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Characterization of a Putative Pathogenicity Island from Bovine 
Staphylococcus aureus Encoding Multiple Superantigens

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Previous studies have demonstrated that a proportion of Staphylococcus aureus isolates from bovine mastitis coproduce toxic shock syndrome toxin (TSST) and staphylococcal enterotoxin C (SEC). In this study, molecular genetic analysis of one such strain, RF122, revealed the presence of a 15,891-bp putative pathogenicity island (SaPIbov) encoding the genes for TSST (tst), the SEC bovine variant (sec-bovine), and a gene (sel) which encodes an enterotoxin-like protein. The island contains 21 open reading frames specifying hypothetical proteins longer than 60 amino acids including an integrase-like gene. The element is bordered by 74-bp direct repeats at the left and right junctions, and the integration site lies adjacent to the 3′ end of the gmp synthase gene (gmps) in the S. aureus chromosome. SaPIbov contains a central region of sequence identity with the previously characterized tst pathogenicity island SaPI1 (J. A. Lindsay et al., Mol. Microbiol. 29:527–543, 1998).

A closely related strain, RF120, of the same multilocus enzyme electrophoretic type, random amplified polymorphic DNA type, and ribotype, does not contain the island, implying that the element is mobile and that a recent insertion/deletion event has taken place. TSST and TSST/SEC-deficient mutants of S. aureus strain RF122 were constructed by allele replacement. In vitro bovine β-specific lymphocyte expansion analysis by culture supernatants of wild-type strains and of tst and sec-bovine allele replacement mutants revealed that TSST stimulates BTB13-specific T cells whereas SEC-bovine stimulates BTB93-specific T cells. This suggests that the presence of SaPIbov may contribute to modulation of the bovine immune response.

Staphylococcus aureus can cause many diseases in humans and animals. It is the most frequent cause of bovine mastitis and is a huge economic problem for the dairy industry worldwide (26). Typically, the disease is of a chronic nature, with subclinical mastitis being the most common form. The organisms may survive for long periods of time in the host without causing overt symptoms of disease. Often, antibiotic therapy merely converts a clinical infection to a subclinical form of the disease. The bacterial factors allowing persistence in the host are poorly understood.

S. aureus can produce several superantigens (SAgs) including toxic shock syndrome toxin 1 (TSST-1) and staphylococcal enterotoxin C (SEC) (6). These exotoxins are involved in modulating the host immune response and may contribute to evasion of host defenses and bacterial persistence (10). Genes encoding SAgs are often associated with mobile genetic elements such as pathogenicity islands, phages, and plasmids (5, 23, 34). Pathogenicity islands are accessory genetic elements that range in size from 10 to 200 kb, contain one or more genes associated with virulence, are bordered by directly repeated sequences, can be deleted en bloc, and may have integrase-like genes (15, 18). Recently Lindsay et al. (23) described a pathogenicity island (SaPI1) in a human clinical S. aureus isolate that contained the gene for TSST-1 (tst) and an open reading frame (ORF) with marked sequence similarity to those encoding SEs. The mobility of SaPI1 was demonstrated by phage-assisted excision, transduction, and site-specific integration into a ΔrecA mutant strain.

Previous studies (12, 19) showed that about 20% of bovine S. aureus strains coproduced TSST-1 and SEC. Since these toxins are rarely produced singly by bovine strains, their genes may be linked. This notion was supported by the observation that tst- and sec-specific probes hybridized to HindIII restriction fragments of the same size in Southern blot analysis.

In this study, we characterized the associated genetic element, named bovine staphylococcal pathogenicity island (SaPIbov), and analyzed the activity of these toxins on bovine lymphocytes.

MATERIALS AND METHODS

Bacterial strains. Strains and plasmids are listed in Table 1. S. aureus strains were grown on tryptic soy agar or in tryptic soy broth and stored as glycerol stocks at −70°C. Where appropriate, the antibiotics erythromycin (10 μg/ml), tetracycline (2 μg/ml), and chloramphenicol (5 μg/ml) were incorporated.

TSST-1 and SEC production. Culture supernatant fluids of S. aureus were tested using reverse passive latex agglutination (RPLA) toxin detection kits for TSST-1 (TST-RPLA; Oxoid Ltd., Basingstoke, England) and SEC (SET-RPLA; Oxoid).

