Molecular Characterization of a Novel *Staphylococcus aureus* Serine Protease Operon

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The present study identified and characterized a unique operon (*spl*) encoding six serine protease-like proteins. In addition, native Spl proteins were isolated and characterized. Typical of most exoproteins, the *spl* gene products contain putative 35- or 36-amino-acid signal peptides. The Spl proteins share 44 to 95% amino acid sequence identity with each other and 33 to 36% sequence identity with V8 protease. They also contain amino acids found in catalytic triads of enzymes in the trypsin-like serine protease family, and SplB and SplC were shown to degrade casein. The *spl* operon is transcribed on a 5.5-kb transcript, but several nonrandom degradation products of this transcript were also identified. Similar to other *S. aureus* exoprotein genes, the *spl* operon is maximally expressed during the transition into stationary phase and is positively controlled by the Agr virulence factor regulator. The Sar regulatory system did not affect *spl* operon expression by PCR analysis revealed the presence of the *spl* operon in 64% of the *S. aureus* isolates tested, although one operon-negative isolate was shown to contain at least two of the *spl* genes. Finally, intraperitoneal injection of an *spl* operon deletion mutant revealed no major differences in virulence compared to the parental strain.

*Staphylococcus aureus* exports a wide variety of exoenzymes, some of which are known virulence factors. Among these enzymes are a variety of proteases, such as metalloprotease, thiolprotease, and the serine proteases V8 and exfoliative toxin A (ETA) and ETB. The best characterized of these, V8 serine protease, was initially isolated from *S. aureus* strain V8 but has since been shown to be expressed by 67% of the *S. aureus* isolates tested (2, 11).

Like most exoproteins produced by *S. aureus*, the Agr regulatory system positively regulates extracellular protease expression in response to increasing cell density (7, 26). In contrast, the Sar regulatory system has a negative effect on protease expression. Recent studies indicate that pleiotropic alterations in exoprotein profiles caused by *sar* mutations are due to the derepression of staphylococcal proteases, which then leads to exoprotein degradation (7, 9). In addition, McGavin et al. (21) reported that V8 protease modifies the fibronectin-binding phenotype of *S. aureus*. Thus, secreted proteases could play an important role in the posttranslational regulation of *S. aureus* exoprotein activity, in addition to modifying host proteins to the benefit of the bacteria.

In this study, we identified a novel *S. aureus* operon (designated *spl*) that encodes six previously uncharacterized serine protease-like proteins. This operon is expressed during the transition to stationary phase and is positively regulated by Agr. In addition, two of the Spl proteins expressed by this operon were shown to exhibit proteolytic activity.

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**Materials and Methods**

*Bacterial strains and plasmids.* The bacterial strains and plasmids used in this study are listed in Table 1. *S. aureus* strains were routinely grown in tryptic soy broth (TSB; Difco Laboratories, Detroit, Mich.) containing erythromycin (5 μg/ml), kanamycin (50 μg/ml), or tetracycline (5 μg/ml) where indicated. *Escherichia coli* strains were grown in Luria-Bertani medium (Fisher Biotech) with ampicillin selection (50 μg/ml). All bacterial cultures were routinely incubated at 37°C and aerated by shaking at 250 rpm.

*DNA manipulations.* *E. coli* plasmid DNA was extracted using the Wizard Plus DNA Purification System (Promega, Madison, Wis.). Restriction endonucleases and T4 DNA ligase were purchased from Gibco-BRL (Gaithersburg, Md.) and used as recommended by the manufacturer. Transformations of *E. coli* strain DH5α and *S. aureus* RN4220 were performed using the procedures described by Inoue et al. (17) and Kraemer and Iandolo (19), respectively. *S. aureus* chromosomal DNA was isolated using the method of Dyer and Iandolo (13), and plasmid DNA was isolated using the Wizard Plus DNA Purification System, except that *S. aureus* cells were converted to protoplasts using lysostaphin (50 μg/ml). Bacteriophage transductions in *S. aureus* were performed as described by Shafer and Iandolo (32) as well as by Sauber and Iandolo (31) in the transducing phase.

