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Sequencing of Leucocidin R from *Staphylococcus aureus* P83 Suggests that Staphylococcal Leucocidins and Gamma-Hemolysin Are Members of a Single, Two-Component Family of Toxins

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A 2,813-bp *Hin*cII-*Cla*I DNA fragment encodes the two S and F components (LukS-R and LukF-R) of leucocidin R (Luk-R) which are secreted by *Staphylococcus aureus* P83. The two genes (*luk*S-R and *luk*F-R) belong to a single operon. Two peptidic sequences were deduced: LukS-R is a 35,721-Da polypeptide of 315 amino acids, including a signal sequence of 29 residues, and LukF-R is a 36,838-Da polypeptide of 325 amino acids, including a signal sequence of 25 residues. LukS-R and LukF-R were expressed in *Escherichia coli* and purified from the periplasmic space. Luk-R exerts biological activities on polymorphonuclear cells and on erythrocytes from various animals. Comparison of the amino acid sequence of LukF-R with that of the B component of gamma-hemolysin (HlgB), those of the F and S components of another recently sequenced staphylococcal leucocidin, and those of a few peptides of the F component from Panton-Valentine leucocidin suggests that all four toxins belong to a single, two-component family of toxins.

Leucocidins are toxins secreted by *Staphylococcus aureus*. They consist of two separate and synergistic components. As emphasized by Gladstone and Van Heyningen (4), leucocidins' specific targets are monocytes, macrophages, and polymorphonuclear cells (PMNs), but these toxins do not induce significant hemolysis with the erythrocytes that are usually tested (27). This characteristic allows us to distinguish between leucocidins and staphylococcal gamma hemolysin (6, 24), since the latter, also acting by means of two separate components, is hemolytic and leukotoxic.

Up to now, two types of leucocidins have been reported. Panton et al. (13) identified a toxin, known as Panton-Valentine leucocidin (Luk-PV), in a supernatant of *S. aureus* V8 which was isolated from a patient suffering from chronic furunculosis. The biological activity of this toxin was studied by Woodin (28, 29), who described the requirement of two exoproteins, F and S (32 and 38 kDa, respectively), which are separate but which nevertheless act synergistically upon human PMNs. The combination of these components (LukS-PV and LukF-PV) is not hemolytic with human erythrocytes. These results, except for the molecular masses of 38 and 32 kDa assigned to the F and S components, respectively, were recently confirmed by Finck-Barbançon et al. (2). The difference in masses was probably due to the purification procedures used.

Noda et al. (11, 12) described extensively a seemingly different leukotoxin also produced by strain V8 and which also acts by means of two components, F and S (31 and 32 kDa, respectively), whose molecular masses were different from those first described by Woodin (29). The activity of this second toxin on erythrocytes has never been clearly established. More recently, Rahman et al. (15, 16) reported the sequences of the S and F components of a leucocidin produced by a methicillin-resistant *S. aureus* (MRSA) strain whose biological properties were not documented. However, Finck-Barbançon et al. (2) reported that many *S. aureus* strains produced exoproteins showing partial identity to Luk-PV when immunoprecipitation was performed with affinity-purified Luk-PV antibodies. These results suggested that at least two kinds of leucocidin could be produced by *S. aureus* strains.

Another leucocidin (leucocidin R) produced by *S. aureus* P83 and isolated from an infected bovine udder was described by Soboll et al. (19, 20) and further studied by Loeffler et al. (9). It has been shown that this toxin, which has never been highly purified, nevertheless has a specificity toward bovine and human PMNs, the latter being less sensitive. The activity of this toxin on erythrocytes has not been assessed. By using affinity-purified Luk-PV antibodies, we observed that a culture supernatant of *S. aureus* P83 contained products not identical but antigenically related to LukS-PV and LukF-PV, as described earlier (2).

An oligonucleotide probe derived from the N-terminal sequence of LukF-PV from *S. aureus* ATCC 49775 (V8 strain) hybridized with the total DNA of strain P83. This probe allowed the purification of genes (*luk*S-R and *luk*F-R) encoding the so-called leucocidin R (Luk-R) on a 2,813-bp DNA fragment. We report here the sequence of these components and their expression in recombinant *Escherichia coli*. The two purified recombinant proteins showed immunoprecipitation lines identical to those obtained from a crude *S. aureus* P83 culture supernatant. They produced, when acting synergistically, leukotoxicity with PMNs from bovines and humans but also hemolysis with erythrocytes from various species.

