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The Carboxyl-Terminal Region of Staphylococcal Enterotoxin Type A Is Required for a Fully Active Molecule

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Staphylococcal enterotoxin type A (SEA) gene (*sea*$^+$) mutations were constructed by exonuclease III digestion or cassette mutagenesis. Five different *sea* mutations that had 1, 3, 7, 39, and 65 codons deleted from the 3′ end of *sea*$^+$ were identified and confirmed by restriction enzyme and nucleotide sequence analyses. Each of these *sea* mutations was constructed in *Escherichia coli* and transferred to *Staphylococcus aureus* by using the plasmid vector pC194. Culture supernatants from the parent *S. aureus* strain that lacked an enterotoxin gene (negative control) and from derivatives that contained either *sea*$^+$ (positive control) or a *sea* mutation were examined for in vitro sensitivity to degradation by monkey stomach lavage fluid, the ability to cause emesis when administered by an intragastric route to rhesus monkeys, and the ability to induce T-cell proliferation and by Western immunoblot analysis and a gel double-diffusion assay with polyclonal antibodies prepared against SEA. Altered SEAs corresponding to the predicted sizes were visualized by Western blot analysis of culture supernatants for each of the staphylococcal derivatives that contained a *sea* mutation. The altered SEA that lacked the C-terminal amino acid residue behaved like SEA in all of the assays performed. The altered SEA that lacked the three C-terminal residues of SEA caused T-cell proliferation but was not emetic; this altered SEA was degraded in vitro by monkey stomach lavage fluid and did not react in the gel double diffusion assay. Altered SEAs that lacked 7, 39, or 65 carboxyl-terminal residues were degraded by stomach lavage fluid in vitro, did not produce an emetic response, and did not induce T-cell proliferation or form a visible reaction in the gel double-diffusion assay.

Staphylococcal enterotoxins are emetic toxins that cause the intoxication staphylococcal food poisoning (4). Five major serological types of enterotoxins have been characterized (types A through E; referred to as SEA through SEE, respectively). SEC is further subdivided into SEC1, SEC2, and SEC3 based on minor differences in antigenic epitopes (4). Each type of enterotoxin gene has been cloned, and its DNA and derived amino acid sequences have been determined (3, 5, 6, 9, 10, 13, 14, 17, 19, 30). There is 50 to 85% nucleotide sequence identity among the different types of enterotoxins (7). Comparison of data obtained by nucleotide and DNA sequence analyses is consistent with each enterotoxin being produced in precursor form. Cleavage of the signal sequences yields the mature extracellular forms of the toxins.

In addition to causing emesis, the enterotoxins are potent stimulators of T-cell proliferation and secretion of cytokines such as interleukin-1, tumor necrosis factor, and gamma interferon (15, 16, 28; for a review, see reference 4). Alber and coworkers reported that the emetic response induced by SEB probably is not mediated solely by its effect on T cells (1). Neither carboxymethylated SEB nor an anti-idiotypic monoclonal antibody (raised against a monoclonal antibody to SEB) was able to induce emesis, but both were able to block the emetic ability of SEB and stimulate T-cell proliferation (1).

Sugiyama and coworkers determined the site of emetic action in the rhesus monkey to be the abdominal viscera, based on studies testing the emetic responsiveness of monkeys after destruction of specific neural structures and/or visceral deafferentation (35). Alber et al. suggested that SEB binds to a receptor on a target cell in the intestinal mucosa and that this target cell most likely contains some structure similar to the T-cell receptor (1).

Little is known about the relationship between the structure of the enterotoxins and their activities. Spero and Morlock reported that the trypsin-derived carboxyl-terminal polypeptide of SEC1 (molecular weight, about 22,000) causes diarrhea but does not cause emesis when administered to rhesus monkeys (34). Bohach and coworkers obtained a similar trypsin-derived SEC1 fragment (calculated molecular weight, 20,659) that was mitogenic and pyrogenic and had some of the antigenic properties of intact SEC1 (8). Pontzer and coworkers demonstrated that a synthetic peptide corresponding to amino acids 1 through 27 of SEA competitively inhibits the ability of SEA to induce T-cell proliferation and production of gamma interferon; presumably, this peptide prevents SEA from forming a complex with either the major histocompatibility complex class II antigen or the T-cell receptor (29). The abilities of all of the trypsin-derived SEC1 polypeptides or the synthetic SEA polypeptide to cause emesis were not evaluated (8, 29).

This study was undertaken to evaluate the importance of the carboxyl-terminal region of SEA for induction of emesis and T-cell proliferation. Deletion mutations of the 3′ region of *sea*$^+$ were constructed by exonuclease III digestion and cassette mutagenesis. The products from each of these genes were characterized. The altered SEA that lacked the carboxyl-terminal residue had the same biological and serological activities as SEA. In contrast, altered SEAs with deletions of three or more residues from the carboxyl-terminal end were not fully active.

MATERIALS AND METHODS

Bacterial strains, plasmids, phage, and media. The bacterial strains, phage, and plasmids used in this study are given

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TABLE 1. Bacterial strains, plasmids, and phages

Strain, phage, or plasmid	Relevant phenotype[a]	Reference or source
E. coli		
Strains		
AB259	Ap^s Sea^-	R. P. Novick (24)
JF626	Ap^s Sea^-	J. Felton (6)
Plasmids		
pGEM3Zf(+)	Ap^r	Promega Corp.
pGEM7Zf(+)	Ap^r	Promega Corp.
pMJB3	Ap^r Sea^+	5
pMJB72	Ap^r Sea^+	This work
pMJB74	Ap^r Sea^+	This work
pMJB156	Ap^r Sea^+	This work
Phages		
M13mp11		23
M13mp19		23, 38
S. aureus		
Strains		
RN4220	Sea^-	R. P. Novick (20)
RN450	Sea^-	R. P. Novick (26)
SA20	Sea^-	S. Iordenescu
Plasmids		
pC194	Cm^r	R. P. Novick (18)
pSK236[b]	Ap^r Cm^r	S. A. Khan
Phage 80α	Generalized transducing phage	P. A. Pattee (25)

[a] Ap, ampicillin; Sea, staphylococcal enterotoxin type A; Cm, chloramphenicol.

[b] pSK236 is a pC194 derivative that contains pUC19 inserted into the *Hin*dIII site.

in Table 1. The *sea* deletion mutations that were constructed are described in Table 2.

The culture conditions for propagation of *Escherichia coli* and *Staphylococcus aureus* (6) cells used for DNA isolation and preparation of competent *E. coli* cells (22) have been described. *E. coli* transformants were selected by using LB agar (22) that contained 75 μg of ampicillin per ml.

