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Synergistic Hemolytic Activity of *Staphylococcus lugdunensis* Is Mediated by Three Peptides Encoded by a Non-*agr* Genetic Locus

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Some strains of the coagulase-negative *Staphylococcus lugdunensis* produce a synergistic hemolytic activity (SLUSH), phenotypically similar to the delta-hemolysin of *S. aureus*. Reverse-phase high-pressure liquid chromatography of supernatants from *S. lugdunensis* 307 yielded three late-eluting peaks of 3.5 kDa with synergistic hemolytic activity. A degenerate oligonucleotide probe was designed from partial amino acid sequences of the 23-amino-acid (aa) tryptic fragments from one of the three peaks and hybridized to a single 2.8-kb *Hin*dIII chromosomal fragment. The relevant portion of this fragment was cloned by PCR, and sequencing showed the presence of three related open reading frames (ORFs), SLUSH-A, SLUSH-B, and SLUSH-C, preceded by an unrelated short potentially coding sequence (ORF-X), cotranscribed on a polycistronic 838-nucleotide mRNA. The amino acid sequences of the peptides from the three peaks align perfectly with the predicted sequences from the three SLUSH ORFs (peak I = SLUSH-B; peak II = SLUSH-C; peak III = SLUSH-A). These three peptides are closely related (amino acid homology, >76%) and do not show significant homology to *S. aureus* delta-hemolysin but do resemble a *Salmonella typhimurium* invasin and the "gonococcal growth inhibitor," a bacteriocin secreted by *Staphylococcus haemolyticus*. The predicted ORF-X gene product is a 24-aa peptide with no homology to the SLUSH peptides.

Staphylococcus lugdunensis is a coagulase-negative staphylococcus (4), which may cause soft tissue abscesses (18, 25) and endocarditis (24). In contrast to *S. aureus*, this species produces only few known potential virulence factors: 25% of clinical isolates produce extracellular slime or glycocalyx (3, 6), 65% produce a fibrinogen affinity factor (3), and 95% secrete a hemolysin with phenotypic properties similar to the *S. aureus* 26-amino-acid (aa) delta-hemolysin (6). It acts synergistically with beta-hemolysin, i.e., by producing a zone of complete hemolysis within the zone of incomplete hemolysis produced by a beta-hemolysin-producing strain, and is thermostable (27). In *S. aureus*, the delta-hemolysin gene (*hld*) is encoded within the accessory gene regulator RNAIII (*agr*-RNAIII), which governs most exoprotein expression (16, 19). Delta-hemolysin does not, however, mediate *agr* regulation of the target genes; untranslated RNAIII is the effector (12, 17). A locus in *S. lugdunensis* homologous to *agr* (termed *agr-sl*) has been described (26) but encodes no potential peptide similar to delta-hemolysin, suggesting that the hemolytic activity is encoded outside *agr-sl*. In this paper, we report the identification and sequencing of three novel 43-amino-acid peptides with synergistic hemolytic activity secreted by *S. lugdunensis* and the corresponding genetic locus. The three peptides are clearly distinct from *S. aureus* delta-hemolysin but show some homology to previously described invasins and bacteriocins.

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MATERIALS AND METHODS

Strains and culture conditions. *S. lugdunensis* 307, also designated RN8160 (27), a producer of synergistic hemolysin, and *S. lugdunensis* N860172, also designated RN8159 (27), a nonproducer of synergistic hemolysin, were provided by the Centre National de Référence des Staphylocoques (Lyon, France). The bacteria were cultured in brain heart infusion at 37°C with agitation at 190 rpm, and growth was monitored spectrophotometrically. Hemolytic activity was assayed as described previously (7) by measuring the synergistic hemolysis with *S. aureus* RN4220 beta-hemolysin on Trypcase soy agar plates supplemented with 5% sheep blood (bioMérieux, Marcy l'Etoile, France).

