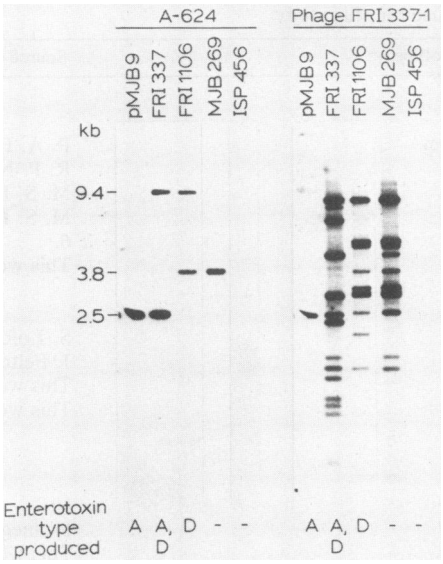


FIG. 1. Southern blot analysis of pMJB9, a recombinant pBR322 containing a 2.5-kbp *Hin*dIII fragment containing *sea*$^+$ and whole-cell DNA from *S. aureus* strains FRI337, FRI1106, MJB269, and ISP456. *Hin*dIII digests of DNA were analyzed by electrophoresis through a 1.0% agarose gel. DNA was transferred onto a nitrocellulose filter and hybridized to ^{32}P-labeled A-624 under low-stringency conditions. Probe A-624 was removed with 0.02 M NaOH, and the test DNAs were rehybridized with ^{32}P-labeled phage FRI337-1. Fragment sizes are given in kilobase pairs. −, No enterotoxin produced.

*Hin*dIII-linearized, alkaline phosphatase-treated *E. coli* vector pGEM-7Zf(+). SED was detected in periplasmic shock fluid obtained from recombinant *E. coli* MJB333 by using a gel double-diffusion assay (data not shown).

The 3.8-kbp *Hin*dIII fragment of FRI1106 that hybridized to probe A-624 (designated *sezA*$^+$) was part of a lysogenic phage. All PFUs obtained by plating UV-induced lysates of strain FRI1106 on *S. aureus* ISP456 hybridized to probes A-624 and *sea*$^+$-containing phage FRI337-1 (data not shown). A single PFU (designated phage FRI1106-1) was purified by three consecutive plaque isolations. Comparisons of *Hin*dIII-digested genomic DNA of strain MJB269 (ISP456 lysogenized with phage FRI1106-1) and ISP456 by Southern blot analysis showed that phage FRI1106-1 had substantial homology to phage FRI337-1 and had a *sezA*$^+$-containing 3.8-kbp *Hin*dIII fragment (Fig. 1). It was unlikely that *sezA*$^+$ was the *sed*$^+$ of FRI1106, because MJB269 did not produce SED, as determined by a gel double-diffusion assay (data not shown).

Characterization of the *sezA*$^+$ gene. An *sezA*$^+$-containing fragment was cloned, and its nucleotide sequence was determined. The 3.8-kbp *Hin*dIII fragment of phage FRI1106-1 was isolated from an agarose gel and ligated into pBR322 that had been linearized with *Hin*dIII and treated with alkaline phosphatase. The ligation mixture was used to transform *E. coli*, and the recombinant clone strain MJB329, which contained pMJB48, was selected for further study. *sezA*$^+$-containing subfragments of pMJB48 were identified by Southern blot analysis using A-624 as a probe (data not shown). These subfragments were cloned into M13 phages mp10 and mp11 for DNA sequence analysis. The nucleotide sequences of both strands were determined by the dideoxy-chain termination procedure of Sanger (Fig. 2). The *sezA*$^+$ gene has 85, 53, 50, 60, and 83% nucleotide sequence identities with the *sea*$^+$ (7), *seb*$^+$ (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 *sezA*$^+$. This derived amino acid sequence had 77, 34, 28, 49, and 79% amino acid sequence identities with the amino acid sequences derived from *sea*$^+$, *seb*$^+$, *sec*$^+$, *sed*$^+$, and *see*$^+$, respectively (comparison with *sea*$^+$ is shown in Fig. 2). Compared with *sea*$^+$ and *see*$^+$, *sezA*$^+$ 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 *sezA*$^+$ with *sea*$^+$). The location of this *sezA*$^+$ 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 or SEE (data not shown).