Phage 80α was maintained by propagation on *S. aureus* RN450 (33). Phage 80α was propagated on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) that contained 5×10^{-3} M $CaCl_2$ by using a soft-agar overlay of Trypticase soy broth plus 0.3% (wt/vol) agar (Difco Laboratories, Detroit, Mich.) (25). *S. aureus* transductants were selected by using brain heart infusion agar (Difco) that contained 0.002 M sodium citrate and 5 μg of chloramphenicol per ml.

TABLE 2. Nomenclature for *sea* deletion mutations constructed in this study

Allele	Description of allele	Mature protein product	Calculated mol wt of mature protein
sea$^+$	Wild-type allele	SEA[a]	27,093
sea-1309	Codons 169 to 233 of *sea*$^+$ deleted	SEA-1309	19,574
sea-1310	Codons 195 to 233 of *sea*$^+$ deleted	SEA-1310	22,465
sea-1315	Codons 227 to 233 of *sea*$^+$ deleted	SEA-1315	26,237
sea-1316	Codons 231 to 233 of *sea*$^+$ deleted	SEA-1316	26,742
sea-1317	Codon 233 of *sea*$^+$ deleted	SEA-1317	27,006

[a] The mature form of SEA is 233 amino acid residues in length (6). The codon corresponding to the amino-terminal residue of the mature form of SEA is considered the first codon.

For electroporation, *S. aureus* cultures were grown in brain heart infusion broth (Difco). The cultures were grown to an optical density of 0.2 to 0.3 at 600 nanometers, chilled on ice for 15 to 30 min, and harvested by centrifugation at 7,000 × *g* for 10 min at 4°C. The cell pellet was washed two times with 30 ml of 500 mM sucrose–1 mM $MgCl_2$–7 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and suspended in 1 ml of the same solution. Cells were divided into aliquots of 40 μl and stored frozen at −70°C. After electroporation, *S. aureus* recipients were selected by using brain heart infusion agar that contained 5 μg of chloramphenicol per ml.

For biological and serological assays, staphylococcal strains were grown in Erlenmeyer flasks that contained a medium consisting of 3% (wt/vol) N-Z-amine type A (Kraft Inc., Norwich, N.Y.) and 1% (wt/vol) yeast extract (Difco). Cultures were grown for 13 to 18 h at 37°C with shaking (200 rpm, Controlled Environmental Incubator; New Brunswick Scientific Co., Inc., Edison, N.J.) and centrifuged at 7,000 × *g* for 30 min at 4°C. Culture supernatants were filtered through Nalgene filters (0.45-μm pore size; Nalge Co., Rochester, N.Y.). For some assays, culture supernatants were concentrated by dialysis against a saturated solution of polyethylene glycol (20 M; Union Carbide Corp.) in 8 mM phosphate buffer (pH 6.6) and then dialyzed against the same buffer at 4°C.

Molecular biological and genetic techniques. Staphylococcal plasmid DNA was isolated from cleared lysates and purified by CsCl-ethidium bromide dye-buoyant density centrifugation (27). *E. coli* plasmid and replicative forms of M13 phage were purified by an alkaline lysis method (2). Procedures for purification of single-stranded M13 phage DNA have been described elsewhere (2). Nucleotide sequences were determined by the dideoxy-chain termination method (32) with an Amersham sequencing kit (Amersham Corp., Arlington Heights, Ill.).

DNA endonuclease restriction, alkaline phosphatase treatment, T4 DNA polymerase treatment, ligation, transformation, and transfection of competent *E. coli* cells were done by standard methods (22).

Derivatives of pC194 that had either *sea*$^+$ or an *sea* mutation were introduced into *S. aureus* RN4220 by electroporation with 40 μl of cells and 0.1 to 1.0 μg of plasmid DNA in Gene Pulser cuvettes (0.2-cm electrode gap; Bio-Rad Laboratories, Richmond, Calif.). The electroporator (Bio-Rad Gene Pulser) was equipped with a pulse controller. The settings for *S. aureus* electroporations were as follows: voltage, 2.5 kV; capacitance, 25 μF; and resistance, 100 Ω. Immediately after electroporation, the cuvettes were rinsed with 1 ml of sucrose-maleate-Penassay medium (11); the cell suspension was placed on ice for 15 min and then incubated for 1 h at 37°C to allow expression of resistance to chloramphenicol. Cells were then plated on antibiotic-containing medium and incubated at 37°C for 24 to 36 h.

pC194 derivatives were transferred from RN4220 to SA20 by transduction. Propagation of phage 80α on donor strains to obtain transducing lysates and transductions were performed as previously described (33). Higher concentrations of altered SEAs were observed in culture supernatants of

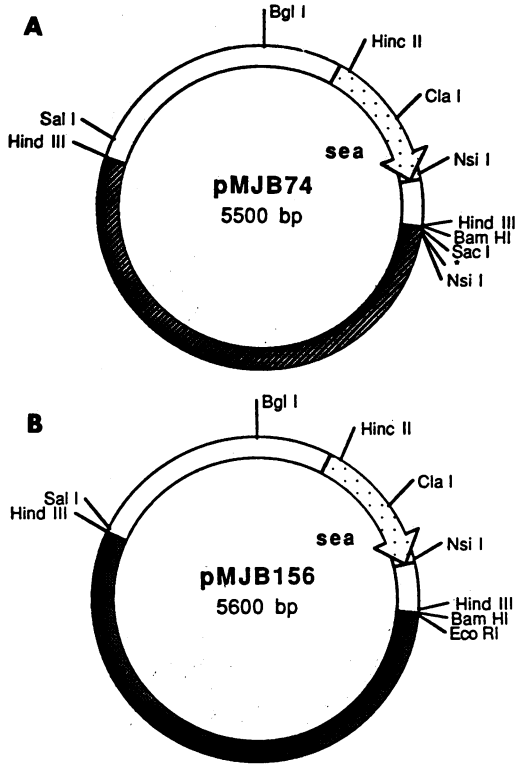

FIG. 1. (A) pMJB74 is a pGEM7Zf(+) (▨) derivative that has a 2.5-kbp *sea*$^+$-containing *Hin*dIII fragment (□, ▩). The location of the SMURFT cartridge is indicated by an asterisk. (B) pMJB156 is a pGEM3Zf(+) (▩) derivative that has a 2.4-kbp *sea*$^+$-containing *Bam*HI-*Sal*I fragment (□, ▩). The location of *sea*$^+$ (▩) is indicated. The direction of transcription is indicated by the arrow. Diagrams are drawn to scale.

SA20 derivatives that contained the larger *sea* deletions compared with those in RN4220 derivatives. Culture supernatants from the SA20 derivatives were used for the biological and serological assays.

