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Characterization of Novel Type C Staphylococcal Enterotoxins: Biological and Evolutionary Implications

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The type C staphylococcal enterotoxins (SEC) are a group of highly conserved proteins with significant immunological cross-reactivity. Although three antigenically distinct SEC subtypes (SEC1, SEC2, and SEC3) have been reported in the literature, we observed that the isoelectric points of SEC from several *Staphylococcus aureus* isolates are different from those of any of these three subtypes. This observation led us to propose that additional SEC molecular variants exist. For assessment of this possibility, the *sec* genes from representative human, animal, and food isolates were cloned and sequenced. The toxins encoded by the 18 isolates used in this study included five unique SEC proteins in addition to SEC1, SEC2, and SEC3. Six of the SEC proteins (including SEC1, SEC2, and SEC3) were produced by human and food isolates. Analysis of seven bovine and ovine isolates showed that isolates from each animal species produced a unique host-specific SEC. All of the SEC caused lymphocyte proliferation, although some of the toxins differed in their ability to stimulate cells from several animal species. An explanation for these results, which is supported by our phenotypic analysis of Sec$^+$ staphylococcal isolates, is that toxin heterogeneity is due to selection for modified SEC sequences that facilitate the survival of *S. aureus* isolates in their respective hosts.

Staphylococcus aureus and *Streptococcus pyogenes* produce a family of related pyrogenic toxins (PT) that includes staphylococcal enterotoxins (SE) (7), pyrogenic exotoxins A and B (48, 50), toxic shock syndrome toxin (TSST) 1 (TSST-1) (8), and streptococcal pyrogenic exotoxins A through C (2, 31, 57). Exotoxins in the PT family have received significant attention, since they share several biological properties that are linked to the pathogenesis of toxic shock syndrome and toxic shock syndrome-like illnesses (12). Although the SE are included in the PT family, they can be distinguished from other PT by their ability to cause emesis following oral ingestion and their causative role in staphylococcal food poisoning (22).

SE are classified on the basis of their antigenic properties; thus far, seven serological variants (SEA, SEB, SEC1, SEC2, SEC3, SED, and SEE) have been reported (7). Although SE and other PT are immunologically distinct, some share cross-reactive antigenic epitopes (13, 29, 54, 55). The type C SE (SEC) are the most closely related PT. SEC1, SEC2, and SEC3 display strong immunological cross-reactivity and share greater than 95% sequence similarity (28).

Recent evidence suggests that diversity within the PT family is more extensive than previously reported. Ho et al. (26) showed that TSST-1 and its variant produced by ovine *S. aureus* isolates can be distinguished by their different isoelectric points (pI). However, both forms of the toxin have the same apparent molecular weight and cross-react immunologically. This observation was similar to those found in early reports describing SEC (1, 18). SEC1, SEC2, and SEC3 exhibit strong immunological cross-reactivity but have unique pI, approximately 8.6, 7.8, and 8.2, respectively (44). The altered charges of SEC and TSST groups of toxins have been explained by a comparison of protein sequence data (17, 28, 34).

Like TSST-1 and its ovine TSST variant, SEC are commonly isolated from both human and animal isolates and have been implicated in a variety of diseases in domestic animals. This study was initiated to determine the degree of molecular heterogeneity among the SEC subtypes and to assess the biological and evolutionary implications of SEC molecular variability. We selected representative Sec$^+$ human, food, and animal isolates, cloned their *sec* structural genes, and compared the predicted amino acid sequences with those previously reported for SEC1, SEC2, and SEC3. In addition, eight SEC molecular variants were purified from their native staphylococcal isolates and compared.

MATERIALS AND METHODS

Bacterial strain characterization. The bacterial strains used in this study and their sources are listed in Table 1. The selection of strains for analysis was random, except for the inclusion of confirmed SEC1, SEC2, and SEC3 producers for comparison. The concentrations of SEC produced by the staphylococcal strains were determined by a quantitative immunological assay described previously (14). SEC-specific hyperimmune antisera were prepared with rabbits by standard protocols (49).

Biotype testing of strains included an assessment of coagulase production (Difco Laboratories, Detroit, Mich.) (35), the API STAPH Trac system (Analytab Products, Plainview, N.Y.), and five additional tests that differentiate *S. aureus* and *S. intermedius* (45). Antibiogram analysis was done by the disk diffusion method of Bauer et al. (3). The antimicrobial disks (Difco) included ampicillin, cephalothin, cloxacillin, erythromycin, gentamicin, neomycin, novobiocin, penicillin, streptomycin, tetracycline, lincomycin, and

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TABLE 1. SEC-producing staphylococcal strains used in this study

Strain	Phenotype[a]	Origin	[SEC] (μg/ml)	Source or reference[b]
MNDon	$Sec1^+$	Human	20.8	P. Schlievert (15)
FRI137	$Sec1^+$	Human	10.4	American Type Culture Collection (18)
FRI361	$Sec2^+$	Food	10.4	M. Bergdoll (1)
FRI913	$Sec3^{+c}$	Food	5.2	M. Bergdoll (44)
FRI909	$Sec3^{+c}$	Human	5.2	M. Bergdoll
MNCopeland	$Sec^+_{MNCopeland}$	Human	20.8	P. Schlievert
740N	Sec^+_{740N}	Human	10.4	R. Gruninger
4446	Sec^+_{4446}	Human	5.2	B. Kreiswirth
3152	Sec^+_{bovine}	Bovine	1.3	B. Kreiswirth
3169	Sec^+_{bovine}	Bovine	1.3	B. Kreiswirth (32)
3170	Sec^+_{bovine}	Bovine	0.3	B. Kreiswirth (32)
5597	Sec^+_{ovine}	Ovine	20.8	B. Kreiswirth (32)
5599	Sec^+_{ovine}	Ovine	2.6	B. Kreiswirth
5610	Sec^+_{ovine}	Ovine	1.3	B. Kreiswirth
5625	Sec^+_{ovine}	Ovine	2.6	B. Kreiswirth (32)

[a] Designations are based on standard nomenclature (10). Phenotypes other than $Sec1^+$, $Sec2^+$, and $Sec3^+$ are provided for this report and may change if newly described toxins are assigned numerical designations. Subscripts indicate strains or origins.

[b] Sources indicated are those providing the strains to the authors and are not necessarily the original sources of isolation: P. Schlievert, University of Minnesota, Minneapolis; American Type Culture Collection, Rockville, Md. (ATCC 19095); M. Bergdoll, Food Research Institute, University of Wisconsin, Madison; R. Gruninger, Henepin County Medical Center, Minneapolis, Minn.; B. Kreiswirth, Public Health Research Institute, New York, N.Y.

[c] On the basis of antibody reactivity assessed at the Food Research Institute, the toxins produced by stains FRI909 and FRI913 were designated SEC3. In view of their sequence differences demonstrated in this study, the future numerical designations of these two toxins are uncertain.

nitrofurantoin. Phage typing was performed and interpreted as previously described (11, 23). The international phage set (21) used included 3A, 6, 29, 42D, 42E, 52A, 53, 75, 78, 84, 102, 107, 116, 117, 118, and 119. Data sets for each strain consisting of 68 biotype, antibiogram, and phage typing characteristics were subjected to single-linkage cluster analysis by use of the Statistical Analysis System (SAS Institute, Inc., Cary, N.C.) with protocols recommended by the manufacturer (47).