*Identification of the spl operon by database analysis.* Several proteins with similar physiochemical properties were purified from *S. aureus* cultures. N-terminal sequence data was used in a BLAST search to screen the *S. aureus* 8325p (the parent strain of RN6390) sequencing database provided by the *Staphylococcus aureus* Genome Sequencing Project (University of Oklahoma Health Sciences Center). The N-terminal sequences used originally matched open reading frames found on two separate contigs. One contig contained the *splA, splB,* and *splC* genes, while the other contig contained part of *splD* and all of *splE* and *splF*. Since a search of the *S. aureus* COL genome database (The Institute for Genome Research) indicated that these six genes make up a single operon, the sequence between the two 8325p contigs was PCR amplified and sequenced. The nucleotide sequence of the entire *spl* operon from 8325p was entered into the GenBank database (accession no. AF271715). The nucleotide sequences of the *splB* and *splC* genes were previously identified and included in the GenBank database as *orf1* and *orf2* (accession numbers U60589 and U63529, respectively).

*Purification and N-terminal sequencing.* *S. aureus* strain RN6390 was grown with shaking at 37°C in 5 liters of Todd-Hewitt broth (Difco Laboratories) to late stationary phase. The culture was precipitated in 4 volumes of ethanol for 1 week at 4°C. The precipitate was collected by centrifugation (13,000 × *g* for 15 min at 4°C) and air dried. Proteins in the pellet were dissolved in 50 ml of water and clarified by centrifugation at 40,000 × *g* at 4°C for 30 min. After dialysis against multiple changes of water (4°C), the volume of the dialysate was reduced to less...
than 100 ml by pervaporation. The dialyze was then subjected to flatbed isoelectric focusing (IEF) in a gradient of ampholytes (pH 3 to 10) in Sephadex (Amersham Pharmacia Biotech, Piscataway, N.J.). The IEF gel was divided into 19 fractions. Proteins were eluted from each fraction and then dialyzed exhaustively at 4°C against multiple changes of water. Proteins in each fraction were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and visualized by staining with Coomassie blue. The SplA, SplB, and SplC triplet was further purified using a fast protein liquid chromatography (FPLC) sizing column (Superose 12 HR 10/30; Amersham Pharmacia Biotech). The purified Spl proteins were then separated on an SDS-PAGE gel containing 0.1% (without mercaptooethanol) and denatured for 15 min at room temperature. Protein sequences was ligated into pCL52.2, generating pSR3. This plasmid was transformed into RN6390 chromosomal to allow a second recombinant event to occur, a single colony was inoculated into antibiotic-free TSB medium containing tetracycline to select for cells in which the plasmid had been integrated into the chromosome by homologous recombination. To allow a second recombinant event to occur, a single colony was inoculated into antibiotic-free TSB medium containing tetracycline to select for cells in which the plasmid had been integrated into the chromosome by homologous recombination. To allow a second recombinant event to occur, a single colony was inoculated into antibiotic-free TSB medium containing tetracycline to select for cells in which the plasmid had been integrated into the chromosome by homologous recombination. To allow a second recombinant event to occur, a single colony was inoculated into antibiotic-free TSB medium containing tetracycline to select for cells in which the plasmid had been integrated into the chromosome by homologous recombination. To allow a second recombinant event to occur, a single colony was inoculated into antibiotic-free TSB medium containing tetracycline to select for cells in which the plasmid had been integrated into the chromosome by homologous recombination.