Construction of deletion mutations. pMJB72 was made by ligation of a DNA cartridge that contained translation termination codons in all reading frames (SMURFT; Pharmacia LKB, Piscataway, N.J.) into pGEM7Zf(+) that had been linearized with *Bst*XI and treated with T4 DNA polymerase to remove 3′ overhangs. Then the 2.5-kbp *sea*$^+$-containing *Hin*dIII fragment of pMJB3 was ligated into the *Hin*dIII site of pMJB72, yielding pMJB74 (Fig. 1A). Insertion of the SMURFT cartridge and orientation of the *sea*$^+$-containing insert in pMJB74 were verified by nucleotide sequence analysis.

pMJB74 was the substrate for generation of large deletions beginning outside of and extending into the 3′ region of *sea*$^+$. Deletions were generated by using the Erase-a-Base System (Promega Corp., Madison, Wis.) according to the manufacturer's instructions. pMJB74 was sequentially digested with *Bam*HI (5′ overhang) and *Sac*I (3′ overhang) and then treated with exonuclease III. The ends were then flushed by reacting with S1 nuclease and the Klenow fragment before ligation and transformation into *E. coli*. To identify *sea* deletion mutations, plasmid DNA isolated from the ampicillin-resistant transformants was examined by restriction enzyme fragment length analysis. The precise extent of several deletions was determined by nucleotide sequence analysis. DNA fragments containing *sea* mutations and the adjacent SMURFT cartridge were subcloned into pSK236, returned to *S. aureus* RN4220, and then transferred to *S. aureus* SA20 by transduction.

Small deletions (3 to 27 bp) in the 3′ region of *sea*$^+$ were constructed by cassette mutagenesis with pMJB156 and synthetic oligonucleotides. pMJB156 is a pGEM3Zf(+) derivative that has the 2.4-kbp *Bam*HI-*Sal*I *sea*$^+$-containing fragment of pMJB74 (Fig. 1B). Synthetic oligonucleotides were obtained from the Biotechnology Laboratories, University of Wisconsin-Madison. The concentrations of the oligonucleotides were determined by monitoring the A_{260} (22). Two complementary oligonucleotides (1 μg of each) were annealed in 10 μl of 10 mM Tris (pH 7.5) at 37°C for 30 min to form a cassette. Each cassette had ends compatible with *Nsi*I and *Bam*HI and contained all but the last one, three, or seven codons of *sea*$^+$ (Fig. 2). In addition, each cassette had a translation stop codon after the *sea* open reading frame to prevent formation of a fusion protein. Bases specifying a unique restriction site were included in each cassette to facilitate screening for *sea* mutations. pMJB156 was digested with *Nsi*I and *Bam*HI and ligated to the oligonucleotide cassettes. Substitution of the cassette for the 290-bp *Nsi*I-*Bam*HI fragment of pMJB156 was verified by DNA sequence analysis. To facilitate the transfer of DNA into *S. aureus*, pMJB156 and its derivatives were digested with *Hin*dIII and ligated to pC194, and the ligation mixtures were transformed into *E. coli*. Then the composite plasmids were isolated from *E. coli* and transferred to *S. aureus* RN4220 by electroporation and then to strain SA20 by transduction.

Serological assays. Polyclonal antibodies prepared against SEA in rabbits were provided by Merlin S. Bergdoll (University of Wisconsin-Madison). The gel double-diffusion assay with antiserum prepared against SEA and purified SEA (Toxin Technology, Madison, Wis.) as a standard has been described previously (31). The concentration of purified SEA was determined by monitoring the A_{277} with an extinction coefficient ($E^{1\%}_{1\ cm}$) of 14.6 (4). Western immunoblot analysis was done with the polyclonal antiserum prepared against SEA and the ProtoBlot kit (Promega) as described by the manufacturer. Samples were electrophoresed through a 15% polyacrylamide–sodium dodecyl sulfate gel with a 6% stacking gel and electrophoretically transferred to nitrocellulose (37). Rainbow markers (Amersham) were used as protein size standards. Concentrations of SEA and SEA-related protein in each of the culture supernatants used in the biological assays were determined by Western blot analysis with known concentrations of purified SEA as standards. Purified SEA was diluted in 0.02 M sodium phosphate–0.15 M NaCl (PBS).

Stomach lavage fluid degradation assay. A sterile saline lavage (15 ml of 0.9% [wt/vol] NaCl; Abbott Laboratories, North Chicago, Ill.) was performed on rhesus monkeys (*Macaca mulatta*) by using nasogastric intubation. This stomach lavage fluid, which was obtained from monkeys that had recently eaten, was used to evaluate the susceptibility of altered SEAs to degradation. Four parts of test sample (culture supernatant) were mixed with one part of stomach lavage fluid or one part of PBS and incubated at 37°C for 1 to 2 h. Then samples were electrophoresed in sodium dodecyl sulfate–15% polyacrylamide gels and analyzed by Western blotting.

Emetic assay. Freshly prepared sterile staphylococcal culture supernatants were stored on ice and administered to monkeys within 2 h of preparation. Rhesus monkeys (2 to 3