Toxin purification. SEC variants were purified to homogeneity by a three-stage procedure. Cultures were grown to the stationary phase with aeration in dialyzable beef heart medium, and the supernatants were recovered by centrifugation. Ammonium sulfate was added to a final concentration of 42.5% to precipitate the majority of contaminating proteins. After 3 h of continuous stirring (4°C), the precipitates were removed by centrifugation, and the concentration of $(NH_4)_2SO_4$ was increased to 52.5%. The second precipitation, which removed the toxins from the supernatants, was allowed to proceed overnight (4°C) to achieve maximum recovery. The toxins were recovered by centrifugation, redissolved in water, and dialyzed exhaustively.

The crude toxin preparations were resolved by preparative flat-bed isoelectric focusing (IEF) as described previously (15, 58). Initially, the proteins were partially purified in a gel bed containing a pI range of 3.5 through 9.5. Additional purification was achieved with ampholyte gradients of pHs 7.0 through 9.0 or 6.0 through 8.0, depending on the toxin pI. Fractions containing the toxins were identified by immunodiffusion (40) with SEC-specific antisera.

Residual protein contaminants were removed by immunoaffinity chromatography. Immunoglobulins were precipitated from hyperimmune SEC rabbit antisera (4°C) with ammonium sulfate at 50% saturation and coupled to Affigel 10 (Bio-Rad Laboratories, Richmond, Calif.). The procedures used for antibody coupling and affinity binding to antigen were those recommended by the manufacturer. Enterotoxins were eluted from the immunoabsorbent with 0.2 M acetic acid containing 0.15 M NaCl, collected in an equal volume of phosphate-buffered saline, dialyzed exhaustively against water, and stored as lyophilized powders.

Analytical electrophoresis. The purity and pI of each toxin after processing as described above were assessed by gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12.5% acrylamide gel slabs by the method of Laemmli (33). Analytical IEF (58) was performed with 5.0% acrylamide gels containing ampholyte ranges of pHs 3.5 through 10.0 and 7.0 through 9.0. Protein standards for SDS-PAGE and IEF were purchased from Diversified Biotechnology Inc. (Newton Centre, Mass.) and Sigma Chemical Co. (St. Louis, Mo.), respectively.

Lymphocyte proliferation assays. The ability of SE to induce lymphocyte proliferation was determined with human, bovine, and ovine cell cultures by a standard 4-day mitogenicity assay (43). Heparinized peripheral blood (10 U/ml) was collected from animals and human volunteers by venipuncture. The mononuclear cell fraction was obtained by centrifugation through a Ficoll-Paque gradient (Pharmacia, Gaithersburg, Md.). Cell cultures (2.0×10^5 cells) were plated in 96-well tissue culture plates and stimulated with each toxin. After 3 days, the cultures were pulsed for 24 h with 1.0 μCi of [^{3}H]thymidine (NEN Research Products, Boston, Mass.). The amount of radiolabel incorporated into cellular DNA was quantitated by liquid scintillation counting. The mitogenic responses induced by the toxins were assessed by a pairwise comparison ($\alpha = 0.05$) by use of the analysis-of-variance Fisher's least-significant-difference procedure (47).

DNA hybridization. Staphylococcal DNA was isolated as described previously (14) and digested with *Cla*I. Following electrophoretic resolution (36), the restriction fragments were transferred to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, N.H.) by the method of Southern (53) and probed with pMIN158, a recombinant plasmid that contains an internal segment of the previously cloned *sec* gene from strain MNDon gene (14, 15). Plasmid DNA was isolated from *Escherichia coli* clones as described by Holmes and Quigley (27) and labelled with [^{35}S]dATP (NEN Research Products) by use of a commercial nick translation kit (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Conditions for the hybridization of radiolabelled

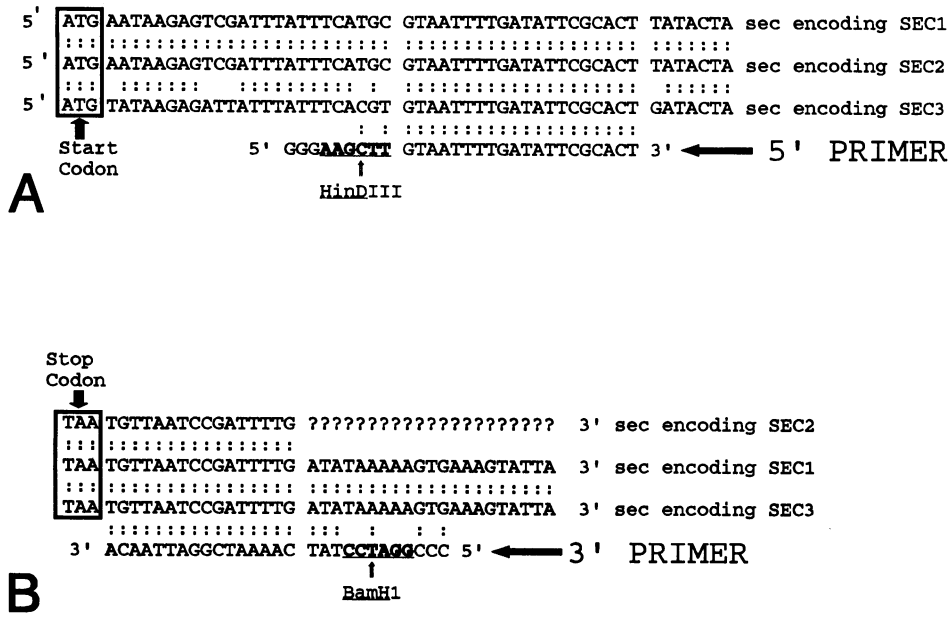

FIG. 1. Strategy used for the construction of PCR primers. Nucleotide sequences for *sec* genes were obtained from references 16, 17, and 28. Shown are regions of the primers homologous to the three *sec* genes as well as restriction site extensions. (A) 5′ primer complementary to a region of *sec* encoding part of the signal peptide. (B) 3′ primer complementary to a conserved region immediately downstream from *sec*.

pMIN158 (2.0×10^6 cpm) with staphylococcal DNA were identical to those described previously (14). The bound probe was visualized by autoradiography on X-ray film (Eastman Kodak Co., Rochester, N.Y.).

Amplification of *sec* genes by PCR techniques. Previously published sequences of three *sec* genes and their flanking regions were used to design oligonucleotide primers with a high probability of hybridizing to the *sec* genes under investigation in this study (Fig. 1). For facilitation of cloning, the 5′ (GGGAAGCTTGTAATTTTGATATTCGCACT) and 3′ (CCCGGATCCTATCAAAATCGGATTAACA) primers each contained a unique restriction site (*Hin*dIII and *Bam*HI, respectively) within a 9-base 5′ extension. Polymerase chain reaction (PCR) amplification was performed with a TempCycler (Coy, Ann Arbor, Mich.) and the following thermal profiles: cycle 1, 97°C for 5 min; cycles 2 through 46, 92°C for 30 s, 40°C for 1 min, and 72°C for 1 min; and cycle 47, 72°C for 5 min. Amplified products were purified by agarose gel electrophoresis, excised from the gels (52), extracted, and stored as ethanol precipitates.

