

## Biotyping of Enterotoxigenic *Staphylococcus aureus* by Enterotoxin Gene Cluster (*egc*) Polymorphism and *spa* Typing Analyses

Giuseppe Blaiotta,<sup>1\*</sup> Vincenzina Fusco,<sup>1</sup> Christof von Eiff,<sup>2</sup> Francesco Villani,<sup>1</sup> and Karsten Becker<sup>2</sup>

*University of Naples Federico II, Department of Food Science, Division of Microbiology, Portici (Naples), Italy,<sup>1</sup> and University of Münster, Institute of Medical Microbiology, Münster, Germany<sup>2</sup>*

Received 3 April 2006/Accepted 23 June 2006

**Thirty-five *Staphylococcus aureus* strains, including 10 reference strains and 25 strains recovered from clinical specimens and food samples, were analyzed by PCR REA (restriction endonucleases analysis) of the *egc* operon and *spa* typing. Nineteen *spa* types and seven different *egc* operons, including four putative new *egc* variants, were revealed. In 13 strains, allelic variants of *sei* and/or *seg* were found. By an analysis of their nucleotide sequence identities, a new homogeneous cluster of a *sei* variant, called the *sei* variant, was detected in six strains. In addition, the prototype *sei* was shown to be more polymorphic than assumed so far. Seven strains possessed the recently described *seg* variant, also exhibiting several nucleotide exchanges. *spa* typing was more effective than REA *egc* grouping as a typing technique. Since, in some cases, the REA typing method was able to discriminate strains showing the same *spa* type, it must be considered for PCR approaches involved in diagnostic procedures and may be useful for epidemiological studies. Hence, the polyphasic approach used in this study can be reliably and advantageously applied for typing *egc*-positive *S. aureus* strains.**

*Staphylococcus aureus* is an extraordinarily versatile pathogen causing a wide spectrum of infections, ranging from mild to severe and life-threatening, in humans as well as economically important infections in animals. In addition to superficial lesions and systemic infections, *S. aureus* is responsible for toxin-mediated diseases, such as toxic shock syndrome (TSS) and staphylococcal food poisoning. The virulence factors causing these toxicoses are members of the family of bacterial pyrogenic toxin superantigens (PTSAgs) comprising the TSS-causing toxins and the staphylococcal enterotoxins (SEs) producing food-borne illness (11, 24). Superantigens bypassing normal antigen presentation stimulate large populations of T cells by binding to a specific variable region of the T-cell antigen receptor beta chain (22). In addition to their nature as superantigens, SEs operate as potent gastrointestinal toxins, causing staphylococcal food poisoning, which has a major public health impact (18, 28). Approximately 1.5 billion dollars are spent annually in the United States because of staphylococcal intoxications (38).

Primarily, five major serological types, SEA through SEE, have been characterized (6). In the past years, many new types of SEs and their coding genes (*seg* through *seu*) have been reported (12, 16, 17, 19, 27, 30–33, 37, 37, 39, 40). However, some of the novel SE homologues were shown to be nonemetic, thus actually lacking the defining property of SEs and consequently designated “staphylococcal enterotoxin-like” superantigens (20). For SEC, minor variants have been reported (23). The staphylococcal PTSAgs constitute a large family of structurally related proteins whose genes are associated with mobile genetic elements. SEB, SEC, SEG, SEI, SEM, SEN, SEO, SEK, SEL,

SEQ, and TSS toxin 1 are encoded by pathogenicity islands (2, 16, 17, 21). SEA, SEE, and SEP are encoded by prophages (7, 10, 17), whereas SED, SEJ, and SER are encoded by a plasmid known as pIB485 (3, 29, 40). The association with mobile genetic elements implies a horizontal transfer of the PTSAg genes between staphylococcal strains and an important role in the evolution of *S. aureus* as a pathogen.

Sequencing of the *seg-sei* intergenic DNA and flanking regions revealed three enterotoxin-like open reading frames related to *seg* and *sei*, designated *sen*, *seo*, and *sem*, and two pseudogenes,  $\phi$ ent1 and  $\phi$ ent2. Moreover, it was shown that these genes belong to an operon designated the enterotoxin gene cluster (*egc*), comprising *seo*, *sem*, *sei*,  $\phi$ ent1- $\phi$ ent2, *sen*, and *seg* (16). In addition, minor variants for *seg* and, limited to one strain, for *sei* were reported recently (1, 8). Due to sequence divergences in the  $\phi$ ent1- $\phi$ ent2 pseudogenes, the *seu* gene (including a variant) was described as a further part of the *egc* cluster (19). Consequently, at least three different *egc* subtypes were suggested: (i) *egc1* (harboring *seo*, *sem*, *sei*,  $\phi$ ent1,  $\phi$ ent2, *sen*, and *seg*), as represented by strain A900322 (GenBank accession number AF285760), (ii) *egc2* (containing *seu* instead of  $\phi$ ent1 and  $\phi$ ent2), as represented by strain FRI 137 (GenBank accession number AY205306), and (iii) *egc3* (containing *sei*, *seu*, *sen*, and *seg* variants), as represented by strain 382F (GenBank accession number AY158703).

Recent studies, comprising isolates recovered from different human and veterinary specimens, showed that *egc* and its carried SE genes are more common in *S. aureus* strains than assumed so far (4, 5, 15, 26, 36).

Increasingly, *S. aureus* typing has become an important tool in the study of strain origin, clonal relatedness, and the epidemiology of outbreaks. Although several different phenotypic and, more recently, molecular techniques are available for differentiating *S. aureus* strains, no method is clearly superior under all conditions.

Previous studies (15–16) have shown that *egc* SE genes are

\* Corresponding author. Mailing address: Dipartimento di Scienza degli Alimenti, Università degli Studi di Napoli Federico II, Via Università, 100 80055 Portici (Naples), Italy. Phone: 39-081-2539410-451. Fax: 39-081-2539407. E-mail: blaiotta@unina.it.