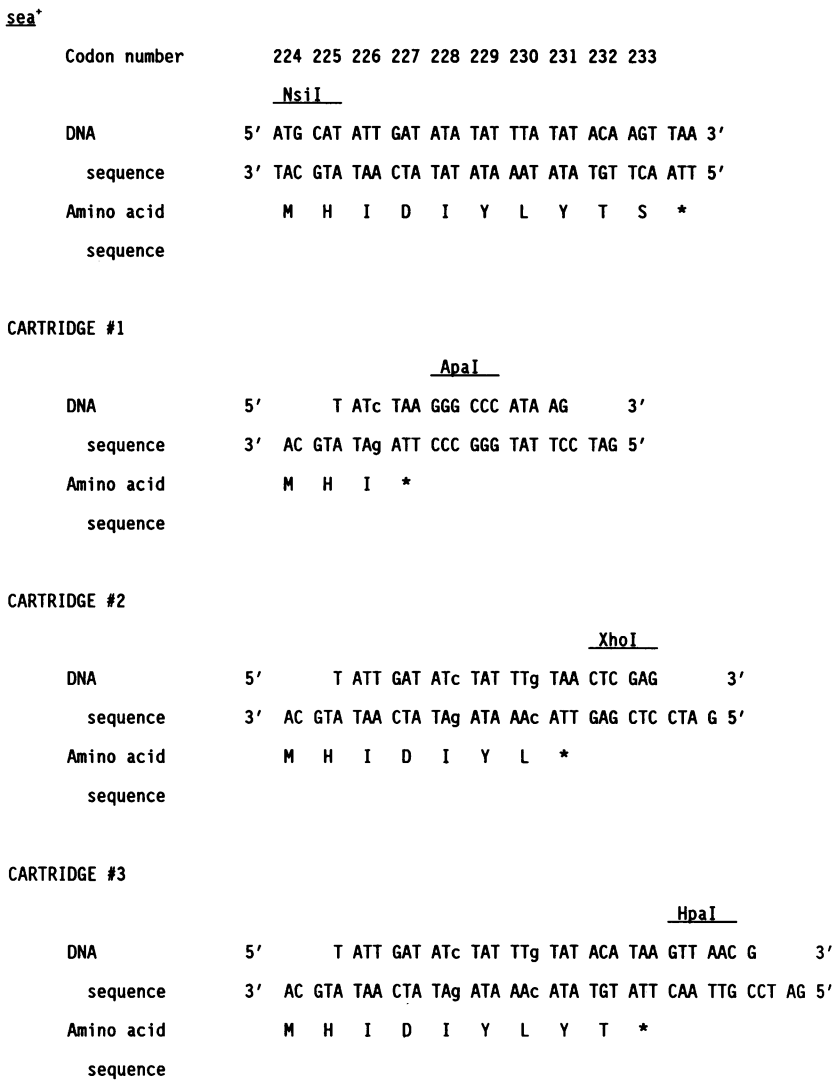

FIG. 2. Double-stranded oligonucleotide cassettes used for construction of *sea*$^+$ deletion mutations. The 3' region of *sea*$^+$ is depicted at the top. The codon numbers correspond to those of *sea*$^+$, with the codon for the amino-terminal residue of mature SEA being designated number 1. The amino acid sequences given for the cartridges are those that are predicted for the SEA open reading frame after the cartridge is substituted for the *Nsi*I-*Bam*HI fragment of pMJB156. Translation termination codons are indicated with an asterisk. The locations of restriction enzyme sites within the cassettes are indicated above the DNA sequences. The lowercase letters within the DNA sequences indicate silent changes that were made to minimize secondary structure formation within the oligonucleotide.

kg) were given about 25 ml of culture supernatant via nasogastric intubation with a size five French infant feeding tube (American Pharmaseal Co., Valencia, Calif.) and observed for 5 h. All assays involving monkeys were performed in collaboration with the Regional Primate Research Center, Madison, Wis.

Mitogenicity assay. T-cell proliferation assays were done with splenocytes obtained from 6- to 8-week-old BALB/c mice. One million mouse splenocytes (150 μl) were seeded in individual wells of a 96-well tissue culture dish (Falcon, Becton Dickinson and Co., Lincoln Park, N.J.) along with 50 μl of test culture supernatant (undiluted or diluted 10^{-1} or 10^{-2}). The cells were incubated 37°C in 5% CO_2 for 72 h and then pulsed for 18 h with 0.5 μCi of [^{3}H]thymidine (Amersham) in 50 μl of complete tissue culture medium (RPMI 1640 [ICN Biomedicals, Inc., Costa Mesa, Calif.] containing 10% fetal bovine serum [HyClone, Logan, Utah], 15 mM HEPES, 3 mM glutamine, 100 U of penicillin, and 100 U of streptomycin [ICN]). The total volume of each well was 250 μl. The contents of the wells were harvested onto glass fiber paper by using a semiautomatic PhD cell harvester (Cambridge Technology, Inc., Watertown, Mass.), and individual filter disks were counted in 3.0 ml of Bio-Safe II scintillation fluid (Research Products International Corp., Mount Prospect, Ill.). Each sample was assayed in triplicate. Data are reported as the mean counts per minute with the standard deviation. SA20 cultures served as the negative control. For some of the experiments that examined the effects of SA20 culture supernatants in the T-cell proliferation assay, a stock (1 mg/ml) of purified SEA was used to spike the SA20 culture supernatant samples. SA20 supernatants were diluted with RPMI. Fifty microliters of each solution was then added to 150 μl of splenocytes. To account for day-to-day variability in the absolute amount of [^{3}H]thymidine incorporated, a

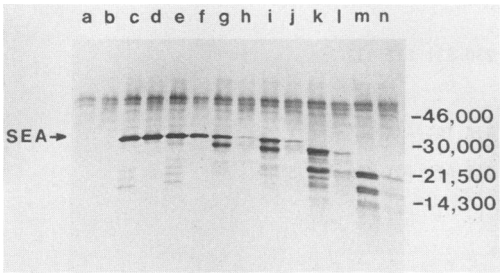

FIG. 3. Comparison of susceptibilities of SEA and altered SEAs to degradation by stomach lavage fluid. Western blot analysis with polyclonal antiserum to SEA was performed with culture supernatants from *S. aureus* derivatives that contained *sea*$^+$ or a *sea* mutation that had been incubated with phosphate-buffered saline (lanes a, c, e, g, i, k, and m) or stomach lavage fluid (lanes b, d, f, h, j, l, and n) for 2 h at 37°C before electrophoresis. Samples were from staphylococcal strains that produced no enterotoxin (lanes a and b; strain SA20, negative control), SEA (lanes c and d), SEA-1317 (lanes e and f), SEA-1316 (lanes g and h), SEA-1315 (lanes i and j), SEA-1310 (lanes k and l), and SEA-1309 (lanes m and n). Positions of molecular weight markers are depicted on the right. The position of the signal corresponding to intact SEA is indicated on the left.

TABLE 3. Characterization of SEA-related products in culture supernatants from *S. aureus* SA20 derivatives

Sample	Western blot[a]	Gel double-diffusion assay[b]	Susceptibility to stomach proteases[c]	Emetic assay[d]	T-cell proliferation assay[e]
SA20	−	−	NA	0/3	−
SEA-1309	+	−	S	0/3	−
SEA-1310	+	−	S	0/3	−
SEA-1315	+	−	S	ND	−
SEA-1316	+	−	S	0/6	+
SEA-1317	+	+	R	3/3	+
SEA	+	+	R	2/3	+

[a] Western blots were reacted with polyclonal antiserum raised against SEA. +, blot contained an SEA-related signal that corresponded to a protein of the predicted size; −, no signal for an SEA-related protein was detected.

[b] Purified SEA was used as the standard. Samples were reacted with polyclonal antiserum that was prepared against SEA. +, sample formed a line of identity with purified SEA; −, no visible reaction was observed.

[c] Western blot analysis was performed on culture supernatants incubated with stomach lavage fluid in vitro for 2 h at 37°C. NA, sample contained no SEA-related proteins (negative control); R, SEA-related protein(s) was resistant to degradation; S, SEA-related protein(s) was susceptible to degradation.

[d] Number of animals that vomited/number of animals in the test group. ND, sample was not tested.

[e] +, culture supernatants stimulated a statistically significant ($P \leq 0.01$) increase in T-cell proliferation compared with that of the SA20 culture supernatant; −, any increase in proliferation was not statistically significant ($P \leq 0.01$).

stimulation index was calculated by dividing the counts per minute obtained from the assay containing the test sample by the counts per minute obtained from the corresponding SA20 supernatants. Statistical analysis (*t* test) was performed on the data from the 10- and 100-fold dilutions from the test and negative control samples with a Minitab Release 7.1 computer package (Minitab, Inc.). The level of significance is $P < 0.01$. Data obtained with culture supernatants that had been diluted 10- and 100-fold were analyzed because they contained enough SEA to fall in the maximal mitogenic range (10^{-2} to 10 μg/ml) (21) but minimized the toxicity of the bacterial culture medium to the T cells.