Purification of hemolytic proteins. After removal of cells by centrifugation at 6,000 × *g* for 15 min, the supernatant from postexponential cultures was filtered (0.45-μm-pore-size Millex-HV; Millipore, Saint Quentin en Yvelines, France) and concentrated for 18 h at 4°C by dialysis against a 60% (wt/vol) solution of polyethylene glycol (Merck, Nogent/Marne, France) in 10 mM Tris-HCl buffer (pH 7). The proteins were separated by nondenaturing polyacrylamide gel electrophoresis (PAGE; see below). Hemolytic activity was detected by applying gel slices to a sheep blood agar plate (bioMérieux) for 2 h at 37°C. Proteins contained in acrylamide slices corresponding to hemolytic zones were eluted into 10 mM Tris-HCl buffer (pH 7.6) at 4°C for 18 h. The hemolytic proteins were then purified by reversed-phase high-pressure liquid chromatography (RP-HPLC) at room temperature on a model 625 LC system (Waters, Saint Quentin en Yvelines, France) with a C_8 Aquapore RP300 column (22 by 0.46 cm; Applied Biosystems, Foster City, Calif.). A 30-μl sample was applied to the column equilibrated with 0.05% trifluoroacetic acid (Pierce, Rockford, Ill.) in 5% acetonitrile (Merck, Darmstadt, Germany). Elution was performed by forming a linear gradient with solvent B (0.05% trifluoroacetic acid in 95% acetonitrile) at 1%/min at a flow rate of 1 ml/min. Peaks were detected by measuring the UV absorption at 214 nm. Fractions were collected and dried in a Speed-Vac concentrator (Savant, Farmingdale, N.Y.), and their hemolytic activity was tested by measuring synergistic hemolysis on blood agar plates as described above.

Trypsin digestion and N terminus sequencing. The purified hemolytic fractions were cleaved with L-(rosylamino-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin (Worthington, Freehold, N.J.) for 5 h at an enzyme/substrate ratio of 1:50 at 37°C. Peptide fragments were separated by RP-HPLC on a C_8 column as described above, and their amino acid sequences were determined by Edman degradation in a gas phase sequenator (470A; Applied Biosystems) on Polybrene-coated fiberglass supports. Phenylthiohydantoin amino acids were identified on line with a 120A PTH analyzer, using the 03RPTH program (Applied Biosystems).

PAGE. Nondenaturing PAGE in 7.5% polyacrylamide gels was performed with buffer lacking sodium dodecyl sulfate (SDS) and β-mercaptoethanol. The apparent molecular mass of the hemolytic proteins was determined by high-resolution Tricine-SDS-PAGE (22) with a 4% stacking gel, a 10% spacer gel, and a 16.5% separating gel for proteins in the range of 5 to 20 kDa. All migrations were performed in a mini-Protean II apparatus (Bio-Rad, Ivry/Seine, France). Low-molecular-weight marker proteins (Pharmacia Biotech, Orsay, France, and Promega, Charbonnieres, France) were used as standards.

Nucleic acid preparations. Plasmid and chromosomal DNAs were isolated from *S. lugdunensis* as previously described (1) and digested with restriction enzymes as specified by the supplier instructions (Boehringer Mannheim, Meylan, France). After electrophoresis on an agarose gel, the DNAs were vacuum transferred to Hybond-N membrane (Amersham, Les Ulis, France) and cross-linked by UV light. RNA was prepared from whole-cell extracts of early-stationary-phase cultures (14). RNA samples from 7.5×10^7 cells were separated for 3 h at 85 V of constant voltage on 1% agarose gels containing 2.3 M formaldehyde and then vacuum transferred to Hybond-N membrane. Complete transfer of nucleic acids was verified in each case by examining the membranes and gels under UV light.