TABLE 1. Source, origin and results of *egc* characterization and *spa* typing of strains analyzed in this study

Strain	Source <sup>b</sup>	Origin <sup>c</sup>	PCR result			REA <i>egc</i> group	<i>spa</i> type
			<i>seφ</i>	<i>seu</i>	<i>sem-seg</i> <sup>d</sup>		
DSM 20231 <sup>Ta</sup>	DSMZ	Human pleural fluid	—	—	—		t011
D4508 <sup>a</sup>	CNTS		—	—	—		t948
ATCC 14458 <sup>a</sup>	ATCC	Feces of child	—	—	—		t008
ATCC 27664 <sup>a</sup> (FRI326)	CNTS	Chicken tetrazzini	—	—	—		t029
A900322	CNTS	Patient with TSS	+	— <sup>e</sup>	+	1	t002
NCTC 9393	CNTS		+	— <sup>e</sup>	+	1	t002
RIMD 31092	CNTS	MRSA strain	+	— <sup>e</sup>	+	1	t002
AS14g	DSAN	NTS (sample A)	+	— <sup>e</sup>	+	1	t209
AS27	DSAN	NTS (sample A)	+	— <sup>e</sup>	+	1	t209
BS4	DSAN	NTS (sample B)	+	— <sup>e</sup>	+	1	t209
DS18g	DSAN	NTS (sample D)	+	— <sup>e</sup>	+	1	t209
SI9	DSAN	MCM (plant A)	+	— <sup>e</sup>	+	1	t164
SI1	DSAN	MCM (plant A)	+	— <sup>e</sup>	+	1	t164
LA14	DSAN	WBRM (plant A)	+	— <sup>e</sup>	+	1	t164
R1	DSAN	WBRM (plant A)	+	— <sup>e</sup>	+	1	t164
ATCC 19095 (FRI137)	CNTS	Leg abscess	+	+	+	2	t352
382F	AFSSA	Unspecified (food)	+	+	+	3	t166
ED-3	DSAN	RPM (sample ED)	+	— <sup>e</sup>	+	4	t078
ED-4	DSAN	RPM (sample ED)	+	— <sup>e</sup>	+	4	t078
105	DSAT	SP (defeathering machine)	+	— <sup>e</sup>	+	4	t002
106	DSAT	SP (defeathering machine)	+	— <sup>e</sup>	+	4	t002
ATCC 25923	ATCC	Clinical isolate	+	+	+	5	t021
AB-8802	DSAN	RPM (sample AB)	+	+	+	6	t021
7645a	IMM	HP (osteomyelitis)	+	+	+	7	t021
109	DSAT	SP (defeathering machine)	+	+	+	7	t012
OM 56/2a	IMM	HP (osteomyelitis)	+	+	+	7	t012
K4644/97	IMM	HP (blood cultures)	+	+	+	7	t949
K6278/97	IMM	HP (blood cultures)	+	+	+	7	t298
A652/99	IMM	HP (nasal swabs)	+	+	+	7	t012
A2586/99	IMM	HP (nasal swabs)	+	+	+	7	t947
A1048/98	IMM	HP (nasal swabs)	+	+	+	7	t363
A2812/98	IMM	HP (nasal swabs)	+	+	+	7	t012
A4178/98	IMM	HP (nasal swabs)	+	+	+	7	t019
10	DSAT	SP (gutting machine)	+	— <sup>c</sup>	(+) <sup>f</sup>		t267
107	DSAT	SP (defeathering machine)	+	— <sup>c</sup>	(+) <sup>f</sup>		t1059

<sup>a</sup> *egc* negative reference strains (used as negative controls).

<sup>b</sup> ATCC, American Type Culture Collection, Rockville, Md.; AFSSA, Agence Francaise de Sécurité Sanitaire des Aliments, Maisons-Alfort, France (kindly provided by P. Fach); DSMZ, Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany; CNTS, Centre Nationale des Toxémies à Staphylococcus, Faculté de Médecine Laennec, Lyon, France (kindly provided by G. Lina); DSAN, Dipartimento di Scienze degli Alimenti, Università degli Studi di Napoli Federico II, Portici, Italy; DSAT, Dipartimento di Scienze degli Alimenti, Università degli Studi di Teramo, Italy (kindly provided by A. Ianieri); IMM, Institut für Medizinische Mikrobiologie, Universitätsklinikum Münster, Münster, Germany.

<sup>c</sup> MRSA, methicillin-resistant *S. aureus*; NTS, "Napoli-Type" salami; WBRM, water buffalo raw milk; MCM, water buffalo Mozzarella cheese manufacturing (natural whey cultures); RPM, raw poultry meat; SP, slaughterhouse for pigeon; HP, strains were isolated from different patients admitted to the University Hospital of Muenster (1997–1999).

<sup>d</sup> PCR using the primer pair SEI-1 (targeting *sem*) and SEG-2 (targeting *seg*) covering a 3,375-bp fragment of the *egc* cluster.

<sup>e</sup> The size of the amplification band was other than expected.

<sup>f</sup> (+), weak PCR amplification result.

arranged in tandem orientation in the *egc* cluster and are coexpressed. *egc*-encoded superantigens from *Staphylococcus aureus* are neutralized by human sera much less efficiently than are classical staphylococcal enterotoxins and toxic shock syndrome toxin (14). Moreover SEG and SEI interact differently with major histocompatibility complex class II and stimulate completely different subsets of human and mouse T cells. These characteristics indicate complementary superantigenic activity and suggest an important advantage to staphylococcal strains in producing both SEG and SEI.

Therefore, analyzing the diversity of staphylococcal PTSAgs may enhance our knowledge of the pathogenicity and evolution of *S. aureus* and improve detection approaches for diagnostic purposes and epidemiological studies.

The purpose of this study was to analyze the genetic

variability within the *egc* cluster by restriction endonucleases analysis (REA) and nucleotide sequencing of a collection of *S. aureus* strains recovered from different human sources and food samples. The discrimination power of this technique was evaluated by comparing its results with those obtained by *spa* typing, which is widely used for biotyping *S. aureus* strains.

#### MATERIALS AND METHODS

**Bacterial strains.** A total of 35 *Staphylococcus aureus* strains comprising 9 clinical, 16 food-derived, and 10 reference strains (including 4 *egc*-negative control strains, DSM 20231<sup>T</sup>, D 4508, ATCC 14458, and ATCC 27664) were investigated. The origins and sources of strains are reported in Table 1.

