

Mutations in the Promoter Spacer Region and Early Transcribed Region Increase Expression of Staphylococcal Enterotoxin A

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Received 12 August 1993/Returned for modification 9 September 1993/Accepted 5 October 1993

The mechanism leading to increased production of staphylococcal enterotoxin type A (SEA) in mutant *Staphylococcus aureus* FRI722 compared within its wild-type parent strain, FRI100, was examined. Sequence analysis revealed two mutations in the upstream promoter region of FRI722 at nucleotides -28 and +3 with respect to the transcriptional initiation site at +1. An *sea* translational fusion of the upstream region of FRI722 to the structural gene from FRI100 showed an increase in *sea* expression by Northern (RNA) analysis and in SEA production by Western (immunoblot) analysis. To independently evaluate the effect of each mutation, site-directed mutagenesis was done and revealed that each mutation was responsible for an increase in SEA production.

Staphylococcal enterotoxin type A (SEA) is one of five characterized, serologically distinct types of staphylococcal enterotoxins (SEA through SEE) produced by *Staphylococcus aureus*. The enterotoxins are the causative agents of staphylococcal food poisoning syndrome (4). Strain-to-strain variation in the amount of extracellular toxin produced has been observed for SEA-producing strains, and this variation is not due to differences in *sea* copy number (7).

The maximal level of SEA production for a wild-type strain is usually less than 8 µg/ml of culture supernatant (3). The only reported strain producing more than 10 µg of SEA per ml is strain 13N-2909, which was obtained over 20 years ago by serial mutagenesis of *S. aureus* 100-8-4 with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine followed by screening for increased production of SEA (10). The authors selected mutants which exhibited a gradual increase in SEA production following each successive round of mutagenesis. Strain 13N-2909 was the final mutant derivative of strain 100-8-4 and was reported to produce nearly 20-fold more SEA than its nonmutant parent. Given the incremental increases in SEA production following successive rounds of mutagenesis, it is likely that some of the mutations were specific for *sea* (e.g., mutations within the *sea* promoter region) and that other mutations were nonspecific (e.g., affecting transport or secretion). We obtained strains 13N-2909 and 100-8-4 (which had been renamed FRI722 and FRI100, respectively) from Merlin S. Bergdoll (University of Wisconsin—Madison). The goal of this study was to determine whether the mechanism leading to increased SEA production in FRI722 was due to mutations in the *sea* promoter region.

The bacterial strains and plasmids used in this study are described in Table 1. SEA-producing *S. aureus* strains were grown in 3% (wt/vol) NZ amine type A (Kraft Inc., Norwich, N.Y.) and 1% (wt/vol) yeast extract (Difco Laboratories, Detroit, Mich.) at 37°C with agitation (200 rpm). Nucleotide sequences were determined with the Sequenase kit (United States Biochemical, Cleveland, Ohio). Polymerase chain reaction (PCR) amplification of *sea*_{FRI722} and sequencing of the amplified product were performed as previously de-

scribed (7). *Escherichia coli* plasmid DNA was obtained by an alkaline lysis method (1). Restriction enzymes and T4 DNA ligase were from New England Biolabs (Beverly, Mass.). Alkaline phosphatase was obtained from Boehringer Mannheim (Indianapolis, Ind.).

Samples for Western blot (immunoblot) analysis were prepared from cultures incubated for 16 to 18 h. Procedures for preparation of culture supernatants, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and Western blot analysis have been described previously (14). For a given gel, samples indicated as having the same dilution factor contained the same amounts of culture supernatants. Densitometry was used to quantify signal intensity (GS 300 densitometer; Hoefer Scientific Instruments, San Francisco, Calif.).