Hybridizations. Bacterial nucleic acids were probed for *S. lugdunensis* synergistic hemolytic activity (SLUSH)-related sequences with two oligonucleotide probes designed from the central portion of tryptic peptide I-9 from RP-HPLC hemolytic peak I. HE3 (5′-GATAAAGATTGGGCHACWATGGGAAC-3′), in the same sense as the predicted mRNA, and HE4c (5′-GTTCCCATWGTDGC CCAATC-3′), in the complementary sense, were synthesized by Eurogentec (Seraing, Belgium). The oligonucleotides were 5′ end labelled with [γ-^{32}P]ATP by using bacteriophage T4 polynucleotide kinase (20) and hybridized under stringent conditions to DNA (hybridization at 50°C in 6× SSPE [1 × SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA, pH 7.7]–1× Denhardt's solution–0.1% [wt/vol] SDS and washing at 37°C in 1× SSPE–0.1% SDS) (23) or RNA (hybridization at 42°C in 1× SSPE–2× Denhardt's solution–2% SDS, washing at room temperature in 1× SSPE–0.5% SDS, then at room temperature in 0.2 × SSPE–1% SDS, and finally at 45°C in 0.1 × SSPE–0.5% SDS) (8). The membranes were then autoradiographed at −80°C with Hyperfilm-MP (Amersham).

PCR-assisted cloning of the *slush* locus. The bacterial DNA restriction fragment hybridizing to ^{32}P-labelled HE3 probe was inserted into pBluescript (Stratagene, Montigny-le-Bretonneux, France) (20), and a portion (1/20) of the ligation mixture was used as template in a PCR with either HE4c or HE3 and T3 oligonucleotides as primers. The resulting amplification product was cloned in *Escherichia coli* (TA cloning kit; Invitrogen, Leek, The Netherlands) and sequenced by the dideoxynucleotide method (21) with T3 and T7 promoter oligonucleotides as primers. The PCR product was also sequenced directly (Genome Express, Grenoble, France).

Mapping. The transcription start point of the *slush* operon was identified by primer extension with a synthetic primer named HE11RT (5′-CCTTTATTGT GTGCCTCTTACTATC-3′). RNAs were prepared as for Northern blotting, with the addition of chloroform-isoamyl alcohol purification and isopropanol precipitation. The primer was 5′ end labelled with [γ-^{32}]ATP. The reaction was carried out on 3 μg of RNA with a hybridization step at 65°C and a 45-min primer extension reaction at 42°C with 4 U of avian myeloblastosis virus reverse transcriptase (Promega) as described previously (15).

Protein secondary-structure prediction. Possible peptide configurations were evaluated by the self-optimized prediction method from the alignment package (http://www.ibcp.fr/predict.html) (5).

RESULTS

The exoproteins from *S. lugdunensis* 307 were screened by nondenaturing PAGE to identify the known hemolytic activity on erythrocytes from various species (27). Hemolytic activity was restricted to a product with an estimated molecular mass of 3.5 to 4 kDa by Tricine-SDS-PAGE (Fig. 1, lane B). On RP-HPLC, this product proved to be complex, with the elution profile shown in Fig. 2. Only proteins from three peaks (I, II, and III), which eluted at 80 to 85% acetonitrile, were shown to be hemolytic, while earlier-eluting fractions had no hemolytic activity. Each hemolytic peak contained a single peptide with a molecular mass of approximately 3.5 to 4 kDa by Tricine-SDS-PAGE (Fig. 1, lanes C to E).

We were initially unable to determine the N-terminal sequence of the purified protein from peak I; this suggested that the N-terminal amino acid was blocked, as in several other staphylococcal exoproteins, including *S. aureus* delta-hemolysin (2). Peptide fragments were generated from peaks I, II, and III by trypsin digestion, separated by RP-HPLC (data not shown), and sequenced. No sequence could be determined

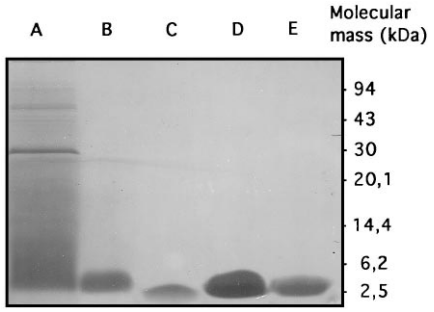

FIG. 1. Tricine-SDS-PAGE. Lanes: A, supernatant of *S. lugdunensis* 307 concentrated by dialysis with polyethylene glycol; B, crude extract obtained after elution of the hemolytic protein from the nondenaturing gel; C to E, RP-HPLC peaks I (C), II (D), and III (E).