Identification was based on growth characteristics on Columbia agar with 5% (vol/vol) sheep blood (at 37°C) and positive catalase production. The clinical

TABLE 2. PCR primers used in this study

Primer	Sequence (5'-3')	<i>egc</i> position <sup>a</sup>	Reference
SEO-1	AGT CAA GTG TAG ACC CTA TT	494-513	8
SEO-2	TAT GCT CCG AAT GAG AAT GA	1027-1008	8
SEM-1	CCA ATT GAA GAC CAC CAA AG	1544-1563	8
SEM-2	CTT GTC CTG TTC CAG TAT CA	2060-2042	8
SEI-1	GAC AAC AAA ACT GTC GAA ACT G	2087-2108	25
SEI-2	CCA TAT TCT TTG CCT TTA CCA G	2716-2695	25
SEI-4	GCC CTA GAG ACT TTA AAA TT	3002-2983	This study
PSE-1	TGA TAA TTA GTT TTA ACA CTA AAA TGC G	2919-2946	19
PSE-2	TAA AAT AAA TGG CTC TAA <u>AAT TGA TGG</u> <sup>b</sup>	3278-3289	19
PSE-4	CGT CTA ATT GCC ACG TTA TAT CAG T	3984-3960	19
SEN-1	ATT GTT CTA CAT AGC TGC AA	3818-3837	8
SEN-2	TTG AAA AAA CTC TGC TCC CA	4499-4480	8
SEG-1	TGC TAT CGA CAC ACT ACA ACC	4758-4778	25
SEG-2	CCA GAT TCA AAT GCA GAA CC	5461-5442	25
SEG-4	AGT TCG AAA CGC ACT TTA TG	5674-5655	This study

<sup>a</sup> According to the sequence of the 6,418-bp DNA fragment of *S. aureus* A900322 harboring the *egc* cluster (GenBank accession number AF285760) (16).

<sup>b</sup> The underlined sequence corresponds to the 15-bp insertion allowing the *seu* gene.

isolates were identified biochemically by the automated ID 32 Staph system (bioMérieux, France). The presence or absence of bound or free coagulase, respectively, was confirmed by latex slide agglutination for clumping factor, protein A, and capsular polysaccharides (Pastorex Staph-Plus; Sanofi Diagnostics Pasteur, France). For all strains, the *S. aureus*-specific *nuc* gene was detected as previously described (9).

**DNA isolation.** Following overnight incubation in brain heart infusion broth (Oxoid, Basingstoke, Hampshire, United Kingdom), staphylococcal cells were streaked on brain heart infusion agar plates and incubated overnight at 37°C. DNA extraction was carried out from a single colony by using the InstaGene matrix (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's recommendations. About 25 ng of DNA was used for PCR amplification.

**PCR amplification of the *S. aureus* *egc*.** The *egc* operon was detected by using PCR primers SEI-1 and SEG-2 as previously described by McLaughlin et al. (25) (Table 2), amplifying a 3,375-bp fragment comprising 47 nucleotides of *sem*, the complete nucleotide sequences of *sei*, *pent1-pent2* (or *seu*), and *sen* followed by 610 nucleotides of *seg*.

PCR amplifications were performed in a 50-μl total volume, including 5 μl of the target DNA, 5.0 μl of *Taq* DNA polymerase 10× buffer (Invitrogen, San Giuliano Milanese, Italy), 2.5 μl of 50 mM MgCl<sub>2</sub>, 0.5 μl of dNTP mix (25 mM each), 0.1 μl of each primer (0.1 mM), and 0.4 μl of *Taq* DNA polymerase (5 U/μl) (Invitrogen). PCR consisted of 30 cycles at 95°C for 10 s and 55°C for 3.5 min and one additional final cycle at 72°C for 10 min. The PCR amplification fragments were resolved by agarose (1% wt/vol) gel electrophoresis at 100 V for 2 h. The gel was stained with ethidium bromide, and the bands were visualized under UV illumination at 254 nm.

**REA of *S. aureus* *egc* cluster.** REA of the amplification fragments of the primer pair SEI-1 and SEG-2 was performed by digestion of about 1 μg (30 to 35 μl) of the PCR product with 20 U of the following restriction enzymes (in a total volume of 50 μl): EcoRI, AluI, TaqI, and CfoI (Promega Italia, Milan, Italy). Restriction digests were resolved by agarose (2% wt/vol) gel electrophoresis at 80 V for 3 h.

**PCR detection of the individual SE genes of the *egc* cluster.** *seo*, *sem*, *sei*, *sen*, and *seg* genes were detected by using the following primer pairs: SEO-1 and

SEO-2, SEM-1 and SEM-2, SEI-1 and SEI-2, SEN-1 and SEN-2, and SEG-1 and SEG-2 (Table 1). The  $\phi$ 1- $\phi$ 2 pseudogenes and *seu* were detected by the use of the primer pairs PSE-1 plus PSE-4 and PSE-2 plus PSE-4, respectively, as previously published by Letertre et al. (19) (Table 2).

**Sequencing procedures of *sei*, and *seg* genes.** The 3,375-bp SEI-1/SEG-2 amplification fragments were used for sequencing the *sei* and *seg* genes. In the case of weak amplification products (Table 3), the primer pairs SEI-1/SEI-4 and SEG-1/SEG-4 (Table 2) were used alternatively. *sei/seg* PCR was performed as described above, while PCR cycling conditions were 94°C for 3 min, followed by 30 cycles of 94°C for 5 s and 53°C for 1 min and finally 72°C for 5 min.

Following purification of the amplification products by using the QIAquick gel extraction kit (QIAGEN, Milan, Italy), the fragments were sequenced, with the same primers used for PCR amplification being applied. DNA sequences were determined by the dideoxy chain termination method using the DNA sequencing kit (Perkin-Elmer Cetus, Emeryville, CA) according to the manufacturer's instructions. The sequences were analyzed, and the GenBank and EMBL databases were queried by using MacDNASIS Pro, version 3.0.7 (Hitachi Software Engineering Europe, Olivet, France).

***spa* typing.** The *x* region of the *spa* gene was amplified by PCR with primers 1095F and 1517R as previously described by Shopsis et al. (35). DNA sequences were obtained by an ABI 377 sequencer (Applied Biosystems, Foster City, Calif.). *spa* types were determined with the software Ridom StaphType (13) by using the Ridom SpaServer (<http://spa.ridom.de/>).

**Nucleotide sequence accession numbers.** All sequences determined in this study were deposited in GenBank under accession numbers AY920260, AY920261, AY920259, AY920257, AY920262, AY920256, AY920258, DQ778337, DQ778338, AY920264, AY920265, AY920263, AY920269, AY920266, AY920267, and AY920268.

