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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. PCR analysis revealed the presence of the *spl* operon in 64% of the *S. aureus* isolates tested, although one *spl* 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, thiol-protease, 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) using ϕ11 as the transducing phage.

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* 8325 (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 8325 contigs was PCR amplified and sequenced. The nucleotide sequence of the entire *spl* operon from 8325 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

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* RN6390 strain	18
ALC135	*agr sar* RN6390 strain	8
ALC136	*sar* RN6390 strain	Ambrose Cheung
KB600	RN6390 *spl* operon null mutant	This study
KB601	RN6390 *splB* mutant	This study
Brittingham	TSS[a] isolate	Amy Bryant
Israel #1	TSS isolate	Amy Bryant
Israel #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[b]	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^r cassette	16
pSR7	pRN5548 derivative used in expression of *splABCD*	This study

[a] TSS, toxic shock syndrome.
[b] MRSA, methicillin-resistant *S. aureus*.

than 100 ml by pervaporation. The dialysate 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 SplF 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 2.0 M urea and transferred to a polyvinylidene difluoride membrane (Millipore Corporation, Bedford, Mass.) according to the method of Matsudaira (20). SplB and SplC (from a separate IEF fraction) were further purified by reverse-phase high-pressure liquid chromatography (HPLC) using a Hewlett-Packard HP1090 (Hewlett-Packard, Waldbronn, Germany) apparatus equipped with a 15-cm C_{18} column (VYDAC, Hesperia, Calif.). Protein was loaded onto the column in 0.1% trifluoroacetic acid and eluted in a gradient of 0 to 60% acetonitrile in 0.1% trifluoroacetic acid with a flow rate of 1 ml/min. The N-terminal amino acid sequences of the purified Spl proteins were determined by Edman degradation (Laboratory for Bioanalysis and Biotechnology, Pullman, Wash.).

Zymographic analysis. *S. aureus* cultures were grown in 10 ml of TSB to late stationary phase. Cultures then were centrifuged at 6,000 × *g* for 5 min (4°C), and the supernatant was filter sterilized through a 0.2-μm-pore-size filter (Pall Corporation, Ann Arbor, Mich.). The exoproteins were concentrated by passage through a Centricon 3 centrifugal filter device (Millipore) at 4°C by following the manufacturer's instructions. Protease activity associated with Spl proteins was detected using zymographic analysis essentially as described by Arvidson et al. (2). A 52.5-μl volume of each sample was mixed with 17.5 μl of 4× sample buffer (without β-mercaptoethanol) and denatured for 15 min at room temperature. The samples were loaded on an SDS–12% polyacrylamide gel containing 0.1% β-casein (Sigma, St. Louis, Mo.), and electrophoresis was performed with a constant voltage of 120 V using buffer precooled to 4°C. Renaturation of proteases was obtained by incubating the gels in 2.5% (vol/vol) Triton X-100 for 30 min with gentle agitation at room temperature. Gels were then placed in development buffer (50 mM Tris, 200 mM NaCl, 5 mM $CaCl_2$, 0.02% Brij 35) for 10 to 12 h with gentle agitation at 37°C and stained with 0.5% Coomassie blue. Zones of hydrolysis were visualized after destaining in a 40% methanol–10% acetic acid solution.

***spl* operon mutagenesis.** The *S. aureus spl* operon was deleted in strain RN6390 by allele replacement as follows. Initially, a 480-bp DNA fragment spanning a region 3′ to *splF* was PCR amplified using *Taq* DNA polymerase (GIBCO-BRL) and the splD3 and splD4 primers (5′-GGTAATAAGCCATCAGGTGAAAGC TCTAGAGG-3′ and 5′-GCGAACGTTGTTCTGCAGTAATAGAC-3′, respectively; see Fig. 2). The DNA product was then digested with *Xba*I and *Pst*I (underlined sequences) and ligated into the *Xba*I and *Pst*I sites of pDG647 downstream of the plasmid-encoded erythromycin resistance (Em^r) cassette. This recombinant plasmid was designated pSR1.

Similarly, a 450-bp DNA fragment spanning a region 5′ to *splA* was PCR amplified using the primers splA1 and splA2 (5′-GTTTCATTGATGAATTCA TATTGGC-3′ and 5′-GTTAAAATAGTTAAAGCAGTGGTACCTTTAACC-3′, respectively; see Fig. 2). This fragment was then digested with *Eco*RI and *Kpn*I (underlined sequences) and ligated into the *Eco*RI and *Kpn*I restriction sites of the plasmid pSR1 upstream of the (Em^r) cassette. This recombinant plasmid, designated pSR2, was then digested with *Pst*I and *Eco*RI, and the resulting fragment containing the Em^r cassette and the two flanking *spl* sequences was ligated into pCL52.2, generating pSR3. This plasmid was transformed into *S. aureus* RN4220 and subsequently transferred into RN6390 by bacteriophage-mediated transduction. This strain was grown at the nonpermissive temperature (43°C) on Trypticase soy agar (TSA) plates containing tetracycline to select for cells in which the plasmid had been integrated into the RN6390 chromosome by homologous recombination. To allow a second recombination event to occur, a single colony was inoculated into antibiotic-free TSB and grown at 30°C for 5 days with 1:1,000 dilution into fresh medium each day. After day 5, the bacteria were transferred to TSA containing erythromycin and isolated colonies were screened for the loss of tetracycline resistance. PCR amplification and Southern blot analysis (data not shown) confirmed the replacement of the *spl* operon. This *spl* mutant strain was designated KB600.