from fragment I-8, suggesting that it was the blocked N-terminal portion, but fragments I-4, II-14, and III-4 yielded distinct but closely related decapeptides, while fragments I-9, II-13, and III-10 were composed of very homologous (>87%) 23-aa chains. Subsequently, fragment II-11 yielded a complete N-terminal sequence of the peptide from peak II (Fig. 3).

The amino acid sequences suggested the presence of several related genetic determinants for synergistic hemolytic activity in *S. lugdunensis*; therefore, oligonucleotide probes (HE3 and HE4c in the complementary sense) were designed from the central portion of fragment I-9 with low degeneracy. Probing of restriction digests of chromosomal DNA from the hemolysin-producing strain *S. lugdunensis* 307 or from the nonproducer, N860172, with these oligonucleotides labelled a single 2.8-kb *Hin*dIII fragment (Fig. 4). Further digestion with *Taq*I shortened the hybridizing fragment to 1.5 kb (data not shown), but the probes did not hybridize to DNA from the single 3.7-kb plasmid from *S. lugdunensis* 307 (Fig. 4). Since no viable hybridizing colonies were obtained after transformation of *E. coli* by the 1.5-kb *Hin*dIII/*Taq*I fragment in pBluescript, the putative locus was amplified directly from the pBluescript ligation mixture, as described above in Materials and Methods.

Using T3 and HE3 as primers, two 0.3- and 0.5-kb products were amplified from the *Hin*dIII/*Hin*dIII fragment, and the larger (CI2) was cloned by using the TA system and sequenced. Similarly, with T3 and HE4c as primers, a 0.9-kb fragment (C6) was cloned from the *Hin*dIII/*Taq*I digest and sequenced. To bridge the gap between these two clones, outward primers were designed from the sequences of clones I2 and 6 and used to amplify an 830-bp DNA fragment (PCR6c/10) by PCR from genomic DNA, which was sequenced directly.

The sequences of clone I2, clone 6, and PCR6c/10 (Fig. 3) overlap widely, and to ensure that the alignment was correct, PCRs were performed on genomic DNA with primers HE10 and HE4c, HE10 and HE5c, HE10 and HE6c, or HE11 and HE6c; in every case the amplification products had the expected size (results not shown). The concatenated sequence shows the presence of three major open reading frames (ORFs), each preceded, 9 nucleotides upstream of the start codon, by a putative ribosome-binding site (AGGAGT, AGG AGCG, and AGGAGCG) (Fig. 5). Each ORF codes for a very similar 43-aa peptide, and the predicted amino acid sequences align perfectly with the sequences of the three hemolytic peaks isolated by RP-HPLC from *S. lugdunensis* 307 culture supernatants (Fig. 3). We designated the three loci *S. lugdunensis* synergistic hemolysin (*slush*) A, B, and C, and their peptide products correspond to RP-HPLC peaks III, I, and II, respectively. Several algorithms predict a predominantly alpha-heli-