RESULTS

Analysis of *sea* mutations generated by exonuclease III digestion. *sea-1309* and *sea-1310* had the two smallest deletions among the *sea* mutations obtained with the exonuclease III digestion. *sea-1309* and *sea-1310* encoded mature protein products that were predicted to be 168 (designated SEA-1309) and 194 (designated SEA-1310) amino acid residues in length, respectively. The biological activities of their protein products were examined. A predominant SEA-related signal visualized by Western blot analysis of culture supernatants of *S. aureus* SA20 derivatives containing *sea-1309* or *sea-1310* corresponded to the predicted sizes of SEA-1309 and SEA-1310 (calculated molecular weights, 19,574 and 22,465, respectively) (Table 2 and Fig. 3). Several smaller breakdown products were also evident (Fig. 3). Culture supernatants containing SEA-1309 or SEA-1310 did not produce a visible precipitin line in a gel double-diffusion assay when reacted with polyclonal antiserum prepared against SEA (data not shown), were degraded by treatment with stomach lavage fluid as evidenced by Western blot analysis (Fig. 3), and did not cause emesis when administered intragastrically to rhesus monkeys (Table 3).

Before the culture supernatants from SA20 derivatives that produced SEA-1309 or SEA-1310 were tested for the ability to induce T-cell proliferation, the possibility that undiluted culture supernatant from SA20 affected the T-cell proliferation assay was examined. SEA at a concentration of 3 μg/ml in tissue culture medium (RPMI) was sufficient to cause a five- to eightfold increase in T-cell stimulation compared with RPMI that contained no SEA (Table 4). However, SA20 culture supernatant that had been spiked with SEA (3 μg/ml in the undiluted culture supernatant) did not cause a significant ($P < 0.01$) increase in T-cell proliferation compared with that of SA20 culture supernatant that contained no SEA (Table 4). Because SA20 culture supernatant that contained 3 μg of SEA per ml did not cause a significant increase in incorporation of [^{3}H]thymidine, results with undiluted culture supernatants were not reported for the altered SEAs.

SA20 culture supernatants that were diluted at least 10-fold with RPMI appeared not to be inhibitory in the T-cell proliferation assay. SA20 culture supernatants that were diluted 10-fold before SEA (3 μg/ml) was added resulted in a stimulation index of 12 to 14 (Table 4). It is not clear why SEA in diluted culture supernatant was more stimulatory than SEA in RPMI; perhaps the diluted SA20 sample had a substance unrelated to SEA that stimulates T-cell proliferation. To compensate for this possibility, the response observed for a test sample that consisted of diluted culture supernatant containing SEA-related protein was compared with the response obtained with the same dilution of an SA20 culture supernatant.

A concentration of SEA in a diluted SA20 supernatant sample that reproducibly produced a positive response in the T-cell proliferation assay was determined. Purified SEA was added to samples of undiluted SA20 culture supernatants to a concentration of 0.4, 2.0, or 10 μg/ml. These SA20 culture supernatants that contained SEA were serially diluted 10-, 100-, and 1,000-fold with RPMI (Table 5). Reproducible positive responses were obtained with all three dilutions for the SA20 culture supernatants that had been spiked with 10 μg of SEA per ml (Table 5). The 100- and 1,000-fold dilutions of the SA20 sample spiked with 2.0 μg of SEA per ml resulted in reproducible positive responses in the T-cell

TABLE 4. T-cell proliferation assay of tissue culture medium or culture supernatants from *S. aureus* SA20 spiked with purified SEA

Sample	Expt 1		Expt 2	
	Mean cpm ± SD[a]	SI[b]	Mean cpm ± SD	SI
RPMI medium[c]	299 ± 191	1.0	1,120 ± 459	1.0
RPMI containing SEA[d]	1,446 ± 956	4.8	8,384 ± 3,972	7.5
SA20 supernatant	156 ± 50	1.0	328 ± 163	1.0
SA20 containing SEA	74 ± 6	0.5	99 ± 53	0.3
Diluted SA20 supernatant[e]	1,240 ± 491	1.0	4,911 ± 1,281	1.0
Diluted SA20 supernatant containing SEA[f]	17,152 ± 5,604	13.8	57,622 ± 4,264	11.7

[a] Samples were assayed in triplicate. Assays were performed on undiluted samples and samples diluted 10-fold as indicated.
[b] A stimulation index (SI) of 1 was assigned to each sample that did not contain SEA. The SIs for samples containing SEA were calculated by dividing the counts per minute for the SEA-containing test sample by the counts per minute obtained for the corresponding control sample.
[c] Tissue culture medium used was RPMI 1640.
[d] Purified SEA was added to 3 μg/ml, and 50 μl of each solution was added to 150 μl of splenocytes.
[e] SA20 culture supernatant was diluted 10-fold in RPMI.
[f] SA20 culture supernatant was diluted 10-fold in RPMI before purified SEA was added to 3 μg/ml.

proliferation assay compared with those of SA20 samples that had been diluted appropriately (Table 5).

Culture supernatants of SA20 derivatives that had *sea-1309* or *sea-1310* contained at least 2 μg of full-length SEA-1309 or SEA-1310, respectively, per ml as judged by Western blot analysis (data not shown). Neither the sample containing SEA-1309 nor that containing SEA-1310 induced a significant response in the T-cell proliferation assay (Table 6).

Analysis of *sea* mutations generated by cassette mutagenesis. *sea* deletion mutations that lacked the last seven, three, or one codon of *sea*$^+$ were constructed by cassette mutagenesis; these *sea* mutations were designated *sea-1315*, *sea-1316*, and *sea-1317*, respectively. Western blot analysis of culture supernatants from SA20 derivatives containing *sea-1315*, *sea-1316*, or *sea-1317* revealed protein products corresponding to the predicted sizes in addition to smaller products (Table 2 and Fig. 3). The signals corresponding to proteins of greater than 30,000 molecular weight, which were visible in all the culture supernatants, including that of SA20, which contained no SEA, are probably due to protein A (Fig. 3).

Culture supernatants containing SEA or SEA-1317 reacted in a gel double-diffusion assay to produce a line of identity with purified SEA (Fig. 4). Culture supernatants containing SEA-1316 or SEA-1315 did not react in the gel double-diffusion assay when tested as unconcentrated samples (Fig. 4) or when concentrated 2.5- to 5-fold (data not shown).

SEA and SEA-1317 were resistant to degradation by stomach lavage fluid. Under identical conditions, SEA-1316 and SEA-1315 were degraded by treatment with stomach lavage fluid as evidenced by Western blot analysis (Fig. 3).