DNA manipulations. Manipulations of DNA were performed by standard techniques (29).

Construction of plasmid pJRFsec:Emr. The 5′ and 3′ parts of the sec gene including 400 and 250 bp, respectively, of flanking sequence were PCR amplified from plasmid pJRF101 using specific primers (Table 1). Primers were designed

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so that the resulting PCR products would include a single Clal site at one end of both fragments. The PCR products were digested with restriction endonuclease Clal and ligated together. The resulting single fragment contained a 150-bp internal deletion compared to the wild-type sec gene. This fragment was cut at natural XbaI and HindIII restriction sites present internal to the 5′ and 3′ ends, respectively, and ligated into the multiple cloning site of pUC18. This resulted in a final insert size of 950 bp. A 1.4-kb TaqI fragment containing the erythromycin resistance determinant from pE194 (16) was cloned into the Clal site in the middle of the sec gene to form pJRF. The temperature-sensitive plasmid vector pTS2 (14, 33), which confers chloramphenicol resistance, was cloned into the HindIII site of the sec gene to form pJRFsec::Em.

Plasmid pRF6684 for construction of the tst knockout carries an in vitro-constructed tut::Tc mutation present in a similar temperature-sensitive plasmid (31).

Construction of allele replacement mutants. Plasmids pRN6684 and pRFare::Em were introduced by electroporation into S. aureus strain RN4220 (2). Once in strain RN4220, the plasmid was transduced into strain RF122 using phage 85 (13). Allele replacement was carried out as described previously (13). The temperature-sensitive phenotype of the plasmids facilitated integration by homologous recombination, and a double-crossover event resulting in a stable mutant was detected by plating on appropriate antibiotics. Loss of TSST-1 or SEC production was detected by plating on appropriate antibiotics.

Experimental procedures. Two or three donors were used to make a size fractionation of a HindIII genomic digest in a sucrose gradient. The fraction containing the 6.5-kb HindIII restriction fragment which hybridized to the constant region of the Cβ chain gene (boCβ) was used for use in PCR assays to analyze boVβ expression. Primers used in PCR assays to analyze boVβ expression were previously described (9).

Quantitative PCR to determine boVβ levels in stimulated lymphocyte cultures. The method described by Kob et al. (20) with the modifications of Deringer et al. (9) was used for assessment of boVβ expression. Primers used in PCR assays to analyze boVβ expression were previously described (9).

Results were expressed as an increase in expansion index compared to unstimulated control cultures. Values below or near 1.0 represent no significant increase in expression.

**TABLE 1. Bacterial strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Properties</th>
<th>Reference(s) or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. aureus strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RF122</td>
<td>Wild-type strain from bovine mastitis (TSST+ SEC+)</td>
<td>12</td>
</tr>
<tr>
<td>RF122-1</td>
<td>tst::Tc mutant</td>
<td>This study</td>
</tr>
<tr>
<td>RF122-2</td>
<td>tst::Tc sec::Em mutant</td>
<td>This study</td>
</tr>
<tr>
<td>RF120</td>
<td>SaPbov-negative strain of same clonal type as RF122</td>
<td>12</td>
</tr>
<tr>
<td>KB103</td>
<td>recA mutant</td>
<td>3</td>
</tr>
<tr>
<td>RN4220</td>
<td>Restriction/modification</td>
<td>21</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pJRF</td>
<td>sec gene (150-bp internal deletion) with 1.4-kb TaqI Em′ insert from pE194 cloned into pUC18</td>
<td>This study</td>
</tr>
<tr>
<td>pJRF101</td>
<td>6.5-kb HindIII fragment in pBluescript KS(+)</td>
<td>This study</td>
</tr>
<tr>
<td>pJRF102</td>
<td>4-kb HindIII fragment from RF122 containing left-hand junction cloned into pUC19</td>
<td>This study</td>
</tr>
<tr>
<td>pJRF103</td>
<td>1-kb PCR product from RF120 containing chromosomal insertion site of SaPbov cloned into pCR2.1-TOPO</td>
<td>This study</td>
</tr>
<tr>
<td>pJRFsec::Em′</td>
<td>pJRF with pTS2 (Cm′) cloned into HindIII site flanking sec gene</td>
<td>This study</td>
</tr>
<tr>
<td>pRN6684</td>
<td>tst::tnm (Tc′) Em′</td>
<td>31</td>
</tr>
<tr>
<td>pCR2.1-TOPO</td>
<td>Vector for cloning PCR products</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pE194</td>
<td>1.4-kb Em′ TaqI fragment</td>
<td>16</td>
</tr>
<tr>
<td>pTS2</td>
<td>Cm′, ts rep; derived from pTV1ts, MCS′ from pBluescript</td>
<td>C. O’Connell (personal communication); 14, 33</td>
</tr>
<tr>
<td>pBluescript KS(+)</td>
<td>Phagemid derived from pUC19; MCS, vector for blue/white colony selection</td>
<td>Stratagene</td>
</tr>
</tbody>
</table>