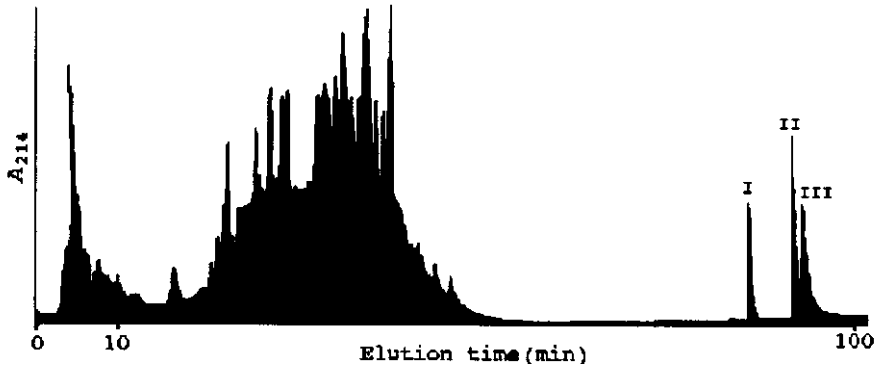

FIG. 2. Tracing of the RP-HPLC elution profile of *S. lugdunensis* 307 supernatant. A linear elution gradient runs from 5% (T = 10 min) to 95% (T = 100 min) acetonitrile in 0.05% aqueous trifluoroacetic acid. I, II, and III, peaks showing synergistic hemolytic activity. A_{214}, absorbance at 214 nm.

cal structure for all three proteins. Comparison of the amino acid sequences of SLUSH-A, SLUSH-B, and SLUSH-C with translated sequences from GenBank revealed >32% homology with aa 382 to 426 of the 68-kDa *Salmonella typhimurium* SipB invasin (9). Somewhat closer homology, from 39 to 48%, was observed with three highly homologous 44-aa proteins which constitute gonococcal growth inhibitor, a bacteriocin secreted by *Staphylococcus haemolyticus* (28) (Fig. 6).

Expression of the *slush* locus was investigated by Northern blotting of RNAs from the hemolytic 307 strain and the nonhemolytic N860172 strain of *S. lugdunensis* and probing with the end-labelled HE4c (antisense) or HE3 (sense) oligonucleotide (the positions of the oligonucleotide probes are shown in Fig. 5). Only strain 307 showed a single 0.8- to 0.9-kb messenger by hybridization with HE4c (Fig. 7), and neither strain hybridized to HE3 (results not shown). This RNA also hybridized to HE5c but not to HE6c (results not shown), suggesting that its 3′ end lies between nucleotides 1115 and 1241, possibly at a 17-nucleotide hyphenated inverted repeat (Fig. 5) which is a potential transcription termination signal. The 5′ end of the transcript was mapped by primer extension at nucleotide 299 (Fig. 8), 838 nucleotides upstream of the putative transcription terminator. The transcription start is preceded by unusual −10 (TTGAGA) and −35 (TACAAT) consensus sequences (Fig. 5). The DNA sequence between the 5′ end of the mRNA and *slush*-A contains an additional ORF (ORF-X) preceded by an AAGGAGG putative ribosome-binding site. The 24-residue translational product of ORF-X shows little homology to any protein in the Swiss-Prot data bank, but its N-terminal sequence contains a putative protein kinase C phosphorylation site, [ST]-X-[RK].