Culture supernatants containing SEA (30 ml) and SEA-1317 (24 ml) administered to monkeys via nasogastric intubation caused emesis (Table 3). Thirty milliliters of culture supernatant from SA20 strains containing no SEA-related material or SEA-1316 did not cause an emetic response in any of the test animals (Table 3). The concentration of intact SEA-1316 in the culture supernatant was determined by Western blot analysis (Fig. 5). The signal corresponding to intact SEA-1316 for the sample that was diluted 1:16 was at least as intense as the signal from the sample containing 0.125 μg of purified SEA per ml (Fig. 5, lanes e and i, respectively). Therefore, the concentration of intact SEA-1316 was at least 2.0 μg/ml, and each animal received 60 μg of SEA-1316. This exceeds the concentration of SEA required to elicit an emetic response. In our system, 10 to 20 μg of purified SEA produced emesis in three of seven test animals (unpublished data).

Each of the culture supernatants tested in the T-cell proliferation assay contained at least 2 μg of full-length

TABLE 5. T-cell proliferation assay of dilutions of culture supernatants from *S. aureus* SA20 spiked with purified SEA

Concn of SEA in supernatant[a] (μg/ml)	Expt	Mean cpm ± SD[b] (SI[c]) with the following sample dilution[d]:		
		10^{-1}	10^{-2}	10^{-3}
0	1	1,240 ± 491 (1.0)	1,223 ± 158 (1.0)	283 ± 68 (1.0)
	2	4,911 ± 1,281 (1.0)	2,989 ± 331 (1.0)	1,050 ± 475 (1.0)
10	1	**15,942** ± 2,434 (12.9)	**13,672** ± 989 (11.2)	**2,661** ± 607 (9.4)
	2	**51,124** ± 10,087 (10.4)	**61,082** ± 10,144 (20.4)	**13,180** ± 3,846 (12.6)
2	1	11,945 ± 4,803 (9.6)	**11,840** ± 869 (9.7)	**704** ± 131 (2.5)
	2	**45,943** ± 6,720 (9.4)	**47,727** ± 2,405 (16.0)	**3,916** ± 669 (3.7)
0.4	1	**8,851** ± 2,793 (7.1)	**4,542** ± 700 (3.7)	265 ± 96 (0.9)
	2	32,604 ± 11,229 (6.6)	19,369 ± 9,151 (6.5)	1,785 ± 738 (1.7)

[a] Culture supernatant from *S. aureus* SA20 was spiked to 0.4, 2.0, or 10 μg of SEA per ml (final concentration in the undiluted sample) and diluted 10-, 100-, or 1,000-fold with RPMI. Samples of 50 μl of each dilution were added to the T-cell stimulation assay.
[b] Samples that resulted in a statistically significant difference ($P < 0.01$) in proliferation compared to the corresponding SA20 sample are in boldface type.
[c] See footnote *b* of Table 4.
[d] Each sample dilution was assayed in triplicate.

TABLE 6. T-cell proliferation assay of culture supernatants from *S. aureus* SA20 derivatives that contain *sea*$^+$ or a mutant allele

Sample	Expt	Mean cpm[a] ± SD (SI[b]) with the following sample dilution[c]:	
		10^{-1}	10^{-2}
SA20[d]	1	3,860 ± 183 (1.0)	1,542 ± 90 (1.0)
	2[e]	3,243 ± 1,321 (1.0)	1,315 ± 337 (1.0)
	3	1,240 ± 491 (1.0)	1,223 ± 158 (1.0)
	4	4,911 ± 1,281 (1.0)	2,989 ± 331 (1.0)
SEA-1309	1	3,214 ± 506 (0.8)	2,053 ± 330 (1.3)
	2	3,639 ± 431 (1.1)	4,244 ± 3,024 (3.2)
SEA-1310	1	2,521 ± 1,004 (0.7)	1,830 ± 435 (1.2)
	2	4,747 ± 929 (1.5)	2,715 ± 1,500 (2.1)
SEA-1315	1	7,313 ± 1,981 (1.9)	**2,859** ± 320 (1.9)
	2	2,811 ± 675 (0.9)	1,130 ± 61 (0.9)
	3	3,534 ± 2,895 (2.9)	**723** ± 46 (0.6)
	4	6,267 ± 2,888 (1.3)	4,045 ± 610 (1.4)
SEA-1316	1	**11,051** ± 906 (2.9)	4,616 ± 1,475 (3.0)
	2	**10,858** ± 985 (3.3)	**6,474** ± 536 (4.9)
SEA-1317	1	**26,911** ± 2,457 (7.0)	**24,594** ± 4,525 (15.9)
	2	**18,772** ± 1,486 (5.8)	**47,102** ± 3,484 (35.8)
SEA	1	**26,388** ± 1,125 (6.8)	**25,569** ± 1,445 (16.6)
	2	**19,924** ± 1,792 (6.1)	**40,837** ± 3,550 (31.1)
	3	**12,131** ± 2,100 (9.8)	**15,936** ± 1,184 (13.0)
	4	**38,000** ± 9,156 (7.7)	**56,423** ± 3,698 (18.9)

[a] Samples that resulted in a statistically significant difference ($P < 0.01$) in proliferation compared with the SA20 sample are in boldface type.
[b] See footnote *b* of Table 4.
[c] Samples were assayed in triplicate.
[d] Culture supernatant from SA20, which contains no *sea*, was used for the negative control.
[e] Culture supernatants were concentrated three- to fivefold.

SEA-related protein per ml, as evidenced by Western blot analysis (representative data shown in Fig. 5 and 6). Compared with appropriately diluted culture supernatants of SA20, culture supernatants containing SEA or SEA-1317 caused a 6- to 36-fold increase in the amount of incorporated [^{3}H]thymidine (Table 6). A reproducible, statistically significant ($P < 0.01$) increase (three- to fivefold) in T-cell proliferation occurred with the supernatant containing SEA-1316 (Table 6). Among the altered SEAs examined, SEA-1315 had the smallest deletion that did not reproducibly cause T-cell proliferation. To verify this observation, two additional experiments were done, each with newly prepared samples that contained SEA-1315 (Table 6, experiments 3 and 4).

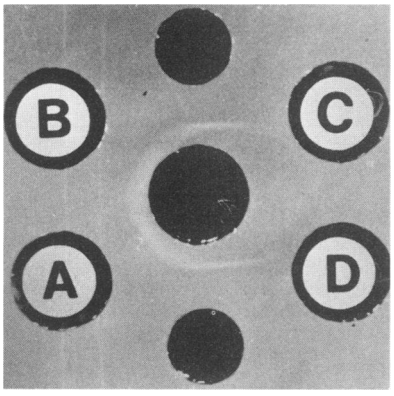

FIG. 4. Gel double-diffusion assay. The center well contained polyclonal antiserum prepared against SEA. The top and bottom wells contained 4 μg of SEA per ml. Wells A through D contained culture supernatant from SA20 strains containing SEA (A), SEA-1317 (B), SEA-1316 (C), and SEA-1315 (D).