a Strains RF120 and RF122 were previously shown to be of the same clonal type by random amplified polymorphic DNA typing, multilocus enzyme electrophoretic typing, and ribotyping analysis (11).

b MCS, multiple cloning site.
plates). Colony blotting using a \( \textit{tst} \)-specific probe was used to identify positive colonies, which were confirmed by PCR using \( \textit{tst} \)-specific primers (Table 2).

**Outward-directed PCR.** This was performed using a Vectorette II kit (Sigma-Genosys) according to the manufacturer's instructions. This system is used to amplify regions of unknown DNA sequence flanking a region of known DNA sequence. Briefly, the target DNA was digested with an appropriate restriction enzyme. Vectorette units were ligated onto the ends of the cleaved target DNA. PCR amplification was carried out with one primer directed to the known sequence (custom primer) and the other primer specific for the Vectorette unit (Vectorette primer). The amplified products were then cloned and sequenced or used as probes in Southern hybridization experiments.

**DNA sequencing and analysis.** DNA sequencing analysis was carried out on both DNA strands by MWG-Biotech, Milton Keynes, United Kingdom. The BLAST algorithm (BLASTN and BLASTX) was used to search for sequence similarities (1).

**Nucleotide sequence accession number.** The SaPIbov sequence shown in Fig. 3 has been assigned GenBank accession number AF217235.

### TABLE 2. PCR primers used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Target or function (reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>tn1</td>
<td>ATCCCTTACTGCAACACAGG</td>
<td>( \textit{tst} ) probe amplification</td>
</tr>
<tr>
<td>tst2</td>
<td>TTCCAAAATAAACACCCGTTTT</td>
<td>( \textit{tst} ) probe amplification</td>
</tr>
<tr>
<td>junct3</td>
<td>GGCTGGAAGACCGCGTAAATA</td>
<td>Integration site, outward PCR</td>
</tr>
<tr>
<td>VL</td>
<td>GGAAATTCGTTGAGATAGAAGTCCACC</td>
<td>LHS 7-kb probe, outward PCR</td>
</tr>
<tr>
<td>VR</td>
<td>GCATCTGTGTTCTGATATAGG</td>
<td>RHS 4-kb probe (outward PCR) and 9-kb RHS region</td>
</tr>
<tr>
<td>JR1</td>
<td>CAGATTAGGATCCGTCGCGG</td>
<td>9-kb RHS region</td>
</tr>
<tr>
<td>pBSr</td>
<td>AACAGCTATAGCACATG</td>
<td>( \text{pJRFsec}::\text{Em} ) construction (5’ sec amplification)</td>
</tr>
<tr>
<td>sec2</td>
<td>CCATCGATTAAAGAAAGTGAAACGCTC</td>
<td>( \text{pJRFsec}::\text{Em} ) construction (5’ sec amplification)</td>
</tr>
<tr>
<td>sec3</td>
<td>CCATCGATGTTATGCTCATCATAACA</td>
<td>( \text{pJRFsec}::\text{Em} ) construction (3’ sec amplification), sec probe amplification</td>
</tr>
<tr>
<td>sec4</td>
<td>AGCTAGTCTTTTAAACAGGC</td>
<td>( \text{pJRFsec}::\text{Em} ) construction (3’ sec amplification), sec probe amplification</td>
</tr>
<tr>
<td>sec5</td>
<td>CGTCTCCACCGTGAAAGG</td>
<td>( \text{seg} ) detection (27)</td>
</tr>
<tr>
<td>sec6</td>
<td>CCAAGTGATTGTCTATTGCTG</td>
<td>( \text{seg} ) detection (27)</td>
</tr>
<tr>
<td>sec7</td>
<td>CAATCTGATTTTTCAACAGTGAC</td>
<td>( \text{sei} ) detection (27)</td>
</tr>
<tr>
<td>sec8</td>
<td>CAGGCACTCCATCTCCGT</td>
<td>( \text{sei} ) detection (27)</td>
</tr>
</tbody>
</table>