RESULTS

**PCR amplification of the *S. aureus* *egc* cluster.** Primers SEI-1 and SEG-2 (Table 2) were used to amplify a 3,375-bp fragment

TABLE 3. PCR *egc* characterization of strains showing SEI-1/SEG-2 weak amplification bands

Strain	Source <sup>a</sup>	PCR result <sup>b</sup>										
		<i>sem-seg</i> <sup>c</sup>					<i>sei</i>		<i>sen</i>	<i>seg</i>		
		<i>sem</i>	<i>seg</i>	<i>seu</i>	<i>seo</i>	<i>sem</i>	SEI-1	SEI-2		SEI-1	SEI-4	SEG-1
10	DSAT	(+)	+	- <sup>e</sup>	+	+	-	+	+	-	-	+
107	DSAT	(+) <sup>d</sup>	+	- <sup>e</sup>	+	+	+	+	+	+	-	+

<sup>a</sup> DSAT, Dipartimento di Scienze degli Alimenti Università degli Studi di Teramo, Italy (kindly provided by A. Ianieri).

<sup>b</sup> If not specified, PCR results are based on the use of previously published primer pairs (8, 19, 25).

<sup>c</sup> PCR using the primer pair SEI-1 (targeting *sem*) and SEG-2 (targeting *seg*) covering a 3,375-bp fragment of the *egc* cluster.

<sup>d</sup> (+), weak PCR amplification result.

<sup>e</sup> The size of the amplification band was other than expected.

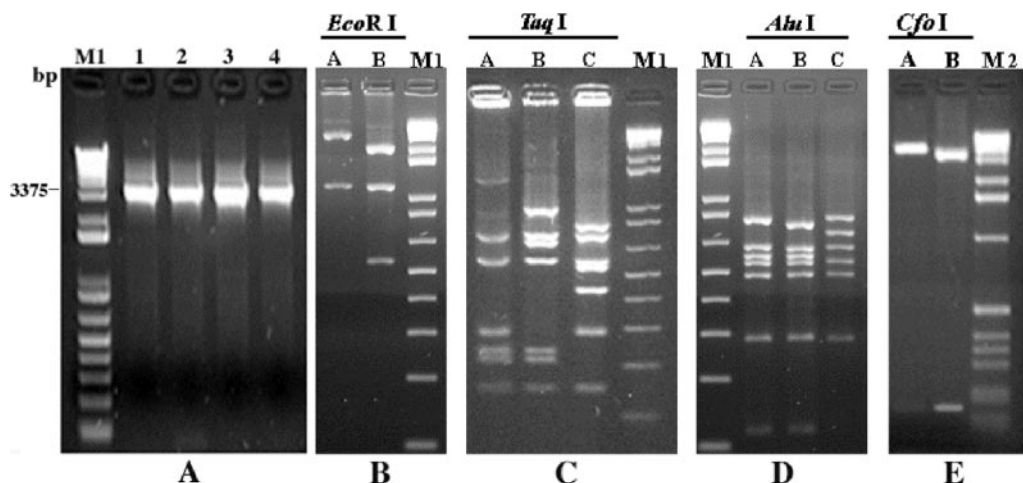


FIG. 1. Specific PCR detection and REA characterization of *S. aureus egc*. Panel A shows the 3,375-bp PCR fragment obtained by amplifying the *S. aureus egc* of strains FRI137 (lane 1), NCTC 9393 (lane 2), RIMD 31092 (lane 3), and AB-8802 (lane 4). Panels B, C, D, and E show different restriction endonuclease patterns obtained from digestions of the 3,375-bp PCR fragment from *egc*-positive strains with EcoRI, TaqI, AluI, and CfoI, respectively. M1, 1-Kb DNA Ladder Plus (Invitrogen SRL); M2, 1-Kb DNA Ladder (Invitrogen SRL).

representing approximately 65% of the nucleotide sequence of the *egc* operon. PCR amplification showed the expected fragment in 29 strains, whereas 2 strains (10 and 107) showed reproducible but weak amplification bands and no amplification signal was obtained by the 4 strains used as negative controls (Table 3).

**REA of *S. aureus egc* cluster.** Fig. 1 (panels B, C, D, and E) shows the different REA patterns of the 29 *egc*-positive strains obtained from digestions of the 3,375-bp PCR fragment using EcoRI, TaqI, AluI, and CfoI. According to the results obtained by REA, the strains were classified into seven groups (Table 4). Three groups were associated with strains possessing the previously described three types of the *egc* operon (Table 1): (i) REA-1, comprising A900322 known to harbor *egc1*, other reference strains, and food-borne isolates, (ii) REA-2, restricted to FRI 137 and known to harbor *egc2*, and (iii) REA-3, restricted to 382F and known to possess *egc3*. Further REA groups (Table 1), such as REA-4, found in veterinary and food origin isolates, REA-5, shown for ATCC 25923, REA-6, observed for AB8802, as well as REA-7, comprising several isolates of human and veterinary origin, represent *S. aureus* strains which may harbor putative novel types of the *egc* operon (designated *egc4*, *egc5*, *egc6*, and *egc7*, respectively).

**PCR detection of the individual SE genes of the *egc* cluster.** Applying the primers PSE-2 and PSE-4, which were previously

reported by Letertre et al. (19) to be specific for the detection of *seu*, a PCR fragment of the expected length was obtained for strains belonging to only REA-2, REA-3, and REA-5 to REA-7, not REA-1 and REA-4 (Table 1). In contrast, a specific PCR fragment was amplified for all *egc*-positive strains using the PSE-1 and PSE-4 primer set (19), flanking a region covering both the pseudogenes  $\phi$ ent1- $\phi$ ent2 and *seu*, respectively.

**Analysis of strains with weak SEI-1/SEG-2 amplicons.** The two strains providing weak SEI-1/SEG-2 amplification results using primers as previously described (8, 25) tested PCR positive for *seo*, *sem*, and *sen*, negative for *seg* and *seu*, and variable for *sei*. Thus, these strains were grouped into two different toxin genotypes differing in the possession of *sei* (Table 3). However, a positive *sei* PCR result was detected in these strains by applying SEI-1 (25) in combination with the newly designed primer SEI-4, which targets an alternative *egc* position. Also, *seg* tested PCR positive for these strains by using a further newly designed primer (SEG-4) along with SEG-1 (25).