S. aureus total cellular RNA was prepared by the method of Sandler and Weisblum (18). Determination of RNA quality and concentration and gel electrophoresis and transfer were performed as previously described (17). For a given gel, samples with the same dilution factor contained the same amount of total cellular RNA. The Ambis Radioanalytic Imaging System (AMBIS Systems, San Diego, Calif.) was used to quantify the radioactive signals on the filter, and the relative amount of RNA hybridized to the probe was determined with data corresponding to sample dilutions that produced signals on the linear portion of the dose (dilution factor)-response (signal intensity) curve. The probe used for detection of *sea* mRNA was derived from pMJB38 and was a 624-bp *Hind*III-to-*Bam*HI fragment designated probe A624 that consisted solely of internal *sea* sequence (6).

Differences in SEA production between FRI722 and FRI100. FRI722 is an SEA high-level-producing mutant strain derived by serial mutagenesis of parent strain FRI100 as described over 20 years ago (10). The mutations responsible for the increased production of SEA in FRI722 were unknown. Friedman and Howard reported an approximately 20-fold increase in SEA production for FRI722 compared with FRI100 on the basis of a crude plate assay (10). Western blot analysis of culture supernatants from FRI100 and FRI722 was performed to more precisely quantitate the difference in SEA production levels between the two strains (Fig. 1). At least eightfold more toxin was produced by FRI722 than by FRI100.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference and/or source
Strains		
<i>S. aureus</i>		
FRI100	<i>sea</i> _{FRI100} ^a	M. S. Bergdoll, Food Research Institute, Madison, Wis.
FRI722	<i>sea</i> _{FRI722}	M. S. Bergdoll (10)
MJB720	RN4220 (pC194 derivative with 3.7-kb <i>EcoRV</i> fragment containing <i>sea</i> _{FRI337})	8
MJB1003	RN4220 (pC194 derivative with 1.4-kb <i>EcoRV</i> fragment containing <i>sea</i> _{FRI722-FRI100})	This work
MJB1007	RN4220 (pC194 derivative with 1.4-kb <i>EcoRV</i> fragment containing <i>sea</i> _{FRI100})	This work
MJB1101	RN4220 (pC194 derivative with 3.7-kb <i>EcoRV</i> fragment containing <i>sea</i> _{FRI337} with A→G mutation at nucleotide -28 ^b)	This work
MJB1102	RN4220 (pC194 derivative with 3.7-kb <i>EcoRV</i> fragment containing <i>sea</i> _{FRI337} with T→C mutation at nucleotide +3)	This work
<i>E. coli</i>		
DH5α	Sea ⁻	11
Plasmids		
pC194	Sea ⁻ Cm ^r	R. P. Novick (15)
pMJB38	<i>sea</i> _{FRI337}	5
pSP73	Sea ⁻	Promega Corp.

^a The subscript indicates the source of *sea*.

^b The nucleotides are numbered with respect to the transcriptional start site for *sea* at nucleotide +1.

Northern (RNA) blot analysis was performed on RNA isolated from strains FRI722 and FRI100 to determine whether the difference in SEA expression would be reflected at the level of *sea* mRNA. The two strains exhibited differences in growth rate (Fig. 2); it was therefore important to compare RNA levels at similar growth phases. Whole-cell RNA samples from FRI722 and FRI100 were isolated at various times throughout growth and subjected to Northern blot analysis with *sea* probe A624 (Fig. 3). Compared with FRI100, FRI722 showed higher expression levels of *sea* mRNA at all time points. With respect to corresponding points in their growth phases, FRI722 and FRI100 were similar in exhibiting maximal *sea* expression during exponential growth and decreased expression during transitional and stationary growth (Fig. 3; data not shown for stationary growth). On the basis of quantification of the signals by an Ambis Imaging System, FRI722 produced at least eightfold more *sea* mRNA than FRI100 at optical densities at 540 nm (OD₅₄₀) of 0.25, 0.5, and 1.0. Maximal expression of *sea* mRNA occurred for both strains at an OD₅₄₀ of 1.0, which corresponded to mid-exponential growth. At an OD₅₄₀ of 2.0, which corresponded to the transition between exponential and stationary growth, the difference in *sea* mRNA expression between FRI722 and FRI100 fell to two- to threefold. Since maximal expression of *sea* mRNA occurred