A specific disruption of *splB* was achieved by PCR amplifying a 320-bp DNA fragment internal to *splB* using the primers splB1 and splB2 (5′-GATACTAAT ATTTTTCCATATACTGG-3′ and 5′-CGCTCACAAGCTTTAGCCCCTGCC

GC-3′, respectively). This amplified fragment was ligated into the *Eco*RV site of pBluescript II KS (Stratagene, La Jolla, Calif.) and subsequently ligated into the *Eco*RI/*Kpn*I sites of pER924 (Table 1). This recombinant plasmid was then electroporated into *S. aureus* strain RN4220, which was spread onto TSA containing erythromycin, and incubated at 30°C overnight. The plasmid was then transferred into RN6390 by phage-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* operon expression construct.** For use in complementation experiments, the *spl* operon (excluding the promoter region) was PCR amplified using primers splA3 (5′-CATTAACTATAAAAATAAGCTTGGAAGGAGG-3′) and splD2 (5′-GTATATTTTGTCAGGATCCGGTGAATGTCTAAG-3′) and then cloned into the *Bam*HI and *Hin*dIII sites (underlined sequences) of pBluescript II KS. This 5.0-kb DNA fragment was then liberated by digestion with *Bam*HI and *Sal*I, ligated to the corresponding sites within pRN5548, and transformed into *S. aureus* RN4220. 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 *spl* operon under the control of the constitutively expressed β-lactamase promoter.

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-ml 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 lysostaphin (50 μg/ml). RNA was isolated using 4 ml of Trizol (GIBCO-BRL) in accordance with the manufacturer's instructions and resuspended in sterile, dimethylpyrocarbonate-treated water containing 0.5% SDS.

Twenty micrograms of RNA (determined by spectrophotometric analysis) from each *S. aureus* strain was incubated at 55°C in denaturing buffer (5% 10× morpholinepropanesulfonic acid [MOPS], 5.5% formaldehyde [37%, wt/vol], 50% formamide) for 15 min and resolved by electrophoresis through a 0.8% agarose gel containing formaldehyde (3). The RNA was transferred to a charged nylon membrane (MSI, Westboro, Mass.) using downward capillary action (29). For dot blot analysis, 20- and 40-μg portions of RNA were denatured as described above and applied to a charged nylon membrane using a dot blot manifold. Immobilized RNA was cross-linked twice on each side of the membrane using a UV Stratalinker 1800 (Stratagene). Next, prehybridization was carried out at 65°C for 1 h in 50 ml of hybridization buffer (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]; 0.02% SDS, 0.1% *N*-laurylsarcosine, 1% blocking stock [10% blocking reagent from Boehringer Mannheim, Indianapolis, Ind.] in maleic acid buffer). Digoxigenin (DIG)-labeled gene-specific DNA probes were generated using a PCR DIG Probe Synthesis Kit (Boehringer Mannheim) with primers specific for each *spl* gene. Hybridization with *spl*-specific probes (see above) was carried out at 65°C in hybridization buffer containing heat-denatured DIG-dUTP-labeled probe for 12 to 16 h. Membranes were washed twice (for 15 min each time) in 20× SSC–0.01% SDS and then twice (for 30 min each time) in 0.5× SSC–0.01% SDS. All washes were carried out at room temperature (with the exception of the second wash in 0.5× SSC–0.1% SDS, which was performed in a water bath at 68°C) with gentle agitation. The remainder of the detection procedure followed the protocol in the *DIG System User's Guide for Filter Hybridization*.

Virulence studies. In an initial study to examine if the *spl* operon contributes to virulence, 29-day-old male Sprague-Dawley rats were given intraperitoneal injections of *S. aureus* RN6390, KB600, and KB600(pSR7). The dosages used were 10^7, 10^8, and 10^9 CFU in a 500-μl volume. The overall health and viability of the rats were assessed over a 3-day period. After this time, the rats were sacrificed and their organs were examined by visual inspection and bacterial culturing.

RESULTS

Purification and physiochemical 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 ENNVTK VKDTNIFPYTGVVAF, EKNVKEITDATKEPY, and ENT VKQITNTNVAPYSG, respectively.