Cloning techniques. For facilitation of cloning of *sec* variants, the genes were first amplified by PCR. The protocol described above generated a single product (approximately 800 bp), which was digested with *Hin*dIII and *Bam*HI. All DNA inserts were ligated to the phagemid pBLUESCRIPT KS$^+$ (Stratagene, La Jolla, Calif.), transformed into *E. coli* TG1, and selected on Luria-Bertani (LB) agar plates containing ampicillin and 5-bromo-4-chloro-β-galactopyranoside (36). Cloning of the structural genes for SEC1, SEC2, and SEC3 from strain FRI913 has been reported elsewhere (16, 17, 28).

Nucleotide sequencing. Recombinant phagemids that were purified in their single-stranded form by use of VCS-M13 helper phage (Stratagene) served as templates for nucleotide sequencing. Dideoxynucleotide sequencing (46) was performed with a commercial kit (Sequenase version 2.0; U.S. Biochemical Corp., Cleveland, Ohio). Nucleotide sequences of *sec* genes were confirmed by sequencing of PCR products generated from each strain in three separate amplifications.

Amino acid sequencing. The N-terminal amino acid sequences of purified SEC proteins were determined by the automated Edman degradation technique of Hewick et al. (25) with a model 475A pulsed-liquid protein sequencer (Applied Biosystems, Foster City, Calif.).

Sequence accession numbers. The nucleotide and amino acid sequence data obtained in this study were deposited with GenBank Submissions, Los Alamos, N.M., under accession numbers L13374 through L13379.

RESULTS

Strain characterization and initial toxin analysis. The 15 strains under investigation included 6 confirmed human strains from staphylococcal infections and 7 strains from cases of bovine or ovine mastitis (Table 1). Two additional strains (FRI361 and FRI913) were isolated from food associated with outbreaks of staphylococcal food poisoning. A phenotypic comparison of the strains was based on an assessment of 68 biotype and antibiogram characteristics in addition to phage typing patterns. Single-linkage cluster analysis results were consistent with the observations of Musser et al. (39). Sec$^+$ strains from humans and food sources were very heterogeneous and did not show a tendency to form a tight cluster. Furthermore, ovine and bovine strains segregated into two distinct groupings, with more diversity being observed among the bovine strains.

The Sec$^+$ phenotype was confirmed for each strain with SEC-specific rabbit antisera. There was a wide range in the quantities of toxins produced by the strains; generally, human strains produced higher amounts (≥5.2 μg/ml) of SEC than animal strains. Only one animal strain (*S. aureus* 5597) produced quantities of SEC similar to those produced by human or food strains. The other six animal strains produced low or very low amounts (≤2.6 μg/ml).

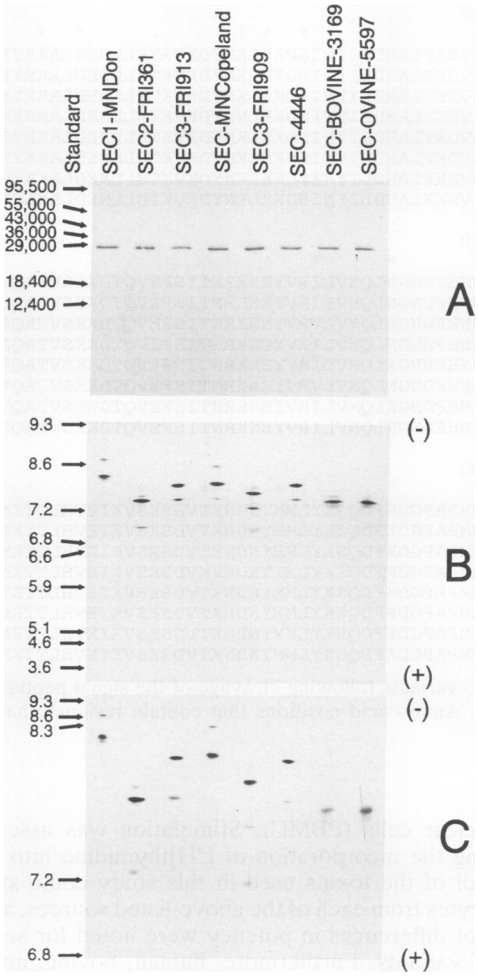

FIG. 2. Analytical gel electrophoresis of SEC variants. (A) SDS-PAGE; molecular masses are expressed in kilodaltons. (B) IEF (pH gradient, 3.5 to 10.0). (C) IEF (pH gradient, 7.0 to 9.0). The pIs of protein standards are indicated in panels B and C (left margin).

Each SEC variant was purified to homogeneity and analyzed by SDS-PAGE (Fig. 2A). The apparent molecular weight of approximately 27,000 for each toxin is consistent with the molecular weights predicted for SEC1, SEC2, and SEC3 on the basis of amino acid composition (28). Despite the presence of a single protein band in SDS-polyacrylamide gels, microheterogeneity typical of the PT family (37) caused several of the toxins to resolve into multiple species in analytical IEF (Fig. 2B and C). pI for SEC1, SEC2, and SEC3 from strain FRI913 were similar to those reported previously (1, 18, 28, 44). SEC1 was found to be a very basic protein, with two major pI forms (8.2 and 8.7), similar to our prior observations (15, 56). The major pI forms of SEC2 and SEC3 from strain FRI913 were 7.8 and 8.1, respectively. However, many of the strains produced toxins with unique IEF patterns. The predominant species of toxins produced by strains MNCopeland and 4446 were similar to SEC3 of strain FRI913. All three of these toxins were more basic than the variety of SEC3 produced by strain FRI909, which had a predominant pI of 8.0. SEC from bovine and ovine strains had identical pI (7.6), were the least basic, and displayed only a minor amount of microheterogeneity.

Southern hybridization. It had been reported previously

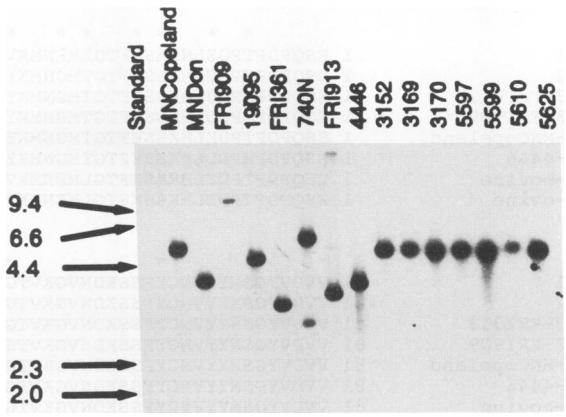

FIG. 3. Southern blot of *Cla*I-digested DNAs from Sec$^+$ strains probed with an internal fragment of the *sec* gene from strain MNDon. Fragment sizes are expressed in kilobases.

that restriction fragments containing *sec* genes are variable in size and that clear differences in hybridization profiles could be demonstrated for human and animal strains (14). To extend these observations to the strains used in this study, we probed *Cla*I-digested genomic DNA from each of the 15 strains. Figure 3 shows the restriction fragments hybridizing to the *sec*-specific probe. Consistent with previous findings, human and food strains displayed a high degree of polymorphisms in their hybridization profiles. Strains MNDon and 4446 contained similarly sized hybridizing *Cla*I restriction fragments (3.6 kb). In the other six human and food strains tested, the *sec* gene was located on restriction fragments of 3.2, 3.4, 4.5, 5.4, 5.8, and 23.6 kb. In contrast, genomic DNA from all seven sheep and cattle strains contained *sec* on a 5.0-kb restriction fragment.