### TABLE 1. Strains and plasmids used in this study

| Strain or plasmid | Relevant phenotype | Reference or source |
|-------------------|--------------------|---------------------|
| **S. aureus**     |                    |                     |
| RN6390            | Wild-type laboratory strain | 23                  |
| RN4220            | Highly transformable strain | 23                  |
| RN6911            | agr sar RN6390 strain | 18                  |
| ALC135            | agr sar RN6390 strain | 8                   |
| ALC136            | arr RN6390 strain | Ambrose Cheung       |
| KB600             | RN6390 spl operon null mutant | This study          |
| KB601             | RN6390 splB mutant | This study           |
| Brittingham       | TSS iso isolate | Amy Bryant           |
| Isracl #1         | TSS isolate | Amy Bryant           |
| Isracl #2         | TSS isolate | Amy Bryant           |
| Newman            | Produces type 5 capsule | Chia Y. Lee         |
| M                 | Produces type 1 capsule | Chia Y. Lee         |
| Wright            | Produces type 8 capsule | Chia Y. Lee         |
| Becker            | Produces type 8 capsule | Chia Y. Lee         |
| Col               | MRSA α | John J. Iandolo      |
| DU4916            | MRSA | John J. Iandolo      |
| Novel             | Bovine mastitis isolate | 33                  |
| 305               | Bovine mastitis isolate | 22                  |
| **E. coli DH5α**  | Highly transformable strain | Bethesda Research Laboratories |
| **Plasmids**      |                    |                     |
| pCL52.2           | Temperature-sensitive cloning vector | 30                  |
| pER924            | Temperature-sensitive cloning vector | 5                   |
| pRN5548           | High-copy gram-positive plasmid | 24                  |
| pDG647            | Source of Em<sup>+</sup> cassette | 16                  |
| pSR7              | pRN5548 derivative used in expression of spl<sub>ABCD</sub> | This study          |

<sup>a</sup> TSS, toxic shock syndrome.  
<sup>b</sup> MRSA, methicillin-resistant S. aureus.
The S. aureus spl operon

Strain was designated KB601. Spl blot analysis confirmed that chromosome via homologous recombination. PCR amplification and Southern blot analysis confirmed that splB had been disrupted (data not shown). This strain was designated KB601.

spl operon expression construct. For use in complementation experiments, the spl operon (excluding the promoter region) was PCR amplified using primers splA3 (5'-CATTAACTATAAAAAATACGTGGAGGAGG-3') and splD2 (5'-GTATATTTTGTCAGGATCGTGAATGTCAGAAG-3') and then cloned into the BamHI and HindIII sites (underlined sequences) of pBluescript II KS. This 5.0-kb DNA fragment was then liberated by digestion with BamHI and SaII, ligated to the corresponding sites within pRN5548, and transformed into S. aureus RN4242. Plasmid DNA from one positive clone was then transferred to S. aureus RN6390. Plasmid DNA from one positive clone was then transferred to various S. aureus strains by electroporation. This plasmid construct, designated pSR7, placed the expression of the operon expression construct.

Northern blot analysis. To examine growth phase-dependent expression, cultures were diluted 1:100 in TSB and grown with aeration at 37°C. At 4, 6, 8, 10, and 12 h after inoculation, 10-μl culture aliquots were mixed with an equal volume of ethanol-acetone solution (1:1, vol/vol) and the cells were stored at −20°C. After sampling was complete, the cell suspensions were centrifuged at 6,000 × g (4°C) for 5 min and the pellets were resuspended in 10 ml of TEN buffer (29). The cells were centrifuged again, and the pellets were resuspended in 1 ml of TEN buffer (containing 2.5 M NaCl). The cells were converted to protoplasts by incubation (37°C, 25 min) in the presence of recombinant lysozymin containing erythromycin, and incubated at 30°C overnight. The plasmid was then transferred into RN6390 by phase-mediated transduction. This strain was grown at the nonpermissive temperature (43°C) in the presence of erythromycin to select for cells in which the plasmid had been integrated into the RN6390 chromosome via homologous recombination. PCR amplification and Southern blot analysis confirmed that splB had been disrupted (data not shown). This strain was designated KB601.

Spl protease like. Unlike V8 protease, the Spl proteins appear to be synthesized using FPLC fraction containing purified SplA, SplB, and SplF separated in the presence of 2.0 M urea to enhance the resolution of these proteins; 5, purified SplC; 6, purified SplB. The molecular size standards used were the BenchMark Protein Ladder (Gibco-BRL). (B) Zymographic analysis of purified SplC (lane 2) and SplB (lane 3) using casein as the substrate. The molecular size markers used (lane 1) were the BenchMark Prestained Protein Ladder (Gibco-BRL).