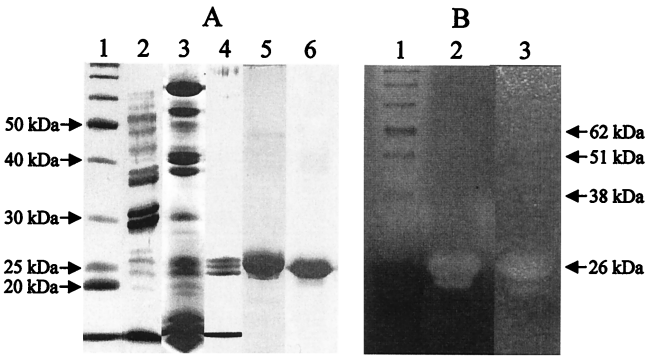

FIG. 1. Purification of the Spl proteins. (A) The Spl proteins from *S. aureus* RN6390 grown to stationary phase were fractionated and then separated by SDS–12% PAGE. Lanes: 1, 10-kDa molecular size markers (Gibco-BRL); 2, exoproteins from an RN6390 culture; 3, partially purified exoproteins after IEF fractionation; 4, 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).

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 epidermolytic toxins. Notably, each gene product contains the conserved amino acids His-74, Asp-113, and Ser-189 (*splA* 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

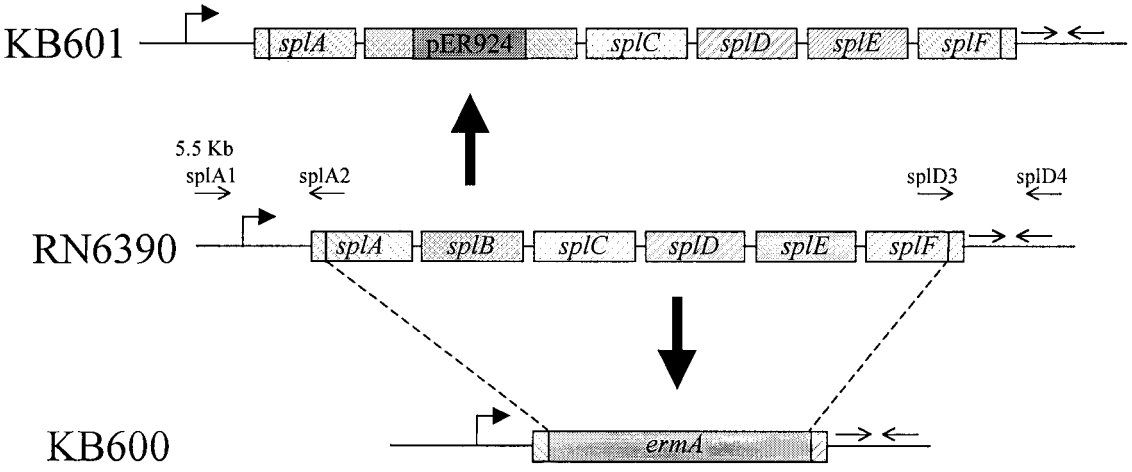

FIG. 2. Schematic representation of the *S. aureus spl* operon (middle) and its disruption by plasmid insertion (top) and allele replacement (bottom). The PCR primers (splA1, splA2, splD3, and splD4) used to generate DNA fragments for allele replacement are indicated. The arrows 5′ and 3′ to the *spl* operon represent the putative position of the transcription start site and a putative factor-independent transcription terminator, respectively.