Amino acid sequences. The primers shown in Fig. 1 directed the amplification of a uniformly sized (approximately 800-bp) PCR product from genomic DNA isolated from each of the Sec$^+$ strains listed in Table 1. The amplified products did not express detectable SEC proteins when cloned into *E. coli*, since they lacked the *sec* promoter and N-terminal residues of the signal peptide. The *sec* genes were sequenced with primers derived from the strain MNDon *sec* sequence reported previously (16). Figure 4 and Table 2 show an alignment of the predicted primary sequences of the mature proteins and summarize their relatedness, respectively.

Sequencing of SEC purified from their native strains confirmed the signal peptide cleavage site predicted in Fig. 4, since each SEC contained a glutamic acid N terminus. All eight varieties of SEC compared in this study contained 239 residues. The amino acid sequence of SEC1 from strain MNDon was identical to that of SEC1 from strain FRI137, the proposed prototype SEC1-producing strain (18). At the nucleotide level, the structural genes differed by one silent alteration. None of the toxin sequences was identical to that of SEC2 from strain FRI361. The most closely related toxin was that isolated from strain MNCopeland, which differed from SEC2 at only two locations, residues 39 and 227. The results obtained for toxins producing a Sec3$^+$ phenotype were complex. Two of three strains previously determined to be Sec3$^+$ (FRI913 and 740N) produced identical proteins. However, although the SEC3 variants of strains FRI909 and FRI913 behaved the same serologically, the two toxins differed at nine positions in their primary sequences. The previously uncharacterized SEC produced by strain 4446

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                                                          40
                          *  *   * *   * *   *    *       *↓             *  *  *            *   *
SEC1              1 ESQPDPTPDELHKASKFTGLMENMKVLYDDHYVSATKVKSVDKFLAHDLIYNISDKKLKNYDKVKTELLNEGLAKKYKDE 80
SEC2              1 ESQPDPTPDELHKSSEFTGTMGNMKYLYDDHYVSATKVMSVDKFLAHDLIYNISDKKLKNYDKVKTELLNEDLAKKYKDE 80
SEC3-FRI913       1 ESQPDPMPDDLHKSSEFTGTMGNMKYLYDDHYVSATKVKSVDKFLAHDLIYNISDKKLKNYDKVKTELLNEDLAKKYKDE 80
SEC3-FRI909       1 ESQPDPMPDDLHKSSEFTGTMGNMKYLYDDHYVSATKVKSVDKFLAHDLIYNINDKKLNNYDKVKTELLNEDLANKYKDE 80
SEC-MNCopeland    1 ESQPDPTPDELHKSSEFTGTMGNMKYLYDDHYVSATKVKSVDKFLAHDLIYNISDKKLKNYDKVKTELLNEDLAKKYKDE 80
SEC-4446          1 ESQPDPMPDDLHKSSEFTGTMGNMKYLYDDHYVSATKVKSVDKFLAHDLIYNISDKRLKNYDKVKTELLNEDLAKKYKDE 80
SEC-bovine        1 ESQPDPTPDELHKASKFTGLMENMKVLYDDRYVSATKVKSVDKFLAHDLIYNISDKKLKNYDKVKTELLNEDLAKKYKDE 80
SEC-ovine         1 ESQPDPTPDELHKASKFTGLMENMKVLYDDRYVSATKVKSVDKFLAHDLIYNISDKKLKNYDKVKTELLNEDLAKKYKDE 80

                                                          120
                                 *           *             ↓      *     *
SEC1             81 VVDVYGSNYYVNCYFSSKDNVGKVTGGKTCMYGGITKHEGNHFDNGNLQNVLIRVYENKRNTISFEVQTDKKSVTAQELD 160
SEC2             81 VVDVYGSNYYVNCYFSSKDNVGKVTGGKTCMYGGITKHEGNHFDNGNLQNVLIRVYENKRNTISFEVQTDKKSVTAQELD 160
SEC3-FRI913      81 VVDVYGSNYYVNCYFSSKDNVGKVTGGKTCMYGGITKHEGNHFDNGNLQNVLVRVYENKRNTISFEVQTDKKSVTAQELD 160
SEC3-FRI909      81 VVDVYGSNYYVNCYFSSKDNVGKVTSGKTCMYGGITKHEGNHFDNGNLQNVLIRVYENKRNTISFEVQTDKKSVTAQELD 160
SEC-MNCopeland   81 VVDVYGSNYYVNCYFSSKDNVGKVTGGKTCMYGGITKHEGNHFDNGNLQNVLIRVYENKRNTISFEVQTDKKSVTAQELD 160
SEC-4446         81 VVDVYGSNYYVNCYFSSKDNVGKVTGGKTCMYGGITKHEGNHFDNGNLQNVLVRVYENKRNTISFEVQTDKKSVTAQELD 160
SEC-bovine       81 VVDVYGSNYYVNCYFSSKDNVGKVTGGKTCMYGGITKHEGNHFDNGKLQNVLIRVYENKRNTISFEVQTDKKSVTAQELD 160
SEC-ovine        81 VVDVYGSNYYVNCCFSSKDNVGKVTGGKTCMYGGITKHEGNHFDNGNLQNVLIRVYENKRNTISFEVQTDKKSVTAQELD 160

                                                          200
                        *                         *        ↓               * *   *    *
SEC1            161 IKARNFLINKKNLYEFNSSPYETGYIKFIENNGNTFWYDMMPAPGDKFDQSKYLMMYNDNKTVDSKSVKIEVHLTTKNGX 240
SEC2            161 IKARNFLINKKNLYEFNSSPYETGYIKFIENNGNTFWYDMMPAPGDKFDQSKYLMMYNDNKTVDSKSVKIEVHLTTKNGX 240
SEC3-FRI913     161 IKARNFLINKKNLYEFNSSPYETGYIKFIENNGNTFWYDMMPAPGDKFDQSKYLMMYNDNKTVDSKSVKIEVHLTTKNGX 240
SEC3-FRI909     161 IKARNFLINKKNLYEFNSSPYETGYIKFIESNGNTFWYDMMPAPGDKFDQSKYLMIYKDNKMVDSKSVKIEVHLTTKNGX 240
SEC-MNCopeland  161 IKARNFLINKKNLYEFNSSPYETGYIKFIENNGNTFWYDMMPAPGDKFDQSKYLMMYNDNKTVDSKRVKIEVHLTTKNGX 240
SEC-4446        161 IKARNFLINKKNLYEFNSSPYETGYIKFIENNGNTFWYDMMPAPGDKFDQSKYLMMYNDNKTVDSKSVKIEVHLTTKNGX 240
SEC-bovine      161 IKARNFLINKKNLYEFNSSPYETGYIKFIENNGNTFWYDMMPAPGDKFDQSKYLMMYNDNKTVDSKSVKIEVHLTTKNGX 240
SEC-ovine       161 IKARSFLINKKNLYEFNSSPYETGYIKFIENNGNTFWYDMMPAPGDKFDQSKYLMMYNDNKTVDSKSVKIEVHLTTKNGX 240
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FIG. 4. Alignment of the predicted primary sequences of the eight known SEC variants following cleavage of the signal peptide. The N terminus of each of the mature proteins was verified by amino acid sequencing. Amino acid positions that contain residues that are not conserved among all SEC are indicated by asterisks.

differed from SEC3 produced by strains FRI913 and 740N by a single conserved residue substitution at position 57.