RESULTS

Purification and physicochemical characterization of novel exoproteins. Fractionation of proteins in S. aureus RN6390 culture supernatants revealed a comigrating group of three extremely basic and similarly sized proteins (SplA, SplB, and SplF) ranging from 26 to 28 kDa (Fig. 1A). Upon preparative IEF, these proteins migrated to the same position in the pH gradients representing pIs of approximately 9.0. Further purification using FPLC resulted in an apparently pure protein preparation containing only these three Spl proteins (Fig. 1A, lane 4). To identify the proteins, they were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and subjected to N-terminal sequencing. The results of this analysis revealed that the N-terminal sequences of the proteins from the upper, middle, and lower bands were ENNYTK VKDTNIFPYTGVVAF, EKKNVEITDATKEPY, and ENT VKQITTNVAPYSY, respectively.

Identification of spl genes in the S. aureus database. Comparison of the N-terminal sequences of the purified proteins to the S. aureus 8325 genome database indicated that these proteins were encoded by genes within a six-gene operon (Fig. 2). The N-terminal sequences of the proteins from the upper, middle, and lower bands (Fig. 1A, lane 4) were identical to the deduced amino acid sequences of the first, second, and sixth gene products, respectively. Interestingly, products of all six genes share 43.9 to 94.6% sequence identity with each other and, as a group, contain significant sequence similarity to known serine proteases (Fig. 3 and 4) such as S. aureus V8 protease and the epidermal lytic toxins. Notably, each gene product contains the conserved amino acids His-74, Asp-113, and Ser-189 (splI gene product numbering), which make up the classic catalytic triad of trypsin-like serine proteases (Fig. 3). Unlike V8 protease, the Spl proteins appear to be synthesized without propeptides. Because of their sequence similarities to serine proteases, the genes encoding these proteins were designated splA, splB, splC, splD, splE, and splF for serine protease like.

Comparison of the SplA, SplB, and SplF N-terminal sequences with the deduced amino acid sequences of the splA, splB, and splF gene products revealed matches starting 35 or 36...
residues downstream of the putative N-terminal methionines, respectively. The product of the splC gene was also detected in IEF gels, purified (Fig. 1A, lane 5), and shown to have an N-terminal sequence (EKNVTQVKDT) that was identical to the deduced amino acid sequence 37 residues downstream of the N-terminal methionine. These data indicate that at least four of the proteins encoded by this operon contain 35- or 36-residue-long signal peptides (Table 2) consistent with secretion in a Sec-dependent manner. The predicted mature protein products ranged in size from 21.9 to 22.4 kDa and, with the exception of the third gene product (SplC), are predicted to be basic proteins having isoelectric points of approximately 9.0 (Table 2). The predicted neutral isoelectric point of the third gene product is consistent with its absence in our initial purified protein preparation of SplA, SplB, and SplF (Fig. 1A, lane 4).

To determine whether the spl operon encodes protease activity, we tested purified preparations of SplB and SplC for the ability to degrade casein. As shown in Fig. 1B, a zymographic analysis using casein as the substrate revealed clear zones of hydrolysis corresponding to the purified proteins, indicating that SplB and SplC exhibit protease activity. Although the other Spl proteins were not tested, it is likely that they also

| SplA | SplB | SplC | SplD | SplE | SplF | V8 |
|------|------|------|------|------|------|----|
| 100  | 100  | 100  | 100  | 100  | 100  | 100 |
| 100  | 100  | 100  | 100  | 100  | 100  | 100 |
| 100  | 100  | 100  | 100  | 100  | 100  | 100 |
| 100  | 100  | 100  | 100  | 100  | 100  | 100 |

FIG. 3. Sequence alignment of SplA to F with staphylococcal V8 serine protease. High sequence conservation throughout the proteins is evident. Identical residues in all five sequences are boxed. The asterisks indicate residues comprising the catalytic triad.

FIG. 4. Sequence identities among the Spl proteins and V8 protease. Shown are the percentages of identical amino acids calculated using paired alignments of the proteins.
exhibit protease activity based on the high degree of sequence conservation exhibited by all of the spl gene products (including the catalytic triads).

**Northern analysis.** Since the expression of most staphylococcal exoprotein genes is activated during the transition into stationary phase and is dependent on the virulence factor regulator Agr, we examined the expression of the spl operon during various stages of growth. RNA was isolated from *S. aureus* RN6390 cultures in 2-h increments beginning in exponential phase (4 h) and extending to stationary phase (12 h). RNA was analyzed by dot blot hybridization using an splB-specific DNA probe. As shown in Fig. 5, maximal expression of splB-specific transcripts was detected at the 8-h time point, which corresponded to early stationary phase (unpublished results).