* LHS, left-hand side; RHS, right-hand side.

### RESULTS

**Detection of the \( \textit{tst} \) and \( \textit{sec} \) genes.** Southern blot analysis was carried out on \( \textit{S. aureus} \) RF122 genomic DNA digested with \( \text{HindIII} \). Probes were generated by PCR amplification with primers specific for the \( \textit{tst} \) and \( \textit{sec} \) genes (Table 2). The probes hybridized to the same 6.5-kb restriction fragment (data not shown), suggesting that the genes lie adjacent to each other.

**Cloning and sequencing of the \( \textit{tst/sec HindIII} \) fragment.** The 6.5-kb \( \text{HindIII} \) fragment carrying \( \textit{tst} \) and \( \textit{sec} \) was cloned into pBluescript, forming pJRF101. DNA sequencing revealed that the \( \textit{sec} \) and \( \textit{tst} \) genes were approximately 2 kb apart and in opposite orientations (Fig. 1). Southern blot analysis using a PCR-generated probe specific for the region between the \( \textit{tst} \) and \( \textit{sec} \) genes indicated that this element was not present in a related strain (RF120) which did not produce TSST-1 or SEC (not shown). Use of plasmid pJRF101 as a probe showed the presence of the 6.5-kb hybridizing fragment in RF122 (Fig. 2) and, surprisingly, a second hybridizing fragment of 3 kb which was also present in RF120. Use of the plasmid vector only as a probe in Southern blot analysis did not demonstrate hybridization with genomic DNA from either strain RF120 or strain RF122 (not shown). This indicates that the 6.5-kb \( \text{HindIII} \) fragment contains sequences with homology elsewhere in the genome.

![FIG. 1. Mapping SaPIbov by PCR and Southern hybridization. Vectorette PCR was performed on DNA flanking the 6.5-kb \( \text{HindIII} \) fragment harboring \( \textit{tst} \) and \( \textit{sec} \). DNA was cleaved with \( \text{BclI} \), ligated with the Vectorette cassette, and subjected to PCR with outward-directed primer VL or VR and a primer specific for the Vectorette unit attached to the end of each fragment. These PCR products provided probes A and B, which were used to analyze genomic DNA of strain RF122 \( \textit{tst sec} \) (lanes 1 and 3) and wild-type strain RF120 (lanes 2 and 4). Primers VR and JR1 were used to amplify the 9-kb right junction fragment. The left and right junctions of SaPIbov are denoted by open boxes.](https://example.com/figure1.png)
was carried out on these outward directed from the 6.5-kb region of known sequence, tst/sec HinI lapped the 6.5-kb pJRF101 indicated that BclI fragments of 11 and 5.5 kb overlapped the 6.5-kb tst/sec HindIII fragment. Vectorette PCR, outward directed from the 6.5-kb region of known sequence, was carried out on these BclI fragments with custom primers VL (left fragment) and VR (right fragment) and a primer specific for the Vectorette units that had been ligated to the ends of each fragment (Fig. 1). This resulted in PCR products of 7 kb (left) and 4 kb (right) (Fig. 1) which were DIG labeled and used as probes A and B in Southern blot analysis of strains RF122 and RF120, a related strain which does not contain the tst/sec element. This was done to determine if either of the Vectorette-amplified fragments contained a junction between the element and adjacent genomic DNA. If the probe contains sequence specific for the region containing the junction, it will hybridize to genomic DNA from both strains.