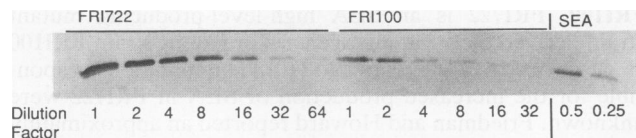


FIG. 1. Western blot analysis with polyclonal antiserum to SEA of culture supernatants (5 µl) from *S. aureus* FRI722 and FRI100. The purified SEA on the right represents concentrations of 0.5 and 0.25 µg/ml as indicated. Strain designations are shown above and dilution factors are shown below each culture supernatant sample. Samples prepared from different strains with the same dilution factor contain the same amount of culture supernatant.

at the same point in growth for both strains and since both strains showed the same overall trend in *sea* expression levels with respect to growth phase, it is unlikely that the increased SEA production of FRI722 is due simply to altered growth phase regulation of SEA production by FRI722.

Sequence analysis of FRI722 promoter region. Since the difference in SEA production between FRI100 and FRI722 was evident at the level of *sea* mRNA, it was conceivable that mutations in the *sea* promoter region contributed to the increased *sea* expression in FRI722. The nucleotide sequence of the promoter region of *sea*_{FRI722} was obtained to determine whether there were differences between *sea*_{FRI722} and *sea*_{FRI100} in this region. A 272-bp fragment that contained the first 55 bp of the *sea* structural gene and 217 bp upstream of its translational start site was obtained by PCR amplification of genomic DNA from FRI722. Oligonucleotide primers (Biotechnology Laboratories, University of Wisconsin—Madison) were chosen on the basis of homology to *sea*_{FRI100}. The upstream primer (5' AGCATACTGCAAG TGAAGTTG 3') hybridized to a region 131 bp upstream of

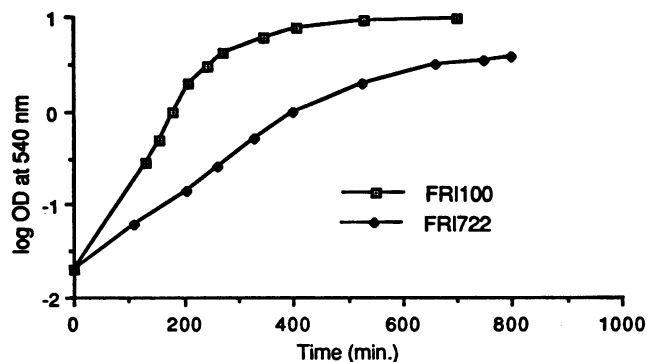


FIG. 2. Growth curves for FRI722 and FRI100. The logarithm of the OD₅₄₀ for the cultures was plotted against the time elapsed since inoculation.