The *sec* genes of three bovine strains and four ovine strains were sequenced. The predicted amino acid sequences were unique for each animal and different from those of toxins from all human and food strains, including SEC1, SEC2, and SEC3. SEC from ovine and bovine strains were more closely related to each other and SEC1 than to either SEC2 or SEC3. The bovine and ovine toxins shared 236 of 239 residues. Three substitutions, at residues 94, 127, and 165, produced changes that were unique to either SEC from ovine strains or SEC from bovine strains and were not observed in any other SEC variant. Of particular interest were SEC from ovine strains, which contained an additional cysteine residue at position 94.

Mitogenicity. Each of the SEC variants was tested for the ability to stimulate lymphocytes in cultures of human (Fig. 5A), bovine (Fig. 5B), and ovine (Fig. 5C) peripheral blood mononuclear cells (PBMC). Stimulation was assessed by measuring the incorporation of [^{3}H]thymidine into cellular DNA. All of the toxins used in this study could stimulate lymphocytes from each of the above-listed sources, although significant differences in potency were noted for several of the SEC variants. Furthermore, human, bovine, and ovine cells displayed species-specific responses.

Human cells, which were stimulated efficiently by all toxins at doses as low as 10^{-4} μg, were the most sensitive. Other than SEC from strain FRI909, which at low doses (10^{-6} through 10^{-4} μg) was more mitogenic, there were no consistent differences in the ability of SEC variants to induce the proliferation of lymphocytes from human volunteers. SEC from ovine strains consistently stimulated both ovine and bovine lymphocytes most efficiently. The proliferation of bovine or ovine PBMC under the same conditions required approximately 10- to 1,000-fold more toxin, and cells from these animals displayed clear differences in their sen-

TABLE 2. Sequence homology among SEC

SEC	% Homology to (divergent residues)[a]:						
	SEC1	SEC2	SEC3 from strain FRI913	SEC3 from strain FRI909	SEC from strain MNCopeland	SEC from strain 4446	SEC from bovine strains
SEC2	97.07 (7)						
SEC3 from strain FRI913	96.23 (9)	98.33 (4)					
SEC3 from strain FRI909	93.31 (16)	95.40 (11)	96.23 (9)				
SEC from strain MNCopeland	97.07 (7)	99.16 (2)	98.33 (4)	95.40 (11)			
SEC from strain 4446	95.82 (10)	97.91 (5)	99.58 (1)	96.23 (9)	97.91 (5)		
SEC from bovine strains	98.74 (3)	96.65 (8)	95.82 (10)	92.89 (17)	96.65 (8)	95.40 (11)	
SEC from ovine strains	98.33 (4)	96.23 (9)	95.40 (11)	92.47 (18)	96.23 (9)	94.98 (12)	98.74 (3)

[a] The percent homology for each pair of toxin variants compared is given for the predicted mature protein. Numbers in parentheses indicate the numbers of divergent residues between each pair of toxin variants compared.

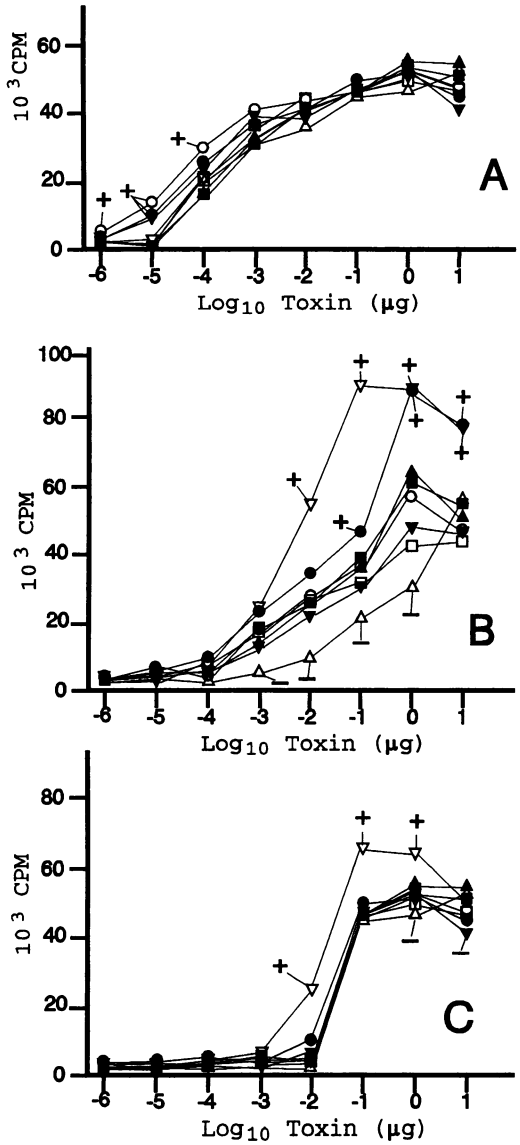

FIG. 5. Stimulation of human (A), bovine (B), and ovine (C) PBMC by SEC variants. Datum points represent the means for three to nine cultures. Data that represent significantly ($\alpha = 0.05$) elevated (+) or reduced (−) responses are indicated. Symbols: ●, SEC1; ▼, SEC2; ■, SEC3 from strain FRI913; ○, SEC3 from strain FRI909; ▲, SEC from strain MNCopeland; □, SEC from strain 4446; ▽, SEC from ovine strains; △, SEC from bovine strains.

sitivity to some of the toxins. Ovine cells were less responsive than human or bovine cells; no effect was observed in culture wells containing less than 0.01 μg of toxin. SEC from ovine strains were the most mitogenic toxins for ovine lymphocytes and induced significantly higher responses at doses of 0.01 through 1.0 μg. In addition, ovine SEC variants were very efficient in the stimulation of bovine lymphocytes and were the most mitogenic toxins in that cell system. SEC1 was also very mitogenic for bovine cells and induced significantly elevated levels of stimulation at several doses. SEC from bovine strains were 10- to 1,000-fold less active than SE from ovine strains and were the least mitogenic toxins in bovine cell cultures.

DISCUSSION

On the basis of immunological reactivity, three antigenic variants of SEC (SEC1, SEC2, and SEC3) had been identified previously (5). However, we conclude from the present study that the SEC group of toxins is significantly more complex. This conclusion is based on a sequence comparison of *sec* genes from 15 randomly chosen strains as well as biological and physicochemical properties of purified SEC proteins.