Southern hybridization analysis (Fig. 1) indicated that the TSST+ SEC+ strain RF122 and the related TSST+ SEC− strain RF120 contained DNA sequences which hybridized with both probes A and B. Probe B hybridized to a single 4.5-kb HindIII fragment in both strains. This and Southern analysis using pJRF101 as a probe (Fig. 2) indicated that there were sequences present elsewhere in the genome with similarity to the tst/sec element. It appears that DNA within the 4.5-kb HindIII fragment is present in the tst/sec element in strain RF122 and also elsewhere in the genome in both strains RF122 and RF120. The absence of a second hybridizing band in RF122 is explained by the likelihood that the 4.5-kb fragment is duplicated elsewhere in the genome and so the two fragments appear as a single (more intense) band on the Southern blot. Accordingly, it was deemed that the right-hand junction may lie outside the 4-kb flanking region. Probe A hybridized to a 2.5-kb HindIII fragment in both strains RF122 and RF120 (Fig. 1). It also hybridized to a second fragment in strain RF122 (4 kb) and in strain RF120 (3.2 kb). This restriction fragment length polymorphism suggested that the left-hand junction lies within the 4-kb HindIII fragment of strain RF122 (Fig. 1). This fragment was cloned into pUC19 to form pJRF102 and sequenced. Southern analysis and sequence information identified where the left-hand junction was likely to be, and a primer (junc3) was designed specific for a region to the left of the junction. Outward-directed Vectorette PCR was carried out on the TSST− SEC− RF120 DNA digested with Alul using this specific primer (Fig. 3), resulting in a 1-kb PCR product which was cloned into pCR2.1-TOPO (Invitrogen) to form pJRF103. Sequence analysis identified the point of divergence of RF120 DNA from RF122 DNA. This product contains the insertion site of the tst element and sequence to the right-hand side of this insertion site. Accordingly, a primer (JRI) specific for a region to the right of the diverged sequence in strain RF120 should be specific for the same region flanking the right-hand junction of the element in strain RF122. PCR primers VR and JRI were then used to amplify the intervening sequence of the tst element by long-range PCR (Fig. 1), resulting in a 9-kb PCR product which was sequenced directly.

**Sequence analysis.** We propose that the inserted DNA element in *S. aureus* strain RF122 is a pathogenicity island referred to here as SaPIbov. Figure 3 shows a map of SaPIbov including 21 ORFs larger than 60 codons. The putative TSST protein showed up to 98% identity with proteins encoded by previously sequenced *tst* genes. It differs at three amino acid residues from *tstO* and seven amino acid residues from *tst1* (22). The *sec* gene product is the SEC-bovine variant (24) which varies at three amino acid residues from SEC1 and is specific for bovine isolates of *S. aureus*. The hypothetical protein product of the staphylococcal enterotoxin (*seI*) gene, lying close to the left-hand junction, has 55% identity with SEI. In addition, there is an integrase-like gene just inside the right-hand junction, the protein product of which has 40% identity with a central region of sequence identity stretching from the *tst* element by long-range PCR (Fig. 1), resulting in a 9-kb PCR product which was sequenced directly.

**Characterization of the flanking regions.** To determine the extent of the putative insertion element containing the *tst* and *sec* genes, the sequences flanking the 6.5-kb HindIII fragment were analyzed by Southern hybridization. Probes specific for the flanking regions were constructed by outward-directed PCR using the Vectorette PCR system. Sequence analysis of pJRF101 identified a single BclI restriction site (Fig. 1) within the cloned 6.5-kb HindIII fragment. Southern blot analysis of strain RF122 genomic DNA cleaved with BclI and probed with pJRF101 indicated that BclI fragments of 11 and 5.5 kb overlapped the 6.5-kb tst/sec HindIII fragment. Vectorette PCR, outward directed from the 6.5-kb region of known sequence, was carried out on these BclI fragments with custom primers VL (left fragment) and VR (right fragment) and a primer specific for the Vectorette units that had been ligated to the ends of each fragment (Fig. 1). This resulted in PCR products of 7 kb (left) and 4 kb (right) (Fig. 1) which were DIG labeled and used as probes A and B in Southern blot analysis of strains RF122 and RF120, a related strain which does not contain the tst/sec element. This was done to determine if either of the Vectorette-amplified fragments contained a junction between the element and adjacent genomic DNA. If the probe contains sequence specific for the region containing the junction, it will hybridize to genomic DNA from both strains.