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

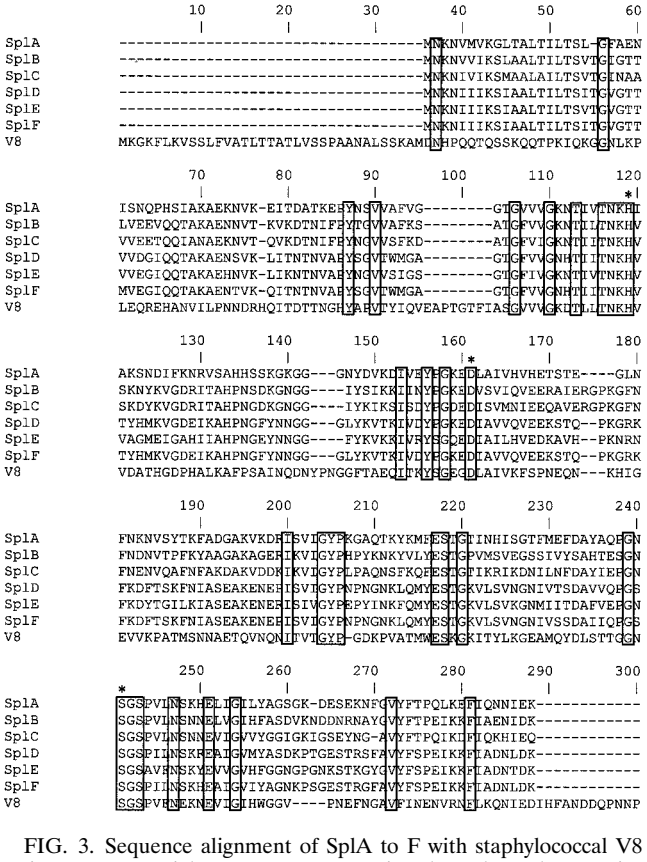

FIG. 3. Sequence alignment of SplA to F with staphylococcal V8 serine protease. High sequence conservation throughout the proteins is evident. Identical residues in all five sequences are boxed. The asterisks indicate residues comprising the catalytic triad.

	SplA	SplB	SplC	SplD	SplE	SplF	V8
SplA	100	47.7	50.0	43.9	43.9	44.8	33.3
SplB		100	62.9	54.4	56.2	53.9	30.4
SplC			100	49.8	50.4	51.0	30.6
SplD				100	67.4	94.6	32.7
SplE					100	67.8	36.4
SplF						100	32.5
V8							100

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.

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	35
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

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.

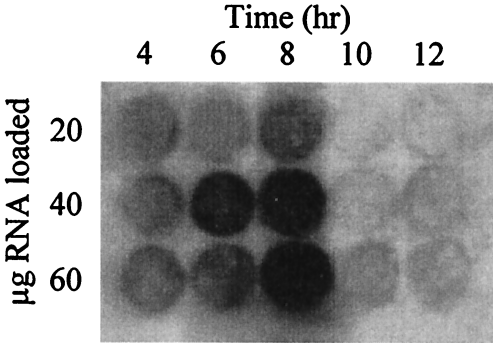

FIG. 5. Temporal regulation of the *spl* operon. Dot blot analysis of total cellular RNA isolated from RN6390 at 4, 6, 8, 10, and 12 h postinoculation and probed with an *splB*-specific probe. The 8-h time point corresponds to the transition into stationary phase.

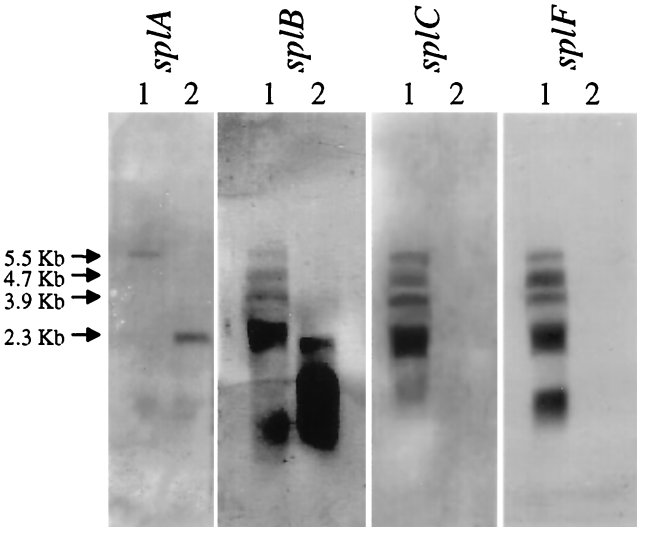

FIG. 6. Northern blot analysis of the *spl* operon. DNA probes specific for *splA*, *splB*, *splC*, and *splF* were hybridized with RNAs isolated from RN6390 (lanes 1) and KB601 (lanes 2). Transcript sizes were determined based on the migration of a 0.24 to 9.5-kb RNA ladder (Gibco-BRL).

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

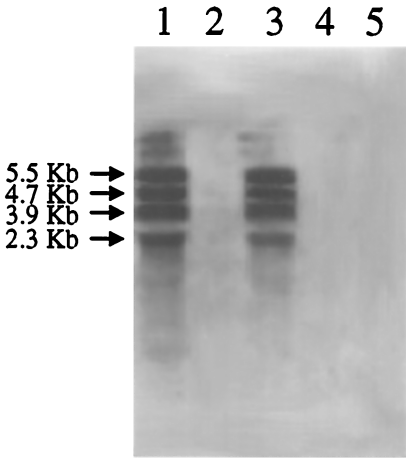

FIG. 7. Transcriptional regulation of the *spl* operon. A DNA probe specific for *splB* was hybridized with RNAs isolated from RN6390 (lane 1), KB600 (lane 2), ALC136 (lane 3), ALC135 (lane 4), and RN6911 (lane 5). Transcript sizes were determined based on the migration of a 0.24 to 9.5-kb RNA ladder (Gibco-BRL).

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^r 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 immunoglobin molecules, and allowing dissemination by tissue destruction (15, 31). V8 protease can cleave and inactivate the heavy chains of all human immunoglobin 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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