**Sequencing of *seg* and *sei* fragments.** The *sei* and *seg* regions of strains giving weak amplification results were sequenced and compared to the sequencing results of the respective sequences of other strains known to harbor *seg* and *sei* (Fig. 2, panel A and B, respectively). The accession numbers of the *sei* and *seg* gene sequences analyzed in this study are given in Fig. 2. Dendrograms, as depicted in Fig. 2, demonstrate the relationships of the sequences. In both panels of Fig. 2, strains were clearly gathered in two separate clusters based on the percentage of identities. In addition to the prototype *seg* and *sei*, variant types, called the *seg* and *sei* variants, were found for both genes. Moreover, a low-grade polymorphism was revealed for *seg*, the *seg* variant, and *sei*, but not for the *sei* variant. In particular, two nucleotide changes were found for *seg* (a G→T substitution at position 5442 and a A→G substitution at position 5450; the nucleotide numbering was previously described by Jarraud et al. [16]) in strains 10 and 107 in the binding site of primer SEG-2, explaining the negative amplification results obtained by the SEI-1/SEG-2 and SEG-1/SEG2 primer sets.

TABLE 4. REA *egc* patterns shown by strains of the same *egc* group

<i>egc</i> group	Pattern obtained by			
	EcoRI	AluI	TaqI	CfoI
1	A	B	B	A
2	A	A	A	A
3	B	B	C	A
4	A	B	C	A
5	B	B	C	B
6	B	C	C	B
7	B	C	B	B



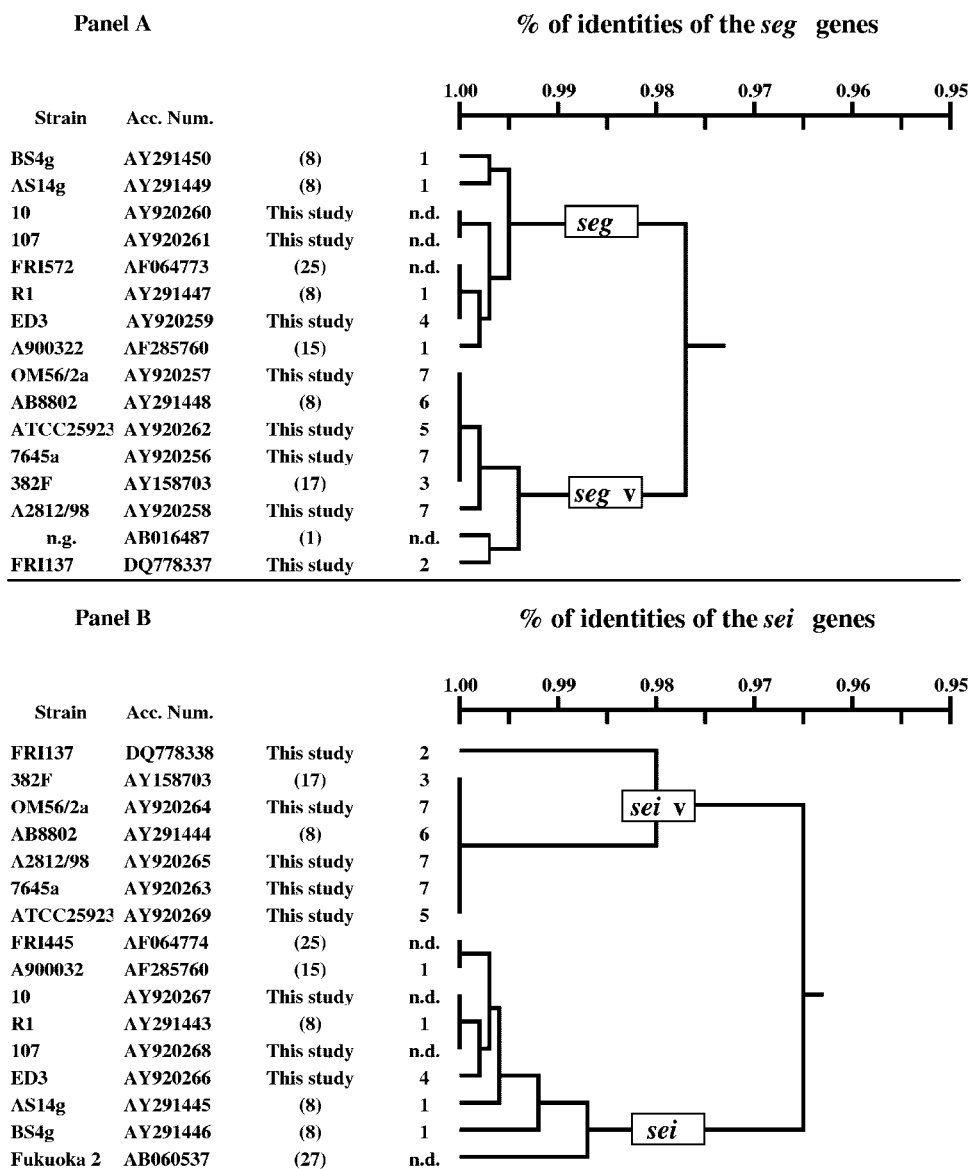


FIG. 2. Nucleotide sequence identities of *seg* (panel A) and *sei* (panel B) genes as analyzed in this study. n.d., not determined; n.g., not given.

Finally, a comparison of amino acid sequences, as obtained by virtual translation of the corresponding genes, showed that the SEI variant presents 4 to 5% (10 to 11 amino acids) of amino acid changes in respect to the SEI of strain FRI 445, while the SEG variant presents about 4% (7 to 8 amino acids) of amino acid changes in respect to the SEG of strain FRI 572.

**spa typing.** A total of 19 *spa* types were obtained by analyzing the 35 strains included in this study (Table 1). In both directions, there was no general concordance between the affiliation to a REA *egc* group and the *spa* type of a given isolate. On one hand, the strains of the REA *egc* groups 1 ( $n = 11$ ), 4 ( $n = 4$ ), and 7 ( $n = 10$ ) displayed three, two, and seven different *spa* types, respectively. On the other hand, some strains exhibiting the same *spa* type were shown to differ by their REA *egc* polymorphism. The three reference strains A900322, NCTC 9393, and RIMD 31092 as well as the two

veterinary strains, 105 and 106, exhibiting the t002 *spa* type, fitted into the two different REA *egc* groups 1 and 4, respectively. Also, the reference strain ATCC 25923, the food-derived strain AB-8802, and the human-derived strain 7645a, harboring the t021 *spa* type, were shown to belong to three different REA *egc* groups (REA-5, -6, and -7, respectively). Moreover, in other cases, as expected, strains isolated from the same source showed the same *spa* type and REA *egc* group: (i) strains AS14 and AS27 were isolated from the same sample A; (ii) strains SI9, SI1, LA 14, and R1 from the same plant A; (iii) strains ED3 and ED4 from the same sample ED; and (iv) strains 105 and 106 from the same machine.