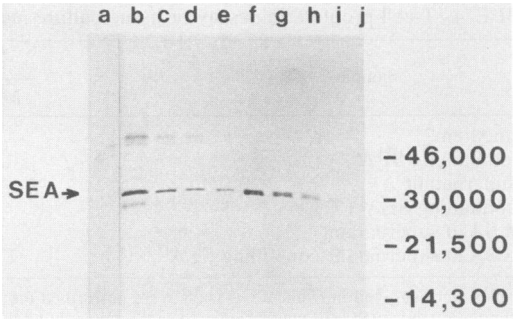

FIG. 5. Western blot analysis with polyclonal antiserum to SEA of culture supernatants (5-μl samples) from *S. aureus* SA20 (undiluted, lane a) or an SA20 derivative that produced SEA-1316 (undiluted [lane b] or diluted 1:4 [c], 1:8 [d], or 1:16 [e]). The following concentrations of purified SEA in PBS (5-μl samples) were analyzed: 1 μg/ml (lane f), 0.5 μg/ml (lane g), 0.25 μg/ml (lane h), 0.125 μg/ml (lane i), and 0.063 μg/ml (lane j).

DISCUSSION

A characteristic of the staphylococcal enterotoxins that contributes to their effectiveness as food intoxicants is their ability to survive the harsh conditions in the gastrointestinal tract (4, 36). Part of this may be explained by their relative resistance to proteolytic cleavage by gastrointestinal proteases. We developed an in vitro assay to compare altered SEAs with unaltered SEAs for their relative sensitivities to degradation by a saline lavage fluid from a monkey's stomach. In this assay, SEA-1317 was as stable as SEA, whereas all the altered SEAs that lacked three or more carboxyl terminal residues (SEA-1316, SEA-1315, SEA-1310, and SEA-1309) were degraded (Fig. 3).

Our data were consistent with resistance to degradation by stomach lavage fluid in vitro being a reliable indication of the ability of an altered SEA to remain in an active form in the

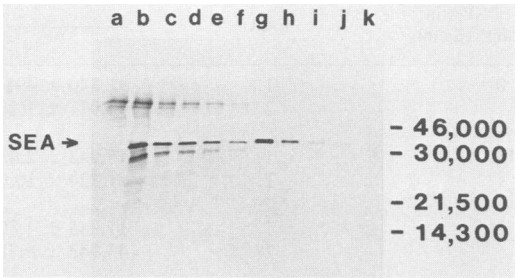

FIG. 6. Western blot analysis with polyclonal antiserum to SEA of culture supernatants (5-μl samples) from *S. aureus* SA20 (undiluted, lane a) or an SA20 derivative that produced SEA-1315 (undiluted [lane b] or diluted 1:4 [lane c], 1:8 [lane d], 1:16 [lane e], or 1:32 [lane f]). Various concentrations of purified SEA in PBS (5-μl samples) were analyzed: 2 μg/ml (lane g), 1 μg/ml (lane h), 0.5 μg/ml (lane i), 0.25 μg/ml (lane j), and 0.125 μg/ml (lane k).

gastrointestinal tract. Such an active form was required for emesis. Specifically, SEA and SEA-1317 were emetic, and none of the altered SEAs that lacked three or more carboxyl terminal residues caused emesis (Table 3). None of six test animals that received 60 μg of SEA-1316 even appeared ill. In contrast, 10 to 20 μg of SEA produced a response by three of seven of our test animals, and the reported dose required to obtain vomiting by 50% of a test group is 2 to 5 μg (4, 12).

Although the possibility that an active site residue(s) was within the terminal three residues has not been ruled out, the inability to cause emesis was more likely due to an altered conformation that results in an increased susceptibility to degradation in the gastrointestinal tract. In addition to the data from susceptibility to stomach lavage fluid in vitro, analysis of culture supernatants containing SEA-1316 by a gel double-diffusion assay was consistent with the hypothesis that SEA-1316 had an altered conformation compared to SEA. SEA-1316 did not form a visible precipitate with antibody produced against SEA in a gel double-diffusion assay (Fig. 2), although the culture supernatant contained at least 2 μg of SEA-1316 per ml. The lower limit of detection for the gel double-diffusion assay we used is 0.5 μg of staphylococcal enterotoxin per ml (31).

Analysis of culture supernatants by Western blotting suggested that the stability in culture supernatants of an altered SEA was affected by the size of the carboxyl-terminal deletion. Examination by Western blot analysis revealed one signal corresponding to intact SEA (Fig. 3, lane c). In contrast, in samples obtained from strains containing *sea* mutations, additional, smaller SEA-related fragments were observed (Fig. 3, lanes e, g, i, k, and m). For the altered SEAs, the amounts of the SEA breakdown products increased with storage at 4°C. However, after 13 days at 4°C, there were still significant concentrations of the intact forms (data not shown). These SEA breakdown fragments may have resulted from digestion by staphylococcal proteases or from spontaneous degradation.

Deletion of the carboxyl-terminal residue had no detectable effect on the ability of SEA to induce T-cell proliferation (Table 6). SEA-1316 stimulated a statistically significant ($P < 0.01$) increase in T-cell proliferation compared with SA20, but not to the same extent as that in the SEA-containing sample (Table 6). Perhaps there was less intact SEA-1316 or the conformation of SEA-1316 was unlike SEA. Further studies are required to determine the reason for the weak activity of SEA-1316 in the T-cell proliferation assay. Altered SEAs with deletions larger than that of SEA-1316 did not reproducibly induce a significant response in the T-cell proliferation assay. Because each of these culture supernatants contained at least as much full-length SEA-related protein as SEA (2 μg/ml) shown to stimulate a positive T-cell response, the inability to induce a T-cell response was probably due not to insufficient protein but rather to loss of the activity.

Based on analysis of SEA-1316, it cannot be concluded that the active sites required for emesis and T-cell proliferation are different. A more likely explanation is that SEA-1316 is more stable in cell cultures used for the T-cell proliferation assay than in the emetic assay, which requires remaining in an active form in the gastrointestinal tract.