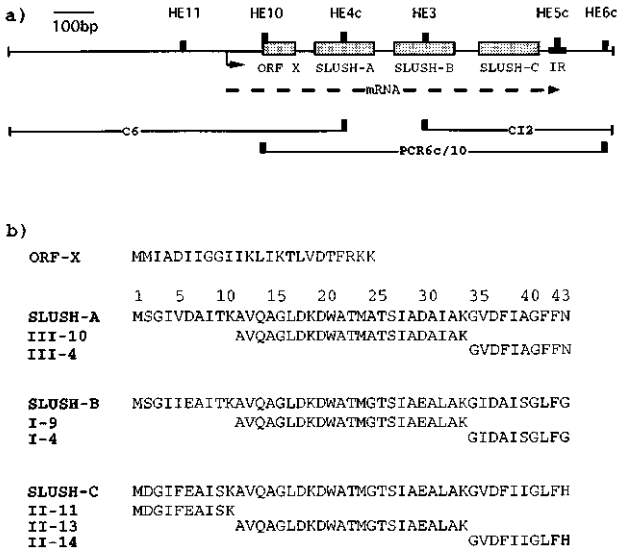

FIG. 3. Organization of the *Taq*I/*Hin*dIII chromosomal fragment containing the *slush* locus from *S. lugdunensis* 307. (a) Gene order and cloning strategy. ▮, primer sites; ⌊→, transcription start; ▬ (IR), inverted repeat. C6, PCR fragment from *Taq*I/*Hin*dIII ligation mixture with T3 and HE4c primers. CI2, PCR fragment from *Hin*dIII/*Hin*dIII ligation mixture with T3 and HE3 primers. PCR6c/10, PCR fragment obtained with two outward primers from CI2 and C6 on genomic DNA. (b) Amino acid composition of ORF-X and comparison of predicted products of *slush*-A, *slush*-B, and *slush*-C with amino acid sequences of tryptic peptides from RP-HPLC peaks III, I, and II, respectively.

DISCUSSION

The synergistic hemolytic activity of *S. lugdunensis* is clearly very distinct from that of *S. aureus*, despite their phenotypic similarity (27). While *S. aureus* delta-hemolysin is a single peptide encoded within the *agr*RNAIII locus (*hld*), *S. lugdunensis* SLUSH comprises three distinct but very similar 43-residue peptides expressed from a locus outside *agr-sl*. The mechanism of hemolysis by such peptides is not clear, and it

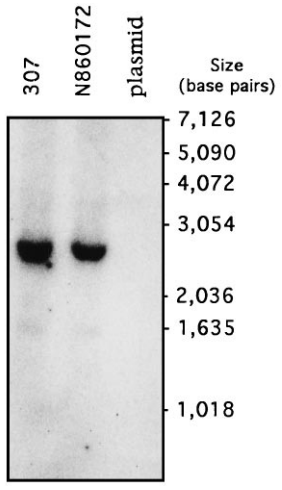

FIG. 4. Southern blot hybridization of *Hin*dIII chromosomal digest from *S. lugdunensis* 307 and *S. lugdunensis* N860172 and uncut plasmid from *S. lugdunensis* 307 with the [γ-^{32}]ATP-labelled oligonucleotide HE3 deduced from the amino acid sequence of peptide I-9 by RP-HPLC.