The six spl genes span a total of 4,916 nucleotides and are separated by 57- to 157-bp spacer regions. Immediately downstream of splF is an inverted repeat sequence that is a potential factor-independent transcription terminator. The absence of inverted repeat sequences downstream of any other spl gene suggests that the six spl genes are cotranscribed, with transcription terminating downstream of splF. As determined by Northern blot analysis (Fig. 6), several transcripts in RN6390 hybridized to splB-, splC-, and splF-specific probes, while only the largest (5.5 kb) of these hybridized to the splA-specific probe. The observation that the splF-specific probe hybridized to all of the transcripts suggests that all of these transcripts have the same 3’ terminus. Assuming that these transcripts terminate just downstream of the splF gene (near the putative transcription terminator), the start of transcription for the 5.5-kb transcript is estimated to be located approximately 400 bp upstream of the splA start codon. Experiments using any of the spl-specific sequences to probe RNA from KB601 (a strain that contained an integrated plasmid in the splB gene) did not detect hybridization to the 5.5-kb RNA. Instead, a 2.3-kb transcript, possibly representing a truncated version resulting from the plasmid integration in splB, was detected only with the splA- or splB-specific probes. Since any promoters downstream of splB would likely be unaffected by the integrated plasmid, these results suggest that the largest transcript spans the entire operon and that the smaller transcripts are nonrandom degradation products.

To examine the roles of Agr and Sar in spl expression, a Northern blot analysis was performed on RNA isolated from RN6911 (agr), ALC135 (agr sar), and ALC136 (sar) when the spl operon was maximally expressed in the parental strain (during the transition from exponential to stationary phase). As shown in Fig. 7, there was no hybridization to RN6911 (agr) and ALC136 (agr sar) RNA using an splB-specific probe (other spl-specific probes gave similar [unpublished] results), demonstrating regulation by Agr. Hybridization of each probe to ALC135 (sar) RNA occurred at levels equivalent to hybridization to RN6390.

**PCR analysis of clinical isolates.** To examine if the spl genes are present in other strains, 11 randomly selected *S. aureus* isolates (Table 1) were analyzed by PCR amplification using splA- and splD-specific primers. PCR amplification of DNA from these isolates using spl operon-specific primers successfully amplified a 2.8-kb DNA fragment (presumably containing splA, splB, splC, and part of splD) in seven (64%) of the isolates tested (data not shown). With the exception of the Brittingham strain, the results of the PCR survey were confirmed by Southern blot analysis. Although the Brittingham strain produced no DNA fragment in PCR amplification using primers specific to the spl operon, Southern blot analysis revealed the presence of

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**Table 2. Characteristics of mature Spl proteins**

| Protein | No. of amino acids | Molecular mass (kDa) | Deduced pI | No. of amino acids in signal sequence |
|---------|--------------------|----------------------|------------|-------------------------------------|
| SplA    | 200                | 21.9                 | 8.6        | 36                                  |
| SplB    | 204                | 22.4                 | 9.1        | 36                                  |
| SplC    | 203                | 22.4                 | 6.4        | 36                                  |
| SplD    | 203                | 22.0                 | 8.9        | 36                                  |
| SplE    | 202                | 22.0                 | 9.2        | 36                                  |
| SplF    | 203                | 21.9                 | 8.9        | 36                                  |

**FIG. 6.** Northern blot analysis of the spl operon. DNA probes specific for splA, splB, splC, and splF were hybridized with RNAs isolated from RN6390 (lanes 1) and KB601 (lanes 2). Transcript sizes were determined based on the migration of a 0.24 to 9.5-kb RNA ladder (Gibco-BRL).

**FIG. 5.** Temporal regulation of the spl operon. Dot blot analysis of total cellular RNA isolated from RN6390 at 4, 6, 8, 10, and 12 h postinoculation and probed with an splB-specific probe. The 8-h time point corresponds to the transition into stationary phase.
sequences that hybridized to splC- and splD/F-specific probes, but not to probes specific for splA and splB (data not shown).