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REFERENCES

1. **Alber, G., D. K. Hammer, and B. Fleischer.** 1990. Relationship between enterotoxic- and T lymphocyte-stimulating activity of staphylococcal enterotoxin B. J. Immunol. **144**:4501–4506.
2. **Ausubel, F. M., B. Roger, R. E. Kingston, D. D. Moore, J. G. Seidman, S. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
3. **Bayles, K. W., and J. J. Iandolo.** 1989. Genetic and molecular analysis of the gene encoding staphylococcal enterotoxin D. J. Bacteriol. **171**:4799–4806.
4. **Bergdoll, M. S.** 1983. Enterotoxins, p. 559–598. *In* C. S. F. Easmon and C. Adlam (ed.), Staphylococci and staphylococcal infections. Academic Press, Inc., New York.
5. **Betley, M. J., S. Lofdahl, B. N. Kreiswirth, M. S. Bergdoll, and R. P. Novick.** 1984. Staphylococcal enterotoxin A gene is associated with a variable genetic element. Proc. Natl. Acad. Sci. USA **81**:5179–5183.
6. **Betley, M. J., and J. J. Mekalanos.** 1988. Nucleotide sequence of the type A staphylococcal enterotoxin gene. J. Bacteriol. **170**: 34–41.
7. **Betley, M. J., M. T. Soltis, and J. L. Couch.** 1990. *In* R. P. Novick and R. Skurray (ed.), Conference proceedings for "Molecular Biology of the Staphylococci," 21 through 25 May 1989, in press.
8. **Bohach, G. A., J. P. Handley, and P. M. Schlievert.** 1989. Biological and immunological properties of the carboxyl terminus of staphylococcal enterotoxin C1. Infect. Immun. **57**:23–28.
9. **Bohach, G. A., and P. M. Schlievert.** 1987. Nucleotide sequence of the staphylococcal enterotoxin C_1 gene and relatedness to other pyrogenic toxins. Mol. Gen. Genet. **209**:15–20.
10. **Bohach, G. A., and P. M. Schlievert.** 1989. Conservation of the biologically active portion of staphylococcal enterotoxins C1 and C2. Infect. Immun. **57**:2249–2252.
11. **Chang, S., and S. N. Cohen.** 1979. High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. Mol. Gen. Genet. **68**:111–115.
12. **Chu, F. S., K. Thadhani, E. J. Schantz, and M. S. Bergdoll.** 1966. Purification and characterization of staphylococcal enterotoxin A. Biochemistry **5**:3281–3289.
13. **Couch, J. L., and M. J. Betley.** 1989. Nucleotide sequence of the type C_3 staphylococcal enterotoxin gene suggests that intergenic recombination causes antigenic variation. J. Bacteriol. **171**: 4507–4510.
14. **Couch, J. L., M. T. Soltis, and M. J. Betley.** 1988. Cloning and nucleotide sequence of the type E staphylococcal enterotoxin gene. J. Bacteriol. **170**:2954–2960.
15. **Fast, D. J., P. M. Schlievert, and R. D. Nelson.** 1988. Nonpurulent response to toxic shock syndrome toxin 1-producing *Staphylococcus aureus*. J. Immunol. **140**:949–953.
16. **Fast, D., P. M. Schlievert, and R. D. Nelson.** 1989. Toxic shock syndrome-associated staphylococcal and streptococcal pyrogenic toxins are potent inducers of tumor necrosis factor production. Infect. Immun. **57**:291–294.
17. **Hovde, C. J., S. P. Hackett, and G. A. Bohach.** 1990. Nucleotide sequence of the staphylococcal enterotoxin C3 gene: sequence comparison of all three type C staphylococcal enterotoxins. Mol. Gen. Genet. **220**:329–333.
18. **Iordanescu, S.** 1975. Recombinant plasmid obtained from two different compatible staphylococcal plasmids. J. Bacteriol. **124**: 597–601.
19. **Jones, C. L., and S. A. Khan.** 1986. Nucleotide sequence of the enterotoxin B gene from *Staphylococcus aureus*. J. Bacteriol. **166**:29–33.
20. **Kreiswirth, B. N., S. Lofdahl, M. J. Betley, M. O'Reilly, P. Schlievert, M. S. Bergdoll, and R. P. Novick.** 1983. The toxic shock syndrome toxin exotoxin structural gene is not detectably transmitted by a prophage. Nature (London) **305**:709–712.
21. **Langford, M. P., G. J. Stanton, and H. M. Johnson.** 1978. Biological effects of staphylococcal enterotoxin A on human

peripheral lymphocytes. Infect. Immun. **22**:62–68.
22. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Messing, J., and J. Vieira.** 1982. A new pair of M13 vectors for selecting either DNA strand of double-digest restriction fragments. Gene **19**:269–276.
24. **Murray, N. E., P. L. Barten, and K. Murray.** 1973. Restriction of bacteriophage by *Escherichia coli* K. J. Mol. Biol. **81**:395–407.
25. **Novick, R. P.** 1963. Analysis by transduction of mutations affecting penicillinase formation in *Staphylococcus aureus*. J. Gen. Microbiol. **33**:121–136.
26. **Novick, R. P., and R. Brodsky.** 1972. Studies on plasmid replication. I. Plasmid incompatibility and establishment in *Staphylococcus aureus*. J. Mol. Biol. **68**:285–302.
27. **Novick, R. P., E. Murphy, T. J. Gryczan, E. Baron, and I. Edelman.** 1979. Penicillinase plasmids of *Staphylococcus aureus*: restriction-deletion maps. Plasmid **2**:109–129.
28. **Parsonnet, J., Z. A. Gillis, and G. B. Pier.** 1986. Induction of interleukin-1 by strains of *Staphylococcus aureus* from patients with non-menstrual toxic-shock syndrome. J. Infect. Dis. **154**: 55–63.
29. **Pontzer, C. H., J. K. Russell, and H. M. Johnson.** 1989. Localization of an immune functional site on staphylococcal enterotoxin A using the synthetic peptide approach. J. Immunol. **143**:280–284.
30. **Ranelli, D. M., C. L. Jones, M. B. Johns, G. J. Mussey, and S. A. Khan.** 1985. Molecular cloning of staphylococcal enterotoxin B gene in *Escherichia coli* and *Staphylococcus aureus*. Proc. Natl. Acad. Sci. USA **82**:5850–5854.
31. **Robbins, R., S. Gould, and M. S. Bergdoll.** 1974. Detecting the enterotoxigenicity of *Staphylococcus aureus* strains. Appl. Microbiol. **28**:946–950.
32. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **12**:5436–5467.
33. **Schroeder, C. J., and P. A. Pattee.** 1984. Transduction analysis of transposon Tn*551* insertion in the *trp-thy* region of the *Staphylococcus aureus* chromosome. J. Bacteriol. **157**:533–537.
34. **Spero, L., and B. A. Morlock.** 1978. Biological activities of the peptides of staphylococcal enterotoxin C formed by limited tryptic hydrolysis. J. Biol. Chem. **253**:8787–8791.
35. **Sugiyama, H., and T. Hayama.** 1965. Abdominal viscera as site of emetic action for staphylococcal enterotoxin in the monkey. J. Infect. Dis. **115**:330–336.
36. **Thompson, N. E., M. S. Bergdoll, R. F. Meyer, R. W. Bennett, L. Miller, and J. D. Macmillan.** 1985. Monoclonal antibodies to the enterotoxins and to the toxic shock syndrome toxin produced by *Staphylococcus aureus*, p. 23–59. *In* A. J. L. Macario and E. C. Macario (ed.), Monoclonal antibodies against bacteria. Academic Press, Inc., New York.
37. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
38. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.