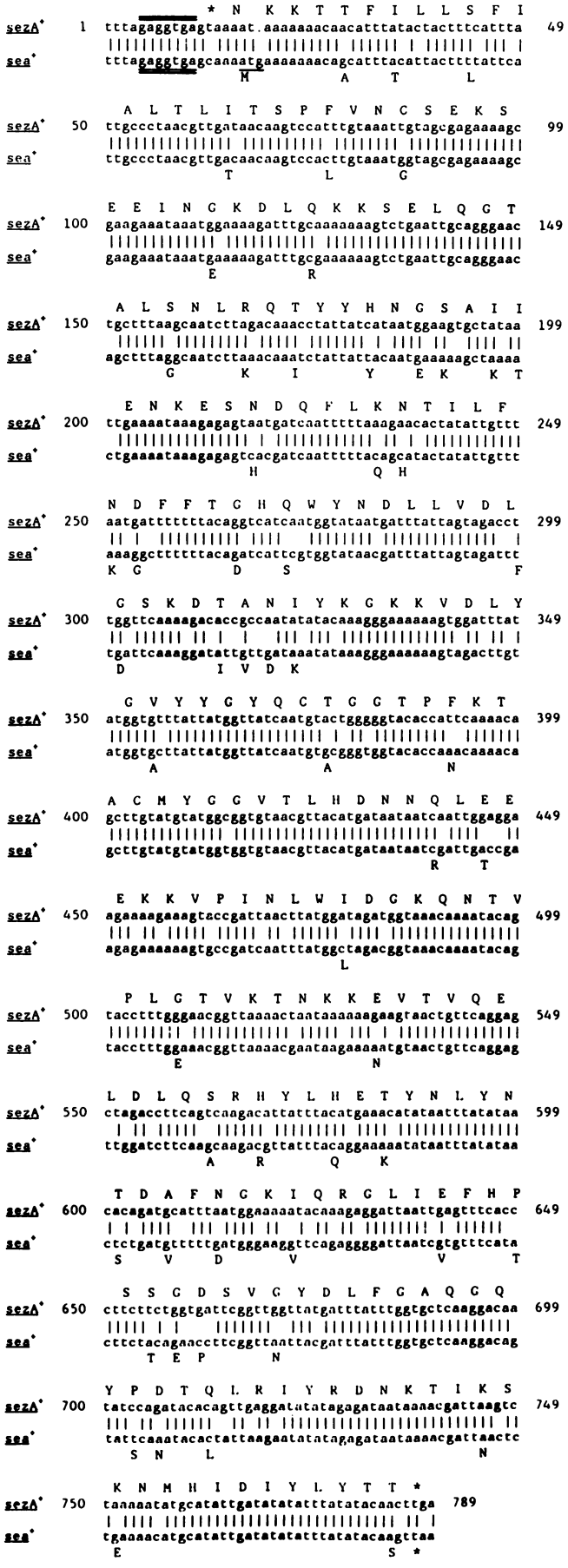
sezA$^+$ appeared to have a functional promoter, because *sezA*$^+$ was transcribed into stable mRNA that was about the same size as *sea*$^+$ mRNA. Transcription of *sezA*$^+$ was demonstrated by Northern (RNA) analysis of RNA isolated from MJB269 and ISP456 by using as probe a 375-bp *Dra*I-*Nsi*I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

DISCUSSION

S. aureus FRI1106, an Sed$^+$ strain, contained two *Hin*dIII fragments (3.8 and 9.4 kbp) that, under low-stringency hybridization conditions, hybridized to A-624, a probe derived from *sea*$^+$. *sed*$^+$ was encoded by a 9.4-kbp *Hin*dIII fragment, as shown by analysis of the recombinant *E. coli* strain, MJB333. Hybridization between probe A-624 and the 9.4-kbp *Hin*dIII fragment was most likely due to the homology between *sea*$^+$ and *sed*$^+$. To date, all Sed$^+$ strains examined (including FRI1106) contain identical *Eco*RI restriction fragment patterns (3; data not shown for plasmid FRI1106-1). This is in contrast to the restriction-fragment-length polymorphisms that occur among *sea*$^+$-containing phages (6).

The 3.8-kbp *Hin*dIII fragment of FRI1106, which hybridized to A-624, contained a gene designated *sezA*$^+$. Of the sequenced enterotoxin genes, *sezA*$^+$ was most closely related to *sea*$^+$ and *see*$^+$, with 85 and 83% nucleotide sequence identities, respectively.

We have no evidence that the *sezA*$^+$ 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 *sezA*$^+$ had 77 and 79% sequence identities with SEA and SEE, respectively. It is reasonable to expect



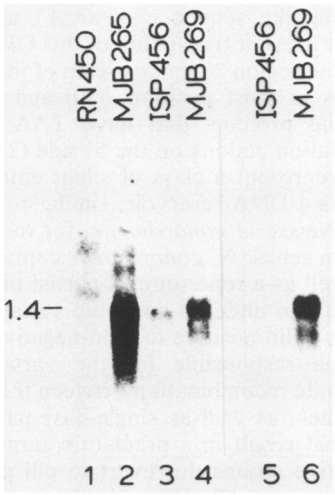


FIG. 3. Northern hybridization analysis of RNA isolated from *S. aureus* strains probed with ^{32}P-labeled A-624 (lanes 1 through 4) or a ^{32}P-labeled 375-bp *DraI-NsiI* fragment that contains only *sezA*$^+$ DNA sequences (lanes 5 and 6). The location of a 1.4-kb fragment is indicated.

such closely related proteins to serologically cross-react, because mature 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 enterotoxinlike *sezA*$^+$ 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]).

sezA$^+$ 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, *sea*$^+$ or *see*$^+$ 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 *sezA*$^+$ and the *sea*$^+$ and *see*$^+$ genes. Both explanations are consistent with the observations that *sezA*$^+$ was transcribed into stable mRNA and that compared with either *sea*$^+$ or *see*$^+$, *sezA*$^+$ had a deletion of one nucleotide which corresponded to the locations of the translation initiation codons for both *sea*$^+$ and *see*$^+$ (Fig. 2). Also, *sezA*$^+$ 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 *sezA*$^+$ and its derived amino acid sequence for the 771-bp ORF with *sea*$^+$ (7) and its derived amino acid sequence. Under the nucleotide sequence for *sea*$^+$ are the amino acid residues in SEA that are different from those derived from the 771-bp ORF of *sezA*$^+$. ═══, 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.

ribosome-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 pili 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 gonococcus 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 pili-negative strains do revert to pili positive) (for a review, see reference 42). Recombination between *sezA*$^+$ and either the *sea*$^+$ or the *see*$^+$ 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*$^+$ of strain FRI1230 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, FRI1106 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, D230, 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 FRI1106-1, which has DNA with 85% nucleotide sequence identity with *sea*$^+$ but is apparently not translated into an enterotoxinlike protein.

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