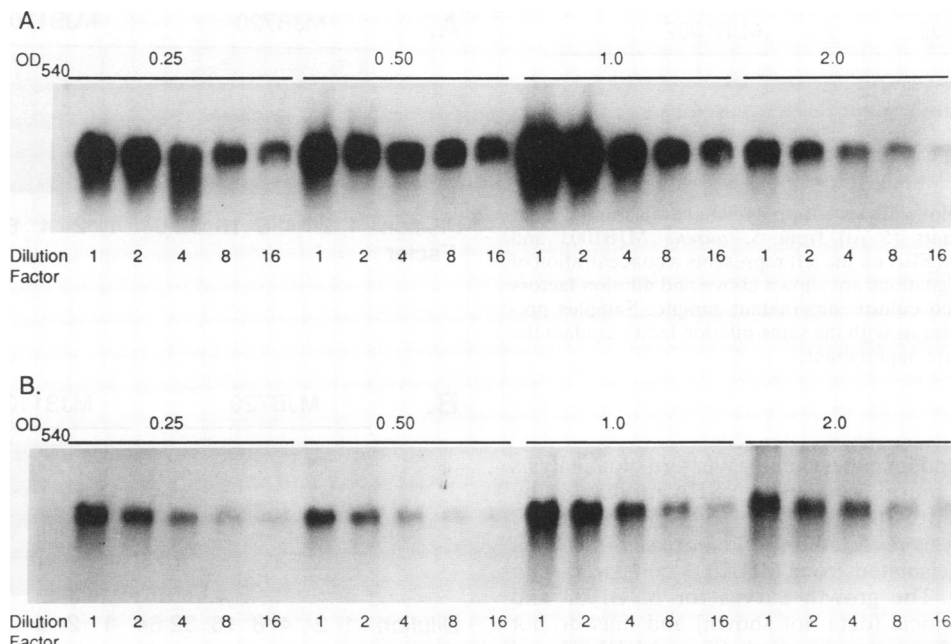


FIG. 3. Northern blot analysis of samples prepared from FRI722 (A) and FRI100 (B). Samples were collected when the OD_{540} of the cultures was 0.25, 0.50, 1.0, and 2.0 as indicated above each set of samples. Total cellular RNA was electrophoresed through a 1% agarose gel, transferred to a Nytran membrane, and reacted with ^{32}P -labeled A624 probe. Different samples with the same dilution factor (shown below each sample) contain the same amount of total cellular RNA. The two blots were normalized by running identical samples of RNA from FRI100 at an OD_{540} of 2.0 on both blots. The signal intensity for the sample was the same for both blots (data not shown), indicating equivalent transfer efficiency and probe hybridization; therefore, samples between the two blots can be directly compared.

the apparent transcriptional start site for *sea* (8), and the downstream primer (5' TGTGTGCAACGTTAGGG 3') hybridized to a region 141 bp downstream of the transcriptional start site. The PCR method used was adapted from a basic protocol previously described (1). Genomic DNA samples were denatured at 94°C for 5 min, and then the mixtures went through 30 cycles of denaturation at 92°C, annealing at 45°C, and extension at 72°C. The amplified DNA was sequenced directly, and FRI722 was found to contain two nucleotide changes with respect to its parent strain, FRI100 (7) (Fig. 4). An A→G transition at nucleotide -28 (with respect to the transcriptional start site at +1) was found in the spacer region between the putative -35 and -10 regions of the promoter, and a T→C transition was located at nucleotide +3 very close to the transcriptional start site for *sea* (8).

Western and Northern blot analysis of MJB1003 and MJB1007. To determine whether the two nucleotide changes

found in FRI722 contributed to increased production of SEA, a translational fusion between the upstream region of FRI722 and the *sea* structural gene from FRI100 was constructed. The 272-bp fragment obtained by PCR amplification of genomic DNA from FRI722 contained the *sea* upstream promoter region flanked by the naturally occurring *EcoRV* site at the 5' terminus and the *HincII* site located 45 bp downstream of the translation initiation site. These sites were used to substitute this fragment for the analogous *EcoRV*-to-*HincII* fragment of *sea*_{FRI100}. A 1.4-kb *EcoRV* fragment containing *sea*_{FRI722-FRI100} was then subcloned into a pC194-pSP73 shuttle plasmid and transformed into *E. coli* DH5 α . A similar construction containing *sea*_{FRI100} was also made. The *sea*-containing plasmids were electroporated (14) into *S. aureus* RN4220 with selection for chloramphenicol resistance (5 μ g/ml). The resulting RN4220 derivatives were MJB1003 (contains *sea*_{FRI722-FRI100}) and MJB1007 (contains *sea*_{FRI100}).