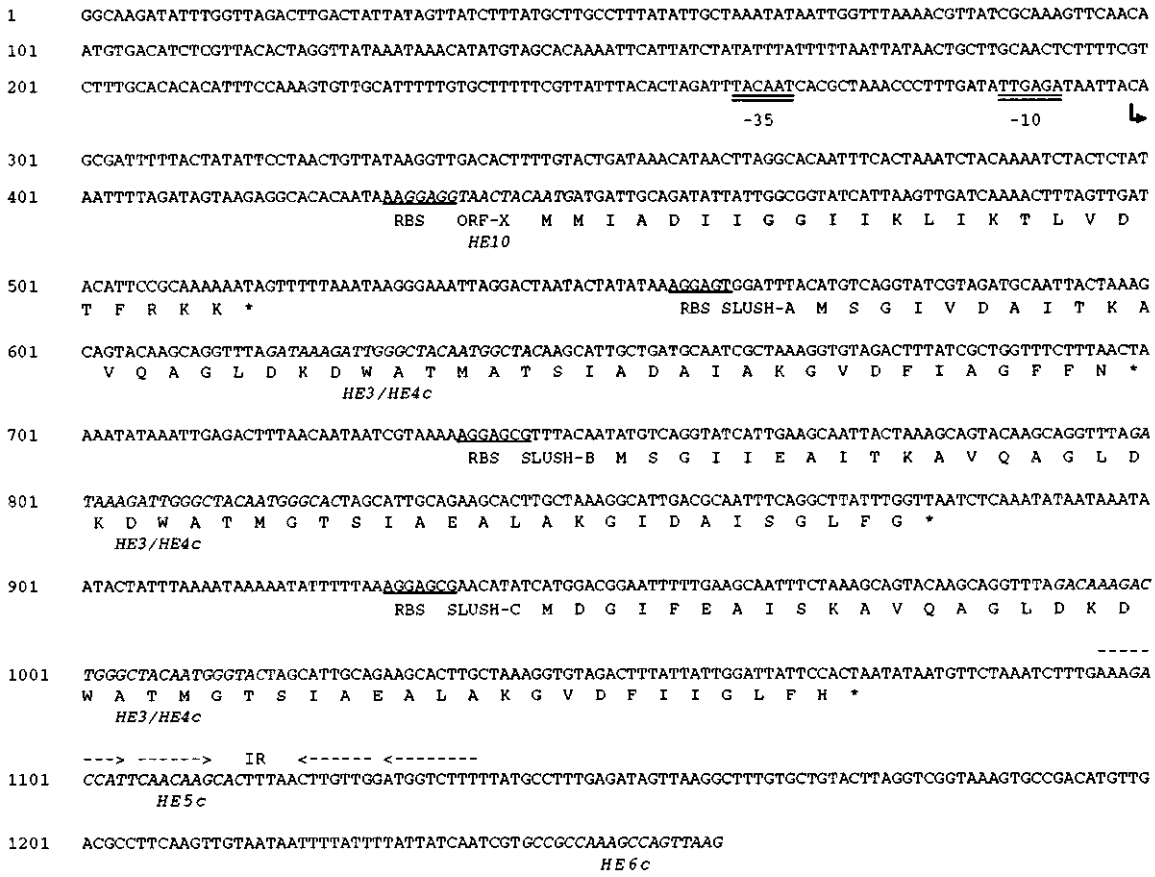

FIG. 5. Nucleotide sequence of the *slush* operon. The deduced amino acid sequences of SLUSH-A, SLUSH-B, SLUSH-C, and ORF-X are indicated below. RBS, putative ribosome-binding site; ═, −10 and −35 consensus sequences; ↳, start point for transcription; ---->, putative transcription end; *, stop codon. Primers for PCR or hybridization are shown in italics. GenBank accession number U73444 has been assigned to this sequence.

may be an accessory phenomenon accompanying other activities. Indeed, a similar exoprotein, made of three 44-aa peptides which inhibit gonococcal growth (28) and also have delta-hemolytic activity, is secreted by *S. haemolyticus*. The SLUSH peptides show 34 to 48% amino acid homology to these molecules, and preliminary experiments indicate that crude extracts from hemolytic, but not nonhemolytic, *S. lugdunensis* exert an inhibitory influence on other staphylococci. They do not appear to affect a wide range of gram-positive bacteria like many bacteriocins (11), perhaps reflecting competition between staphylococci for the same ecological niche. The amount of protein purified by RP-HPLC was insufficient to conduct trials of the synergism of the three SLUSH peptides on hemolysis or bacterial inhibition, and, while we have shown that the three peptides belong to the same operon, it is possible that they are not translationally coupled. Differential posttranscriptional regulation of the ORFs, perhaps by translational attenuation, could affect the final molar ratio of the three peptides.

The amino acid sequences of extracellular SLUSH peptides show that there is no removal of a signal peptide consequent on secretion, suggesting the involvement of an associated export system. SLUSH peptides share a 32% homology with a part of the central domain (aa 300 to 450) of SipB invasin protein from *Salmonella typhimurium*, which contains a potential membrane-spanning region (13) and which is significantly similar to the *Shigella* invasin IpaB and to YopB, a protein secreted by *Yersinia* spp. (13). These conserved regions may reflect parts of the proteins required for secretion and/or in-