**DISCUSSION**

Originally, *seg* and *sei* genes were identified in two separate strains (FRI 572 and FRI 445) by Munson et al. (27). Subse-

quently it was shown that, when present, *seg* and *sei* genes coexist in all clinical isolates of *S. aureus* examined (4, 15), and they are arranged in tandem orientation on the same 3.2-kb DNA fragment (15). Sequence analysis of the *seg-sei* intergenic DNA and flanking regions revealed three enterotoxin-like open reading frames related to *seg* and *sei*, formerly designated *sek*, *sel*, and *sem*, and two pseudogenes,  $\phi$ ent1 and  $\phi$ ent2, (16). Furthermore, reverse transcription-PCR experiments revealed that all of these genes belong to one operon, designated the enterotoxin gene cluster (*egc*) (16). To rule out possible confusion with SEs described at the same time (12, 32), the *sek* and *sel* genes were renamed *sen* and *seo*, respectively, as published in a correction note (16).

Mempel et al. showed that *egc* occurs in about 48% of the SE-positive *S. aureus* strains isolated from atopic eczema (26). Recently, we detected this cluster in *S. aureus* strains isolated from food samples (8). Moreover, genes of the *egc* cluster were found in isolates recovered from nasal and blood specimens in high percentages (4, 5). Recently, by applying the microarray technology for simultaneous detection of the SE genes, Sergeev et al. (34) estimated that about 92% of *S. aureus* strains contain multiple SE genes (especially the *egc* cluster genes). Surveys of the *egc* distribution in *S. aureus* strains of animal origin also demonstrated a high frequency of *egc*-like genes (36).

However, based on several studies, it has been assumed that differences in the possession of individual genes or nucleotide variations may occur for the SE genes of the *egc* cluster (4, 5, 8, 14, 16, 19). In particular, Letertre et al. (19) found a 15-bp insertion in  $\phi$ ent1 and some point mutations in both  $\phi$ ent1 and  $\phi$ ent2 of the strains FRI 137 and 382F with respect to these two pseudogenes of strain A900322 originally described by Jarraud et al. (16). Instead of allowing translation of the pseudogenes ( $\phi$ ent1 and  $\phi$ ent2), this insertion allowed a putative 261-nucleotide open reading frame (named *seu*) to be translated (19). In addition, it was demonstrated that *egc* of strain 382F is characterized by variants of the *sei*, *seu*, *sen*, and *seg* genes (19). This *seg* allele was shown to be identical to the previously characterized *seg* variant (1). A further SEG<sub>L29P</sub> variant was reported by Jarraud et al. (16).

We recently showed that some *egc*-positive strains, derived from food samples, were negative using PCR assays designed for *sei* and/or *seg* (8). Those strains displayed a *egc* restriction pattern compared to those of other *egc*-positive strains. Partial nucleotide sequencing of the *sei* and *seg* genes in strain AB-8802 (*egc*<sup>+</sup>, *sei* negative, and *seg* negative) confirmed the mispriming of both reverse *sei* primer (SEI-2) and forward *seg* primer (SEG-1) used in the PCR amplifications. We also found that approximately 30% of the *S. aureus* strains tested showed a positive *sei* amplification result by PCR, but they failed in subsequent hybridization reactions with *sei*-specific probes using a generic DNA enzyme immunoassay system (4). In this study, two strains (10 and 107) of veterinary origin showed weak amplification using primers SEI-1 and SEG-2 but tested PCR positive using newly designed *egc* primers, suggesting that the binding sites of the original primer may be polymorphic. The last hypothesis was corroborated by the sequence analysis of *sei* and *seg* genes of 10 and 107 *S. aureus* strains. The full *sei* gene sequences of AB-8802 and 7645a *S. aureus* strains showed 100% identity with the *sei* variant gene

of strain 382F (GenBank accession number AF064774 [19]), while their full-nucleotide *seg* gene sequences displayed 99% identity (AB-8802) with the *seg* variant gene as previously described by Abe et al. (1).

In addition, we analyzed a part of the *egc* operon (*sem-seg* segment) of food-borne, human, veterinary, and reference *S. aureus* strains by REA using EcoRI, CfoI, AluI, and TaqI, allowing the differentiation of at least seven different *egc* operons. Thus, REA of the *egc* operon was shown to be successful in typing and analyzing *egc*-positive *S. aureus* strains. Sequence analysis of the putative new *egc* types may provide further insight into the evolution of *egc*, which was recently identified as a highly prevalent operon of enterotoxin genes, forming a putative nursery of superantigens in *S. aureus*.

The high level of polymorphism of the *S. aureus egc*, as detected by REA analysis of the SEI-1/SEG-2 PCR fragment and confirmed by DNA sequencing, was shown. The *egc* polymorphism seems to be unrelated to the evolution of *S. aureus*, as demonstrated by *spa* typing. In addition to the recently described three *egc* operons, four further variants were characterized. In particular, a variant of *sei*, called the *sei* variant, detectable in several *S. aureus* strains was delineated. Furthermore, the previously described *seg* variant was shown to occur frequently in a defined *S. aureus* strain collection. The observed polymorphism was due to point mutations causing loss or generation of restriction endonuclease sites or DNA insertions as described elsewhere. Moreover, this distinct polymorphism may explain the existence of strains possessing only some of the *egc*-carried genes or pseudogenes. Furthermore, failures in the detection of the *egc* cluster may occur, especially if the primer pair SEI-1/SEG2 is used. This should be considered, particularly for PCR approaches for diagnostic purposes and epidemiological studies, although it may be avoided by the application of the PSE-1/PSE-4 primers (19), which target regions representing highly conserved parts of the cluster.