Southern hybridization analysis (Fig. 1) indicated that the TSST+ SEC+ strain RF122 and the related TSST+ SEC− strain RF120 contained DNA sequences which hybridized with both probes A and B. Probe B hybridized to a single 4.5-kb HindIII fragment in both strains. This and Southern analysis using pJRF101 as a probe (Fig. 2) indicated that there were sequences present elsewhere in the genome with similarity to the tst/sec element. It appears that DNA within the 4.5-kb HindIII fragment is present in the tst/sec element in strain RF122 and also elsewhere in the genome in both strains RF122 and RF120. The absence of a second hybridizing band in RF122 is explained by the likelihood that the 4.5-kb fragment is duplicated elsewhere in the genome and so the two fragments appear as a single (more intense) band on the Southern blot. Accordingly, it was deemed that the right-hand junction may lie outside the 4-kb flanking region. Probe A hybridized to a 2.5-kb HindIII fragment in both strains RF122 and RF120 (Fig. 1). It also hybridized to a second fragment in strain RF122 (4 kb) and in strain RF120 (3.2 kb). This restriction fragment length polymorphism suggested that the left-hand junction lies within the 4-kb HindIII fragment of strain RF122 (Fig. 1). This fragment was cloned into pUC19 to form pJRF102 and sequenced. Southern analysis and sequence information identified where the left-hand junction was likely to be, and a primer (junc3) was designed specific for a region to the left of the junction. Outward-directed Vectorette PCR was carried out on the TSST− SEC− RF120 DNA digested with Alul using this specific primer (Fig. 3), resulting in a 1-kb PCR product which was cloned into pCR2.1-TOPO (Invitrogen) to form pJRF103. Sequence analysis identified the point of divergence of RF120 DNA from RF122 DNA. This product contains the insertion site of the tst element and sequence to the right-hand side of this insertion site. Accordingly, a primer (JRI) specific for a region to the right of the diverged sequence in strain RF120 should be specific for the same region flanking the right-hand junction of the element in strain RF122. PCR primers VR and JRI were then used to amplify the intervening sequence of the tst element by long-range PCR (Fig. 1), resulting in a 9-kb PCR product which was sequenced directly.

**Identification of the chromosomal integration site.** A 74-bp direct repeat occurs at the junction of the inserted element in strain RF122. This sequence occurs in the SaPIbov− strain RF120 and marks the chromosomal integration site which lies adjacent to the GMP synthase gene (*gmps*) in the *S. aureus* chromosome (Fig. 3).
gene to ORF11 containing six ORFs with up to 97% identity with ORFs from SaPI1. In addition, ORF15 has 71 and 100% identity with ORF12 and ORF13 from SaPI1, respectively. The direct repeats characteristic of pathogenicity islands are different in the two elements, with SaPI1 containing 17-bp repeats and SaPIbov containing 74-bp repeats. There was a single copy of the 74-bp repeat in strain RF120, which is closely related to strain RF122. BLAST analysis of the unfinished S. aureus genome databases of strains COL and 8325-4 found that only 24 of the 74 bases appeared to be present, while strain EMRSA-16 contained 27 of the 74 bases. Nonetheless, this still represents a recognition target which could potentially direct integration of the element into the genomes of these strains. The site of integration of SaPI1 lies near the tyrB gene, unlike that of SaPIbov, which lies at one end of the gmps gene. The deduced amino acid sequences of the integrase genes show about 40% identity. The five amino acids thought to be essential for integrase function are conserved (data not shown).

**Mobility experiments.** To examine if phage could mobilize the pathogenicity island, we used transducing phages 80a, 85, and 11 to attempt to transduce SaPIbov marked with the tst::Tcr mutation to the recA strain KB103 (Table 1). No transductants were identified, which suggests that none of these phages could mobilize the element as has been reported for phages 13 and 80a with SaPI1.

**Confirmation of allele replacement by Southern hybridization.** The occurrence of a double-crossover event leading to the presence of a single copy of the mutated tst allele in strain RF122-1 and a single copy of the sec mutated allele in strain RF122-2 was confirmed by Southern hybridization (Fig. 4). A DIG-labeled PCR product specific for a region encompassing the SauI insertion site of the tet marker interrupting the tst gene was constructed and used to probe HindIII-cut genomic DNA from wild-type (RF122) and mutant (RF122-1 and RF122-2) strains. The presence of a HindIII site within the tet locus resulted in two hybridizing bands in the wild type. In the tst sec double mutant (RF122-2), the 5.8-kb fragment was replaced by a fragment of 7.2 kb due to insertion of the 1.4-kb ermC fragment. This also confirmed that the genes are closely linked.