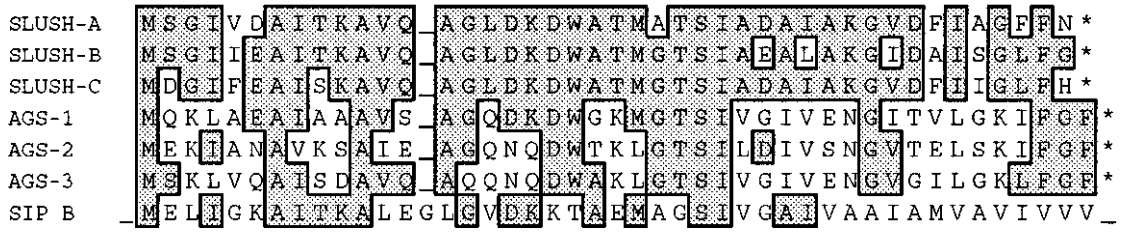

FIG. 6. Comparison of the amino acid sequences of SLUSH-A, SLUSH-B, SLUSH-C, the antigonococcal substances (AGS-1, AGS-2, and AGS-3) secreted by *S. haemolyticus*, and aa 382 to 426 of the SipB protein produced by *Salmonella typhimurium*; identical amino acids are boxed.

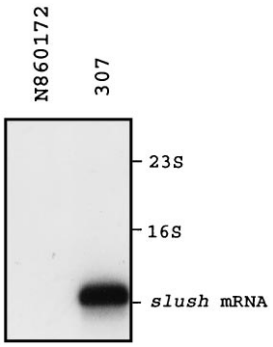

FIG. 7. Northern blot analysis of RNA samples from early-stationary-phase cultures of *S. lugdunensis* N860172 and 307. RNA samples were probed with antisense *slush*-specific oligonucleotide (HE4c) labelled with [γ-^{32}]ATP. 16S and 23S rRNAs were used as size markers.

vasion (10). If the SLUSH peptides are independently active, as suggested by the hemolytic activity of each RP-HPLC peak, some protection would be required against self-toxicity. Indeed, we were unable to clone the locus in *E. coli*, perhaps because of such activity. Any detoxifying "*trans* chaperone" activity is unlikely to reside in the ORF-X gene product, since this ORF was present in the construct introduced into *E. coli*; however, this peptide, if expressed, may have a regulatory or addressing function. The simplicity of the *slush* locus contrasts with the organization of bacteriocin loci in gram-positive bacteria, which usually constitute operon-like gene clusters that also harbor genes encoding proteins for immunity, processing, maturation, regulation, and translocation functions (11). The ABC transporter genes involved in their secretion are generally present on an adjacent locus, but we saw no additional mRNA from *S. lugdunensis* that could hybridize to C6, which extends for a considerable distance upstream of the *slush* locus.

The three coding regions for SLUSH-A, SLUSH-B, and SLUSH-C are unlikely to have arisen from a recent duplication event, since their 3′ and 5′ sequences show little homology and 10 conserved codons have accumulated synonymous changes in their third base (Fig. 5). The overall base composition of the *slush* locus shows a G+C content of 32%, compared with 37% for all *S. lugdunensis* sequences available, suggesting that the sequence has not arisen by transposition. The SLUSH system of *S. lugdunensis* might be a convenient model for studies of the conformation and interaction of bacteriocins and invasins with liposomes or cell membranes.

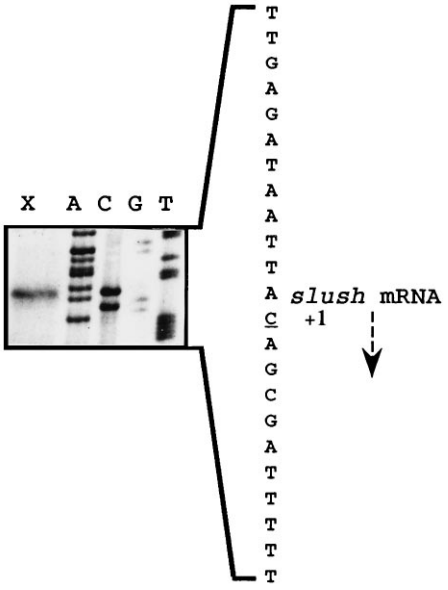

FIG. 8. Determination of the *slush* transcription start. Primer extension from *S. lugdunensis* 307 with the HE11RT primer is shown. Lanes: X, extension fragment; A, C, G, and T, sequencing reactions.

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