Shortly after the description of SEC by Bergdoll et al. (6), two molecular variants of the toxin were found to be produced by strains FRI137 and FRI361 (1, 18). The immunologically similar toxins produced by these strains are the prototypes of SEC1 and SEC2, respectively. SEC1 is a commonly produced toxin, and $Sec1^+$ *S. aureus* strains are often isolated from human infections (14). In our random selection of eight human and food strains, one other strain, MNDon, produced an SEC that was identical to SEC1 of FRI137. However, none of the toxins sequenced in this study was identical to SEC2 of FRI361. This result was unexpected since, chronologically, SEC2 was the second subtype to be identified. On the basis of the similarity of sequences, the SEC most closely related to SEC2 was produced by strain MNCopeland. However, these two toxins are quite different physicochemically, as a result of charge substitutions at positions 39 and 227. Substitutions of lysine and arginine at these positions in strain MNCopeland cause a significantly more basic pI. However, both toxins are identical at positions 20, 22, and 26. The residues at these three positions are critical for the formation of an antigenic epitope that distinguishes SEC1 and SEC2, and the SEC from strain MNCopeland reacts with a monoclonal antibody that recognizes the SEC2-specific epitope (56).

Reiser et al. (44) proposed a third subtype, SEC3, to describe a toxin that was antigenically distinct from SEC1 and SEC2. This toxin, produced by strain FRI913, was later sequenced and shown to have residues that were previously considered SEC1 specific and SEC2 specific, as well as three SEC3-specific residues (28). The present study shows that this SEC variant and very closely related toxins are apparently quite common. Another strain (strain 740N) produced the same toxin with complete sequence homology at the amino acid and structural gene levels, except for a single silent substitution. A similar SEC variant produced by strain 4446 differed from prototype SEC3 by only one conservative substitution (lysine → arginine) at position 57.

The classification of toxins into the SEC3 subtype on the basis of antigenicity does not ensure that their amino acid sequences are identical. Some SEC variants are indistinguishable from SEC3 from strain FRI913 in immunological assays but have different amino acid sequences and biochemical properties. This fact was first demonstrated by Couch and Betley (20), who cloned and sequenced the *sec* gene from strain FRI1230. Despite the toxin from this strain previously being identified as SEC3 because of its immunological relatedness to SEC3 from strain FRI913 (44), its structural gene was identical to the gene for SEC2. The immunological reactivity that distinguished SEC from strain FRI1230 from SEC2 was suspected to arise from strain-specific processing of the signal peptide. In the present study, SEC3 produced by strain FRI909 was characterized and found to be unique. Although SEC3 from strain FRI909 was more related to SEC3 from strain FRI913 than to SEC1 or SEC2, the two forms of SEC3 have nine divergent residues and different pI.

If available, a subtype-specific monoclonal antibody could facilitate the classification of SEC3-related toxins. However, despite attempts by us and other investigators (55), we are unaware of the production of antibodies that recognize only SEC3. All SEC3-related toxins characterized thus far share residues 20, 22, and 26 with SEC2 and react with monoclonal antibodies that recognize the SEC2-specific epitope (11a, 55). Therefore, the antigenic properties of SEC3 that distinguish it from both SEC1 and SEC2 (44, 55) are likely due to conformational differences influenced by other regions of the molecule.

SEC from ovine and bovine strains represent unique forms of SEC that have properties significantly different from those of previously described subtypes, especially SEC2 and SEC3. On the basis of sequence analysis, they are most related to each other and to SEC1. Although they are identical to SEC1 in the region that reportedly contains the SEC1-specific epitope (residues 20 through 26), the pI of the ovine and bovine strain toxins are substantially lower because of nonconserved substitutions in other regions of the proteins. The finding that SEC from ovine and bovine strains differ from each other by 3 residues is analogous to that reported for TSST from bovine and ovine strains (34). However, unlike TSST from bovine strains, which is identical to TSST-1 produced by human strains, SEC from bovine strains are different from all human strain toxins. Furthermore, SEC from bovine and ovine strains do not display the extensive degree of sequence heterogeneity observed with SEC variants produced by human strains. All three SEC from bovine strains had the same sequences and pI, as did the four SEC from ovine strains.

The host-specific production of SEC variants by bovine and ovine strains and the diversity of SEC variants from human strains are significant considering that PT are virulence factors for *S. aureus*. This family of toxins is suspected to cause immunosuppression and shock through class II major histocompatibility complex binding and stimulation of T cells in a Vβ-dependent manner (19, 38). Considering the wide host range of *S. aureus*, it is unlikely that a single protein could efficiently interact with the toxin receptors in all potential hosts. Lee et al. (34) found that TSST-1 and TSST from ovine strains interact differentially with T cells in a host-specific manner. The ovine variety of TSST is less efficient at stimulating human or bovine lymphocytes than ovine cells. These investigators proposed that one mechanism of adaptation by staphylococci involves the acquisition of *tst* by gene transfer from other strains. Modification of the toxin through mutation and selective pressures to retain toxin variants that modulate the immune response could explain the emergence of toxins with maximum efficiency in the respective hosts of the strains.

SEC variants also demonstrate host specificity in their stimulation of T cells. The greater ability of SEC from ovine strains than of SEC from bovine strains and other SEC variants to stimulate ovine cells suggests that the toxins have adapted specifically to their hosts. However, the lack of the same apparent host specificity of bovine lymphocytes for SEC from bovine strains is noteworthy. Instead, a relatively weak proliferative response was observed. One possible explanation for these results is that the different response patterns observed are a consequence of a more recent acquisition of *sec* by bovine strains (from ovine strains). This suggestion is consistent with the demonstration of *sec* structural genes differing by only 3 residues on a *Cla*I restriction fragment that is the same size in both bovine and ovine strains. Unlike the *sec* genes, the *tst* genes in bovine and ovine strains display restriction length polymorphisms and are less well conserved (32, 34). The possibility that SEC from ovine and bovine strains recently diverged is interesting, since Sec^+ ovine and bovine strains probably represent different clones. Although bovine and ovine strains belong to biotype C (24), they are clearly unique on the basis of genetic relatedness, as measured by multilocus enzyme electrophoresis (39), and are segregated into different clusters in our analysis.

An alternate explanation for the different mitogenicity results for SEC from bovine and ovine strains is that each SEC variant is already highly adapted to its host. In this case, the mitogenicity of mixed in vitro cultures does not adequately reflect the levels of immunosuppression and virulence in vivo. This possibility must be considered in view of the cadre of T-cell populations in rosette-enriched cultures. Effective stimulation of at least one cell population could have a negative stimulatory role leading to depressed levels of cellular proliferation. A likely candidate for this activity is the $BoCD8^+$ subpopulation observed in mammary gland secretions during staphylococcal mastitis (41). This population has been shown to downregulate the proliferation of $BoCD4^+$ cells in cultures (42).