**BoVβ analysis.** Initial screening of S. aureus RF122 indicated that the strain expressed SEC and TSST-1 (12). Therefore, it was of interest to determine whether this finding correlated with SAg properties of culture supernatant con-

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**FIG. 3.** The 15,891-bp SaPIbov. Black arrows indicate ORFs of unknown function. White arrows indicate genes referred to in the text: sec, sel, int, tst, and gmps. The hatched boxes indicate positions of the direct repeats (DR), the sequence of which is given. Open boxes represent the erythromycin (erm) and tetracycline (tet) cassettes used to construct the TSST and TSST SEC mutants. Primer junclf was used in Vectorette PCR to amplify DNA containing the SaPIbov integration site in strain RF120 after digestion with AluI.

**FIG. 4.** Southern blot hybridization analysis of tst and tst sec mutants. Genomic DNA from wild-type strain RF122 (lane 1), mutant RF122-tst (lane 2), and double mutant RF122-2-tst sec (lane 3) was cleaved with HindIII, separated by agarose electrophoresis, and transferred to a nylon membrane. It was hybridized with a probe specific for tst covering the site of insertion of the tet marker.
centrates derived from this isolate. Culture supernatant concentrates from wild-type RF122 stimulated expression of boVβ BTB13 but not boVβ BTB35 (Fig. 5), typical of strains producing SEC-bovine as reported by Deringer et al. (9). Also, the sec mutant RF122-2 failed to activate expression of boVβ TB13 RNA. Interestingly, wild-type RF122 strongly activated expression of three additional boVβs (BTB18, BTB27, and BTB93) not shown previously to be associated with SEC-bovine. The activity of the RF122 tst::Tcr supernatant implicated TSST-1 as being responsible for expansion of boVβ BTB93 but not the other boVβs. Expression of boVβ BTB18 and boVβ BTB27 remained elevated even when cells were stimulated with the supernatant from the tst and the tst sec double mutants of strain RF122. This indicated that strain RF122 expresses SAgs in addition to SEC-bovine and TSST-1. The residual SAg activity expressed by the RF122 tst and sec-bovine mutants suggested that the strains expressed SAgs other than SEC-bovine and TSST-1. The other expanded boVβs are only distantly related to those activated by SEC-bovine or TSST-1 and represent more divergent Vβ subgroups. For example, boVβ BTB18 and boVβ BTB27 TCRs are most related to human Vβs 1 and 7, which are classified in subgroups 1 and 2, respectively (9). Since expansion of neither of these could be attributed to either SEC-bovine or TSST-1, this activity was presumed to be caused by other SAgs. Molecular characterization of the pathogenicity island encoding the tst and sec-bovine genes revealed the presence of a novel enterotoxin-like gene (sel), the product of which is 55% identical to SEI. PCR analysis of RF122 genomic DNA revealed the presence of the recently identified seg and set genes. We have shown that the recombinant SEL protein is expressed in S.
The present study reveals that the sec-bovine gene is associated with a pathogenicity island. This is the first study to characterize the genetic element encoding the sec gene. It is likely that the other sec variants may lie on related mobile genetic elements. Indeed, it will be interesting to ascertain the diversity of the elements encoding superantigens in S. aureus in general. In the near future, it is likely that a plethora of such elements will be characterized. The presence of superantigen genes on mobile elements facilitates their horizontal spread between strains of S. aureus. Moreover, the relatedness of the SaPIbov integrase to the integrase of S. pyogenes phage T270, which expresses SpeA, suggests that such elements may have crossed the genus barrier at some time.

Although not essential for virulence, SAGs may play an important role in host immune response evasion and survival. Overall, it is clear from this study that the presence of SaPIbov in strain RF122 enables it to produce SAGs which specifically activate bovine lymphocyte populations. Thus, through modulation of the immune response it may confer a survival advantage in the bovine host.

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