During this study, the possibility of exploiting the *egc* polymorphism for epidemiological studies was evaluated by comparing results obtained by REA of the *egc* operon with those by *spa* typing. Although more limited than REA by pulsed-field gel electrophoresis analysis in discriminatory power, *spa* typing is often used as a screening method for typing *S. aureus* strains due to its shorter turnaround time, ease of use, and inherent advantages of sequence analysis, storage, and information sharing (35).

Our results reveal higher discriminatory power of *spa* typing in biotyping *S. aureus egc*-positive strains than that of REA *egc*. On the other hand, the REA *egc* analysis' capability to discriminate, in some cases, among strains showing the same *spa* type, even though they were geographically related, makes it a reliable typing tool. Therefore, by using a polyphasic approach, including both REA of *egc* and *spa* typing analyses, the biotyping of *egc*-positive *S. aureus* strains can be reliably achieved, furnishing, at the same time, knowledge of great consequence about the potential toxicity of *S. aureus* strains.

#### ACKNOWLEDGMENTS

We thank G. Lina of the CNTS (Lyon, France), P. Fach of the AFSSA (Maisons-Alfort, France), and A. Ianieri of the DSA (Teramo, Italy) for kindly providing strains used in this study.

Financial support was provided by the MIPAF of Italy (project SQUALTECA) and the German Network of Competence "Pathogenomics," Alliance "Gram-Positive Cocci."