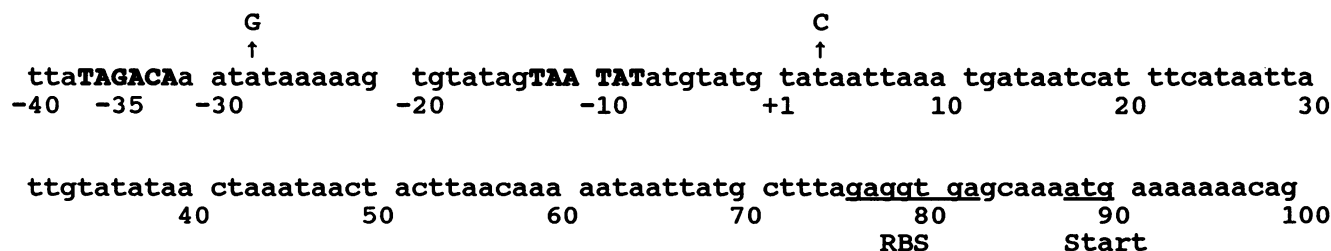


FIG. 4. Nucleotide sequence of the upstream region of *sea*_{FRI100} (7) (*sea*_{FRI100} has the same sequence as *sea*_{FRI337} [6]). The nucleotide corresponding to the 5' end of *sea* mRNA is indicated at position +1, and the putative -10 and -35 regions of the promoter are capitalized and in boldface (8). The translational start site (Start) and putative ribosome binding site (RBS) are underlined beginning at positions +88 and +76. The two mutations present in FRI722 are indicated above the sequence with arrows.

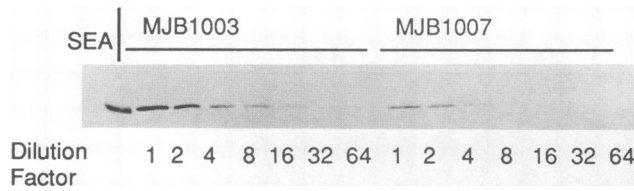


FIG. 5. Western blot analysis with polyclonal antiserum to SEA of culture supernatants (5 μ l) from *S. aureus* MJB1003 and MJB1007. The purified SEA on the left represents a concentration of 0.5 μ g/ml. Strain designations are shown above and dilution factors are shown below each culture supernatant sample. Samples prepared from different strains with the same dilution factor contain the same amount of culture supernatant.

In several independent experiments, Western blot analysis revealed at least a fourfold increase in SEA production by MJB1003 compared with MJB1007 (Fig. 5). Similarly, Northern blot analysis revealed fourfold more *sea* mRNA in total cellular RNA isolated from MJB1003 compared with MJB1007 (Fig. 6). The growth curves for MJB1003 and MJB1007 were identical (data not shown) and thus do not account for the differences observed. Since MJB1003 and MJB1007 are isogenic except for the two mutations found in the *sea* upstream region of FRI722 at nucleotides -28 and $+3$, these results suggested that one or both of the mutations present in the upstream region of FRI722 contributed to the increased production of SEA by this strain.

Analysis of single-site substitutions at nucleotides -28 and $+3$. To evaluate the individual effects of the mutations at -28 and $+3$, in vitro mutagenesis was used as previously described (8) to obtain each mutation as a single-site substitution. Western blot analysis was then used to compare SEA production between the two resulting strains and an isogenic strain containing no mutations. In several independent experiments, Western blot analysis revealed that levels of SEA production were at least twofold greater for MJB1101 (pC194 derivative with *sea* containing A \rightarrow G mutation at nucleotide -28) and MJB1102 (pC194 derivative with *sea* containing T \rightarrow C mutation at nucleotide $+3$) than for MJB720 (pC194 derivative with wild-type upstream *sea* sequence [Fig. 7]). These results indicated that the mutations at -28 and $+3$

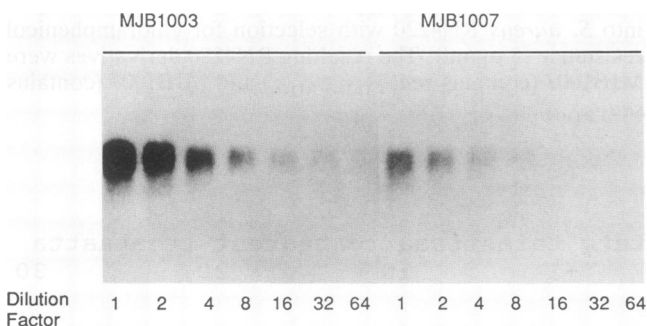


FIG. 6. Northern blot analysis of samples prepared from MJB1003 and MJB1007. Samples were collected when the OD₅₄₀ of the cultures was 1.0. Total cellular RNA was electrophoresed through a 1% agarose gel, transferred to a Nytran membrane, and reacted with ³²P-labeled A624 probe. Different samples with the same dilution factor (shown below each sample) contain the same amount of total cellular RNA. Photographs of MJB1003 and MJB1007 RNA samples are from the same autoradiogram.