Substitution of amino acid residues in key positions of SEC proteins has important implications for toxicity as well as for biochemical properties. For instance, although they differ by only 7 residues, SEC1 and SEC2 have different mitogenic potencies for rabbit lymphocytes (56). Presumably, this characteristic is the result of different binding efficiencies resulting from unique protein structures which interact with either class II major histocompatibility complex molecules or T-cell receptors. One may propose that the different stimulatory abilities of other SEC variants, such as the ovine and bovine variants, are also the result of one or more substitutions. Of particular interest is the substitution to cysteine at position 94 in SEC from ovine strains. The effects of this substitution, producing two tandem cysteine residues and three overall, are currently unknown but could have important structural implications. To date, these SEC are the only SE demonstrated to have a deviation from the typical two cysteine residues per toxin molecule.

We could not detect any obvious pattern for sequence divergence among the various forms of SEC proteins. Our observations are consistent with the proposal by Couch and Betley (20) that diversity among PT arose from ancestral genes whose descendants diverged through recombination between toxins with extensive similarity but also with variability arising through random mutations. Additional selective pressures promoting PT variability may have resulted from the widespread dissemination of PT variants, and additional selective pressures may have been imposed by the transfer of PT structural genes among multiple genera. This transfer could have been facilitated by the acquisition of toxin genes by a variety of mobile genetic elements (4, 9, 30, 51).

SEC- and TSST-related toxins are both produced by a heterogeneous group of *S. aureus* strains. Musser et al. (39) reported that the production of TSST-1 is characteristic of strains representing the entire breadth of genotypic diversity among *S. aureus* and suggested that the *tst* gene did not recently evolve in this species. Although the scope of the present study is limited to the 15 strains whose *sec* sequences have been determined, the considerable sequence divergence, restriction length polymorphisms, and widespread transmission of *sec* genes among staphylococcal strains representing a broad host range with heterogeneous

phenotypes suggest that SEC production is also a primitive trait of *S. aureus*. A more encompassing study of Sec^+ strains would be necessary to confirm this suggestion.

The results of this study emphasize the need for a more encompassing SEC classification system and methods for the analysis of subtypes produced by diverse staphylococcal strains. In addition, it is unclear whether other SE types display a similar degree of variability in sequence, physicochemical properties, and biological activities. Although sequence and toxicity analyses, combined with screening by pI, are important for answering these basic questions, routine analysis may be done more efficiently by immunological techniques. Present work in our laboratory includes the production of monoclonal antibodies specific for each antigenic variant. Once these reagents are available, the correlation between antigenicity and other relevant properties may be able to be determined.

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REFERENCES

1. **Avena, R. M., and M. S. Bergdoll.** 1967. Purification and some physicochemical properties of enterotoxin C, *Staphylococcus aureus* strain 361. Biochemistry **6**:1474–1480.
2. **Barsumian, E. L., C. M. Cunningham, P. M. Schlievert, and D. W. Watson.** 1978. Heterogeneity of group A streptococcal pyrogenic exotoxin type C. Infect. Immun. **20**:512–518.
3. **Bauer, A. W., W. M. M. Kirby, J. C. Sherris, and M. Turck.** 1966. Antibiotic susceptibility testing by a standardized disk method. Am. J. Clin. Pathol. **45**:493–496.
4. **Bayles, K. W., and J. J. Iandolo.** 1989. Genetic and molecular analyses of the gene encoding staphylococcal enterotoxin D. J. Bacteriol. **171**:4799–4806.
5. **Bergdoll, M. S.** 1983. Enterotoxins, p. 559–598. *In* C. S. F. Easmon and C. Adlam (ed.), Staphylococci and staphylococcal infections. Academic Press, Inc. (London), Ltd., London.
6. **Bergdoll, M. S., C. R. Borja, and R. M. Avena.** 1965. Identification of a new enterotoxin as enterotoxin C. J. Bacteriol. **90**:1481–1485.
7. **Bergdoll, M. S., R. N. Robbins, K. Weiss, C. R. Borja, I. Y. Huang, and F. S. Chu.** 1973. The staphylococcal enterotoxins: similarities. Contrib. Microbiol. Immunol. **1**:390–396.
8. **Bergdoll, M. S., and P. M. Schlievert.** 1984. Toxic-shock syndrome toxin. Lancet **ii**:691.
9. **Betley, M. J., and J. J. Mekalanos.** 1985. Staphylococcal enterotoxin A is encoded by bacteriophage. Science **229**:185–187.
10. **Betley, M. J., P. M. Schlievert, M. S. Bergdoll, G. A. Bohach, J. J. Iandolo, S. A. Khan, P. A. Pattee, and R. R. Reiser.** 1990. Letter. ASM News **56**:182.
11. **Blair, J. E., and R. E. O. Williams.** 1961. Phage typing of staphylococci. Bull. W.H.O. **24**:771–784.

11a. **Bohach, G. A.** Unpublished results.

12. **Bohach, G. A., D. J. Fast, R. D. Nelson, and P. M. Schlievert.** 1990. Staphylococcal and streptococcal pyrogenic toxins involved in toxic shock syndrome and related illnesses. Crit. Rev. Microbiol. **17**:251–272.
13. **Bohach, G. A., C. J. Hovde, J. P. Handley, and P. M. Schlievert.** 1988. Cross-neutralization of staphylococcal and streptococcal pyrogenic toxins by monoclonal and polyclonal antibodies. Infect. Immun. **56**:400–404.
14. **Bohach, G. A., B. N. Kreiswirth, R. P. Novick, and P. M. Schlievert.** 1989. Analysis of toxic shock syndrome isolates producing staphylococcal enterotoxins B and C1 with use of Southern hybridization and immunological assays. Rev. Infect. Dis. **11**(Suppl. 1):S75–S82.
15. **Bohach, G. A., and P. M. Schlievert.** 1987. Expression of staphylococcal enterotoxin C1 in *Escherichia coli*. Infect. Immun. **55**:428–432.
16. **Bohach, G. A., and P. M. Schlievert.** 1987. Nucleotide sequence of the staphylococcal enterotoxin C1 gene and relatedness to other pyrogenic toxins. Mol. Gen. Genet. **209**:15–20.
17. **Bohach, G. A., and P. M. Schlievert.** 1989. Conservation of the biologically active portions of staphylococcal enterotoxins C1 and C2. Infect. Immun. **57**:2249–2252.
18. **Borja, C. R., and M. S. Bergdoll.** 1967. Purification and characterization of enterotoxin C produced by *Staphylococcus aureus* strain 137. Biochemistry **6**:1467–1473.
19. **Choi, Y., B. Kotzin, L. Herron, J. Callahan, P. Marrack, and J. Kappler.** 1989. Interaction of *Staphylococcus aureus* toxin "superantigens" with human T cells. Proc. Natl. Acad. Sci. USA **86**:8941–8945.
20. **Couch, J. L., and M. J. Betley.** 1989. Nucleotide sequence of the type C3 staphylococcal enterotoxin gene suggests that intergenic recombination causes antigenic variation. J. Bacteriol. **171**:4507–4510.
21. **Davidson, I.** 1972. A collaborative investigation of phages for typing bovine staphylococci. Bull. W.H.O. **46**:81–98.
22. **Dolman, C. E., and R. J. Wilson.** 1938. Experiments with staphylococcal enterotoxin. J. Immunol. **35**:13–30.
23. **Fox, L. K., C. T. Hutton, and D. D. Hancock.** 1990. Typing *Staphylococcus aureus* to determine sources of intramammary infection (IMI). J. Dairy Sci. **73**(Suppl. 1):257.
24. **Hajek, V., and J. Schindler.** 1981. Cluster analysis of coagulase-positive staphylococci, p. 27–35. *In* J. Jeljaszewicz (ed.), Staphylococci and staphylococcal infections. Gustav Fischer Verlag, Stuttgart, Germany.
25. **Hewick, R. M., M. W. Hunkapillar, L. E. Hood, and W. J. Dreyer.** 1981. A gas-liquid solid phase peptide and protein sequenator. J. Biol. Chem. **256**:7990–7997.
26. **Ho, G., W. H. Campbell, M. S. Bergdoll, and E. Carlson.** 1989. Production of a toxic shock syndrome variant by *Staphylococcus aureus* strains associated with sheep, goats, and cows. J. Clin. Microbiol. **27**:210–212.
27. **Holmes, D. S., and M. Quigley.** 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114**:193–197.
28. **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.
29. **Hynes, W. L., C. R. Weeks, J. J. Iandolo, and J. J. Ferretti.** 1987. Immunologic cross-reactivity of type A streptococcal exotoxin (erythrogenic toxin) and staphylococcal enterotoxins B and C1. Infect. Immun. **55**:837–838.
30. **Johnson, L. P., M. A. Tomai, and P. M. Schlievert.** 1986. Bacteriophage involvement in group A streptococcal pyrogenic exotoxin A production. J. Bacteriol. **166**:623–627.
31. **Kim, Y. B., and D. W. Watson.** 1970. A purified group A streptococcal pyrogenic exotoxin. Physicochemical and biological properties including the enhancement of susceptibility to endotoxin shock. J. Exp. Med. **131**:611–628.
32. **Kreiswirth, B. N., S. J. Projan, P. M. Schlievert, and R. P. Novick.** 1989. Toxic shock syndrome toxin 1 is encoded by a variable genetic element. Rev. Infect. Dis. **11**(Suppl. 1):S83–S89.
33. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
34. **Lee, P. K., B. N. Kreiswirth, J. R. Deringer, S. J. Projan, W. Eisner, B. L. Smith, E. Carlson, R. P. Novick, and P. M. Schlievert.** 1992. Nucleotide sequences and biologic properties of toxic shock syndrome toxin 1 from ovine and bovine associated *Staphylococcus aureus*. J. Infect. Dis. **165**:1056–1063.