## REFERENCES

- Abe, J., Y. Ito, M. Onimaru, T. Kohsaka, and T. Takeda. 2000. Characterization and distribution of a new enterotoxin-related superantigen produced by *Staphylococcus aureus*. *Microbiol. Immunol.* **44**:79–88.
- Baba, T., F. Takeuchi, M. Kuroda, H. Yuzawa, K. Aoki, A. Oguchi, Y. Nagai, N. Iwama, K. Asano, T. Naimi, H. Kuroda, L. Cui, K. Yamamoto, and K. Hiramatsu. 2002. Genome and virulence determinants of high virulence community-acquired MRSA. *Lancet* **359**:1819–1827.
- Bayles, K. W., and J. J. Iandolo. 1989. Genetic and molecular analyses of the gene encoding staphylococcal enterotoxin D. *J. Bacteriol.* **171**:4799–4806.
- Becker, K., A. W. Friedrich, G. Lubritz, M. Weilert, G. Peters, and C. von Eiff. 2003. Prevalence of genes encoding pyrogenic toxin superantigens and exfoliative toxins among strains of *Staphylococcus aureus* isolated from blood and nasal specimens. *J. Clin. Microbiol.* **41**:1434–1439.
- Becker, K., A. W. Friedrich, G. Peters, and C. von Eiff. 2004. Systematic survey on the prevalence of genes coding for staphylococcal enterotoxins SEIM, SEIO, and SEIN. *Mol. Nutr. Food Res.* **48**:488–495.
- Bergdoll, M. S. 1989. *Staphylococcus aureus*, p. 463–523. In M. P. Doyle (ed.), *Foodborne bacterial pathogens*. Marcel Dekker, Inc., New York, N.Y.
- Betley, M. J., and J. J. Mekalanos. 1985. Staphylococcal enterotoxin A is encoded by phage. *Science* **229**:185–187.
- Blaiotta, G., D. Ercolini, C. Pennacchia, V. Fusco, A. Casaburi, O. Pepe, and F. Villani. 2004. PCR detection of staphylococcal enterotoxin genes in *Staphylococcus* spp. strains isolated from meat and dairy products. Evidence for new variants of *seG* and *seI* in *S. aureus* AB-8802. *J. Appl. Microbiol.* **97**:719–730.
- Brakstad, O. G., K. Aasbakk, and J. A. Maeland. 1992. Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the *nuc* gene. *J. Clin. Microbiol.* **30**:1654–1660.
- 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.
- Dinges, M. M., P. M. Orwin, and P. M. Schlievert. 2000. Exotoxins of *Staphylococcus aureus*. *Clin. Microbiol. Rev.* **13**:16–34.
- Fitzgerald, J. R., S. R. Monday, T. J. Foster, G. A. Bohach, P. J. Hartigan, W. J. Meaney, and C. J. Smyth. 2001. Characterization of a putative pathogenicity island from bovine *Staphylococcus aureus* encoding multiple superantigens. *J. Bacteriol.* **183**:63–70.
- Harmsen, D., H. Claus, W. Witte, J. Rothganger, H. Claus, D. Turnwald, and U. Vogel. 2003. Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for *spa* repeat determination and database management. *J. Clin. Microbiol.* **41**:5442–5448.
- Holtfreter, S., K. Bauer, D. Thomas, C. Feig, V. Lorenz, K. Roschack, E. Friebe, K. Selleng, S. Lovenich, T. Greve, A. Greinacher, B. Panzig, S. Engelmann, G. Lina, and B. M. Broker. 2004. *egc*-encoded superantigens from *Staphylococcus aureus* are neutralized by human sera much less efficiently than are classical staphylococcal enterotoxins or toxic shock syndrome toxin. *Infect. Immun.* **72**:4061–4071.
- Jarraud, S., G. Cozon, F. Vandenesch, M. Bes, J. Etienne, and G. Lina. 1999. Involvement of enterotoxins G and I in staphylococcal toxic shock syndrome and staphylococcal scarlet fever. *J. Clin. Microbiol.* **37**:2446–2449.
- Jarraud, S., M. A. Peyrat, A. Lim, A. Tristan, M. Bes, C. Mougel, J. Etienne, F. Vandenesch, M. Bonneville, and G. Lina. 2001. *egc*, a highly prevalent operon of enterotoxin gene, forms a putative nursery of superantigens in *Staphylococcus aureus*. *J. Immunol.* **166**:669–677. (Correction, **166**:4260.)
- Kuroda, M., T. Ohta, I. Uchiyama, T. Baba, H. Yuzawa, I. Kobayashi, L. Cui, A. Oguchi, K. Aoki, Y. Nagai, J. Lian, T. Ito, M. Kanamori, H. Matsumaru, A. Maruyama, H. Murakami, A. Hosoyama, Y. Mizutani-Ui, N. K. Takahashi, T. Sawano, R. Inoue, C. Kaito, K. Sekimizu, H. Hirakawa, S. Kuhara, S. Goto, J. Yabuzaki, M. Kanehisa, A. Yamashita, K. Oshima, K. Furuya, C. Yoshino, T. Shiba, M. Hattori, N. Ogasawara, H. Hayashi, and K. Hiramatsu. 2001. Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. *Lancet* **357**:1225–1240.
- Le Loir, Y., F. Baron, and M. Gautier. 2003. *Staphylococcus aureus* and food poisoning. *Genet. Mol. Res.* **2**:63–76.
- Letertre, C., S. Perelle, F. Dilasser, and P. Fach. 2003. Identification of a new putative enterotoxin SEU encoded by the *egc* cluster of *Staphylococcus aureus*. *J. Appl. Microbiol.* **95**:38–43.
- Lina, G., G. A. Bohach, S. P. Nair, K. Hiramatsu, E. Jouvin-Marche, R. Mariuzza, and the International Nomenclature Committee for Staphylococcal Superantigens. 2004. Standard nomenclature for the superantigens expressed by *Staphylococcus*. *J. Infect. Dis.* **189**:2334–2336.
- Lindsay, J. A., A. Ruzin, H. F. Ross, N. Kurepina, and R. P. Novick. 1998. The gene for toxic shock toxin is carried by a family of mobile pathogenicity islands in *Staphylococcus aureus*. *Mol. Microbiol.* **29**:527–543.
- Llewellyn, M., and J. Cohen. 2002. Superantigens: microbial agents that corrupt immunity. *Lancet Infect. Dis.* **2**:156–162.
- Marr, J. C., J. D. Lyon, J. R. Roberson, M. Lupher, W. C. Davis, and G. A. Bohach. 1993. Characterization of novel type C staphylococcal enterotoxins: biological and evolutionary implications. *Infect. Immun.* **61**:4254–4262.
- McCormick, J. K., J. M. Yarwood, and P. M. Schlievert. 2001. Toxic shock syndrome and bacterial superantigens: an update. *Annu. Rev. Microbiol.* **55**:77–104.
- McLaughlin, J., G. L. Narayanan, V. Mithani, and G. O'Neill. 2000. The detection of enterotoxins and toxic shock syndrome toxin genes in *Staphylococcus aureus* by polymerase chain reaction. *J. Food Prot.* **63**:479–488.
- Mempel, M., G. Lina, M. Hojka, C. Schnopp, H. P. Seidl, T. Schafer, J. Ring, F. Vandenesch, and D. Abeck. 2003. High prevalence of superantigens associated with the *egc* locus in *Staphylococcus aureus* isolates from patients with atopic eczema. *Eur. J. Clin. Microbiol. Infect. Dis.* **22**:306–309.
- Munson, S. H., M. T. Tremaine, M. J. Betley, and R. A. Welch. 1998. Identification and characterization of staphylococcal enterotoxin types G and I from *Staphylococcus aureus*. *Infect. Immun.* **66**:3337–3348.
- Olsen, S. J., L. C. MacKinnon, J. S. Goulding, N. H. Bean, and L. Slutsker. 2000. Surveillance for foodborne-disease outbreaks—United States, 1993–1997. *Morb. Mortal. Wkly. Rep. CDC Surveill. Summ.* **49**:1–62.
- Omoe, K., D. L. Hu, H. Takahashi-Omoe, A. Nakane, and K. Shinagawa. 2003. Identification and characterization of a new staphylococcal enterotoxin-related putative toxin encoded by two kinds of plasmids. *Infect. Immun.* **71**:6088–6094.
- Omoe, K., M. Ishikawa, Y. Shimoda, D. L. Hu, S. Ueda, and K. Shinagawa. 2002. Detection of *seg*, *seh*, and *sei* genes in *Staphylococcus aureus* isolates and determination of the enterotoxin productivities of *S. aureus* isolates harboring *seg*, *seh*, or *sei* genes. *J. Clin. Microbiol.* **40**:857–862.
- Orwin, P. M., J. R. Fitzgerald, D. Y. Leung, J. A. Gutierrez, G. A. Bohach, and P. M. Schlievert. 2003. Characterization of *Staphylococcus aureus* enterotoxin L. *Infect. Immun.* **71**:2916–2919.
- Orwin, P. M., D. Y. Leung, H. L. Donahue, R. P. Novick, and P. M. Schlievert. 2001. Biochemical and biological properties of staphylococcal enterotoxin K. *Infect. Immun.* **69**:360–366.
- Ren, K., J. D. Bannan, V. Pancholi, A. L. Cheung, J. C. Robbins, V. A. Fischetti, and J. B. Zabriskie. 1994. Characterization and biological properties of a new staphylococcal exotoxin. *J. Exp. Med.* **180**:1675–1683.
- Sergeev, N., D. Volokhov, V. Chizhikov, and A. Rasooly. 2004. Simultaneous analysis of multiple staphylococcal enterotoxin genes by an oligonucleotide microarray assay. *J. Clin. Microbiol.* **42**:2134–2143.
- Shopsin, B., M. Gomez, S. O. Montgomery, D. H. Smith, M. Waddington, D. E. Dodge, D. A. Bost, M. Riehnman, S. Naidich, and B. N. Kreiswirth. 1999. Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. *J. Clin. Microbiol.* **37**:3556–3563.
- Smyth, D. S., P. J. Hartigan, W. J. Meaney, J. R. Fitzgerald, C. F. Deobald, G. A. Bohach, and C. J. Smyth. 2005. Superantigen genes encoded by the *egc* cluster and SaPIbov are predominant among *Staphylococcus aureus* isolates from cows, goats, sheep, rabbits and poultry. *J. Med. Microbiol.* **54**:401–411.
- Su, Y.-C., and A. C. L. Wong. 1995. Identification and purification of a new staphylococcal enterotoxin. *H. Appl. Environ. Microbiol.* **61**:1438–1443.
- Su, Y.-C., and A. C. L. Wong. 1997. Current perspectives on detection of staphylococcal enterotoxins. *J. Food Prot.* **60**:195–202.
- Yarwood, J. M., J. K. McCormick, M. L. Paustian, P. M. Orwin, V. Kapur, and P. M. Schlievert. 2002. Characterization and expression analysis of *Staphylococcus aureus* pathogenicity island 3: implications for the evolution of staphylococcal pathogenicity islands. *J. Biol. Chem.* **277**:13138–13147.
- Zhang, S., J. J. Iandolo, and G. C. Stewart. 1998. The enterotoxin D plasmid of *Staphylococcus aureus* encodes a second enterotoxin determinant (*sej*). *FEMS Microbiol. Lett.* **168**:227–233.