**Virulence studies.** The role of virulence factors during the course of infection is most easily determined by generating mutant strains and then assessing their ability to cause disease in an animal model of infection. Accordingly, the entire spl operon was replaced in RN6390 with an Em' determinant (see Materials and Methods), resulting in strain KB600. SDS-PAGE analysis of the exoproteins produced by this strain confirmed that it failed to produce the Spl proteins (unpublished data). Furthermore, transformation of KB600 with the spl operon expression plasmid pSR7 resulted in restoration of spl expression. To assess the effects of the spl operon on staphylococcal pathogenesis, three dosages (10^7, 10^8, and 10^9 CFU) of RN6390, KB600, and KB600(pSR7) were intraperitoneally injected into 29-day-old rats. Examination of these animals revealed no differences in the 50% lethal dose or the time required to cause death. Furthermore, no differences in the lesions produced by these strains were observed. Thus, the spl operon plays no obvious role in virulence as determined by this animal infection model.

**DISCUSSION**

The results presented here demonstrate the presence of a novel *S. aureus* six-gene operon (designated spl) that encodes protease activity. Members of the serine protease family, including V8 protease and the ETs, contain a well-conserved active site, known as a catalytic triad (4, 10, 11). This motif is characterized by the presence of a highly reactive serine residue within hydrogen-bonding distance of an imidazole ring nitrogen of histidine and a transition state-stabilizing aspartate residue (6, 14). Similarly, the Spl proteins contain the catalytic triad and exhibit significant sequence similarity to V8 protease and the ETs. Although only the SplB and SplC proteins were purified to homogeneity and shown by zymographic analysis to exhibit protease activity (Fig. 1B), we speculate that all of the Spl proteins are proteases.

The splC gene product was identified previously using antisera from *S. aureus* endocarditis patients to screen an expression library for genes that are expressed in vivo (28). Analysis of the deduced amino acid sequence of this antigen (designated ORF2) also demonstrated significant sequence similarity to the serine proteases V8 and the ETs. Furthermore, the presence of a catalytic triad within ORF2 was also noted by these authors, leading them to speculate that ORF2 is a protease. The fact that patients’ sera reacted with ORF2 indicates that this protein is expressed in *S. aureus* endocarditis and thus may be an important virulence factor during the course of this infection. In an initial assessment of virulence, a murine intraperitoneal injection model was used in this study to compare the effects of *S. aureus* RN6390 and its isogenic spl mutant. Although we were unable to observe effects on virulence in this model, more extensive studies using animals more sensitive to *S. aureus* are ongoing.

It has been presumed that various staphylococcal proteases might function as virulence factors through a variety of mechanisms, including inactivation of antimicrobial peptides, cleavage of human immunoglobulin molecules, and allowing dissemination by tissue destruction (15, 31). V8 protease can cleave and inactivate the heavy chains of all human immunoglobulin classes in vitro (1, 23) and modify the fibronectin-binding phenotype of *S. aureus* (21). The degradation of adhesins in the postexponential growth phase could allow the bacteria to spread from initial sites of infection (21). Metalloprotease is required for cleavage of V8 protease from an inactive precursor to a mature protease (12), and the ability of metalloprotease to activate prothrombin in human plasma suggests a role in the disseminated intravascular coagulation that sometimes occurs after systemic infection (34). ETA and ETB, the etiological agents of staphylococcal scalded-skin syndrome, exhibit 25 to 30% similarity to V8 protease. Like V8 protease, the ETs belong to the serine protease family (10) but are dissimilar in their high substrate specificity (25). Epidermolytic activity is lost in mutant toxins with alterations in the catalytic triad region, suggesting that proteolytic activity is essential (27). Recently, Rago et al. (25) revealed that β-melanocyte-stimulating hormone is a substrate of ETA and that both ETA and ETB cleave α-melanocyte-stimulating hormone. These revelations provide important new insight into the mechanisms of the skin desquamation that is associated with these toxins. Whether the spl gene products have a specific substrate like the ETs or whether they are nonspecific proteases involved in tissue destruction is currently under investigation in our laboratories.

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