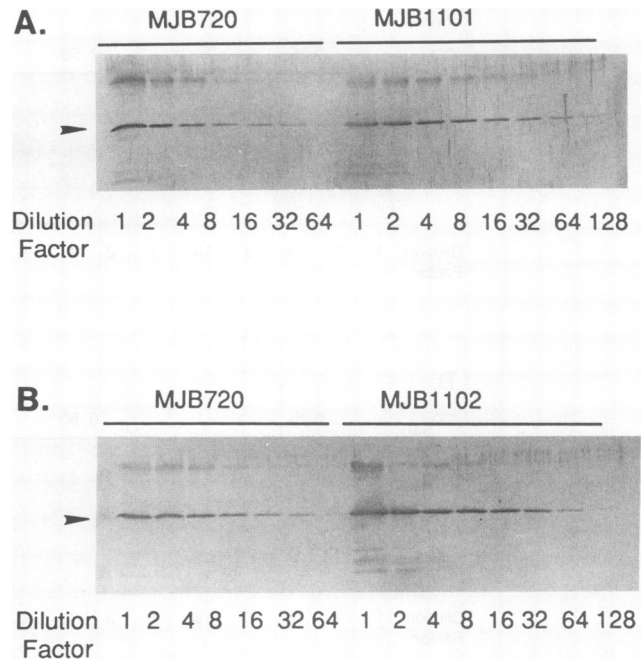


FIG. 7. Western blot analysis with polyclonal antiserum to SEA of culture supernatants (5 μ l) from *S. aureus* MJB720, MJB1101, and MJB1102. Strain designations are shown above and dilution factors are shown below each culture supernatant sample. For a given gel, samples prepared from different strains with the same dilution factor contain the same amount of culture supernatant. The arrowheads indicate the position of SEA.

both led to increased SEA production in FRI722 and MJB1003. Furthermore, the effects of the two mutations appeared to be additive. Since FRI722 was obtained by 13 rounds of mutagenesis, it is possible that the two mutations arose independently during the course of sequential mutagenesis, and each was selected as increasing SEA production.

The mechanism by which the mutations at nucleotides -28 and $+3$ increase *sea* expression is not known, but there is growing recognition that bases outside of the classical -35 and -10 promoter regions are often important determinants of promoter activity and strength (9, 13, 16). Variation in spacing between the -35 and -10 regions of the promoter has been shown to affect promoter activity (12); however, there is no consensus sequence for nucleotides in the spacer region. It is known that information required for promoter recognition and for stable complex formation resides in the promoter region upstream of position -4 (2, 16). It is possible that the A \rightarrow G mutation at nucleotide -28 in *sea*_{FRI722} has a slight effect on promoter recognition or stable complex formation, thereby increasing *sea* expression. Researchers using the *E. coli lacUV5* promoter as well as *E. coli* synthetic promoters have found that nucleotides in the early transcribed region (nucleotides $+1$ to $+20$) affect promoter strength with magnitudes ranging from twofold to more than 10-fold (13, 16). The mutation at $+3$ in FRI722 may affect promoter strength by affecting the rate of transcription initiation or promoter clearance; however, the effect could also occur at the level of mRNA stability.

Nucleotide sequence accession number. The GenBank accession number for the sequence of the upstream region of *sea*_{FRI100} is L22565.

D.W.B. was supported with funds from NIH Cellular and Molecular Biology Training Grant T32 GM07215 and by the Shaw Scientist program. M.J.B. is a Shaw Scientist.

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