35. **Mac Faddin, J. F.** 1976. Coagulase test, p. 41–52. *In* J. F. Mac Faddin (ed.), Biochemical tests for identification of medical bacteria. The Williams & Wilkins Co., Baltimore.
36. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 167. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Metzger, J. F., A. D. Johnson, and L. Spero.** 1975. Intrinsic and chemically produced microheterogeneity of *Staphylococcus aureus* enterotoxin type C. Infect. Immun. **12:**93–97.
38. **Mollick, J. A., R. G. Cook, and R. R. Rich.** 1989. Class II MHC molecules are specific receptors for staphylococcus enterotoxin A. Science **244:**817–820.
39. **Musser, J. M., P. M. Schlievert, A. W. Chow, P. Ewan, B. N. Kreiswirth, V. T. Rosdahl, A. S. Naido, W. Witte, and R. K. Selander.** 1990. A single clone of *Staphylococcus aureus* causes the majority of cases of toxic shock syndrome. Proc. Natl. Acad. Sci. USA **87:**225–229.
40. **Ouchterlony, O.** 1962. Diffusion-in-gel methods for immunological analysis. Prog. Allergy **6:**30–54.
41. **Park, Y. H., L. K. Fox, M. J. Hamilton, and W. C. Davis.** 1982. Bovine mononuclear leukocyte subpopulations in peripheral blood and mammary gland secretions during lactation. J. Dairy Sci. **75:**998–1006.
42. **Park, Y. H., L. K. Fox, M. J. Hamilton, and W. C. Davis.** 1993. Suppression of proliferative response of BoCD4$^+$ T lymphocytes by activated BoCD8$^+$ T lymphocytes in the mammary gland of cows with *Staphylococcus aureus* mastitis. Vet. Immunol. Immunopathol. **36:**137–151.
43. **Poindexter, N. J., and P. M. Schlievert.** 1985. Toxic-shock syndrome toxin-1 induced proliferation of lymphocytes: comparison of the mitogenic response of human, murine, and rabbit lymphocytes. J. Infect. Dis. **151:**65–72.
44. **Reiser, R. F., R. N. Robbins, A. L. Noleto, G. P. Khoe, and M. S. Bergdoll.** 1984. Identification, purification, and some physicochemical properties of staphylococcal enterotoxin C3. Infect. Immun. **45:**625–630.
45. **Roberson, J. R., L. K. Fox, D. D. Hancock, and T. E. Besser.** 1992. Evaluation of methods for differentiation of coagulase-positive staphylococci. J. Clin. Microbiol. **30:**3217–3219.
46. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
47. **SAS Institute, Inc.** 1985. SAS/STAT guide for personal computers, ed. 6. SAS Institute, Inc., Cary, N.C.
48. **Schlievert, P. M.** 1980. Purification and characterization of staphylococcal pyrogenic exotoxin type B. Biochemistry **9:**6204–6208.
49. **Schlievert, P. M., and D. A. Blomster.** 1983. Production of staphylococcal pyrogenic exotoxin type C: influence of physical and chemical factors. J. Infect. Dis. **147:**236–242.
50. **Schlievert, P. M., K. J. Schoettle, and D. W. Watson.** 1979. Purification and physicochemical and biological characterization of staphylococcal pyrogenic exotoxin. Infect. Immun. **23:**609–617.
51. **Shafer, W. M., and J. J. Iandolo.** 1979. Genetics of staphylococcal enterotoxin B in methicillin-resistant isolates of *Staphylococcus aureus*. Infect. Immun. **25:**902–911.
52. **Smith, H. O.** 1980. Recovery of DNA from gels. Methods Enzymol. **65:**371–380.
53. **Southern, E.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:** 503–517.
54. **Spero, L., and B. A. Morlock.** 1979. Cross-reactions between tryptic polypeptides of staphylococcal enterotoxins B and C1. J. Immunol. **122:**1285–1289.
55. **Thompson, N. E., M. J. Ketterhagen, and M. S. Bergdoll.** 1984. Monoclonal antibodies to staphylococcal enterotoxins B and C: cross-reactivity and localization of epitopes on tryptic fragments. Infect. Immun. **45:**281–285.
56. **Turner, T. N., C. L. Smith, and G. A. Bohach.** 1992. Residues 20, 22, and 26 determine subtype specificities of staphylococcal enterotoxins C1 and C2. Infect. Immun. **60:**694–697.
57. **Watson, D. W.** 1960. Host-parasite factors in group A streptococcal infections. Pyrogenic and other effects of immunologic distinct exotoxins related to scarlet fever toxins. J. Exp. Med. **111:**255–284.
58. **Winter, A., H. Perlmutter, and H. Davis.** 1975. Preparative flat-bed electrofocusing in a granulated gel with the LKB 2117 Multiphor. LKB Instruments, Stockholm.