MATERIALS AND METHODS

Bacterial strains. *S. aureus* P83 producing leucocidin R was a generous gift from N. L. Norcross (Cornell University, Ithaca, N.Y.). *S. aureus* V8 was kindly supplied by S. Thornley (Wellcome Laboratories, London, United Kingdom) and deposited in the American Type Culture Collection (ATCC 49775) because of its ability to produce Panton-Valentine leucocidin. *E. coli* NM 522 [*supE thi hsdR* Δ(*lac-*

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proAB) (F' *proAB lacI*^{q} ZM15)] was used as a host strain for recombinant plasmid pUC19. The *E. coli* K-12 strain TG1 [Δ(*lac-pro*) *supE thi hsdR* (F' *traD36 proAB lacI*^{q} ZM15)] was used as a host for the replication of the bacteriophages M13mp18 and M13mp19.

DNA screening and sequence determination. Restriction endonucleases and enzymes modifying DNA were used as recommended by the manufacturers (GIBCO-Bethesda Research Laboratories; Appligène, Strasbourg, France). For the cloning of *luk*F-R, *Cla*I DNA restriction fragments of total DNA from *S. aureus* P83 spanning from 1.5 to 2.5 kb were inserted into the *Acc*I-linearized plasmid pUC19. For the cloning of *luk*S-R, *Hin*cII-*Acc*I DNA restriction fragments of total DNA from the same strain ranging from 1.5 to 2.2 kb were inserted into the *Sma*I–*Acc*I-linearized pUC19. *E. coli* NM522 was transformed (10) by the constructed plasmids, and the recombinant *luk*F-R and *luk*S-R clones were screened by a previously described dot blot method (17) with a 5'-labeled degenerated oligonucleotide probe. This oligonucleotide probe (5'-GTAATTTTATC A/T AC C/T TT C/T TT C/T TC-3') was deduced from the underlined region of the N-terminal sequence of LukF-PV (AQHIT PVSEKKVDDKI) and was complementary to the mRNA. Hybridizations were performed overnight at 55°C in 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7])–5× Denhardt's solution (10)–0.1% (wt/vol) sodium dodecyl sulfate (SDS). Then, membranes were washed twice for 10 min at 45°C in 1× SSPE–0.1% (wt/vol) SDS before being fluorographed (−80°C) with 3M films (type RII). The nucleotide sequences of the cloned DNA fragments were established by the dideoxy method with T7 DNA polymerase (23). Both DNA strands were sequenced with oligonucleotide primers deduced from previous sequencing steps. Nucleotide sequences were examined and compared by using DNASTAR software (DNASTAR, Ltd., London, United Kingdom). Consensus sequence research was performed by using the Consensus program from University of Wisconsin Genetics Computer Group software.

Southern and Northern (RNA) blot analysis. Southern blot analysis was performed as described earlier (21). For the analysis of mRNAs, total RNA was prepared from *S. aureus* P83 as described by Kornblum et al. (7), with modifications. Briefly, a volume of culture medium containing 7.5×10^8 bacteria (late-exponential-phase growth) was centrifuged at 4,000 × *g* for 10 min at 4°C, and the pellet was washed once in 5 ml of TES buffer (20 mM Tris-HCl [pH 8.0], 10 mM EDTA-Na_2, 50 mM NaCl). Bacteria were lysed in 250 μl of TES buffer containing 20% (wt/vol) sucrose, 100 μg of lysostaphin (Sigma) per ml, and 40 U of RNase inhibitor (Appligène) for 30 min at 0°C. After two phenol acid extractions at 65°C, one phenol-chloroform-isoamylic alcohol (49:50:1) extraction, and one ethanol precipitation, DNA was removed by a 30-min digestion procedure at room temperature with 50 μl of DNase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$ [pH 7.5]) containing 40 U of RNase inhibitor and 1 U of DNase (RNase free) (Appligène). Total RNA was then ethanol precipitated, and separation of the RNAs by agarose gel electrophoresis was performed as previously described (25).

Protein analysis. Recombinant clones were grown overnight in 100 ml of 2× YT liquid medium (10) at 37°C. Bacteria were harvested after 5 min of centrifugation at 4,000 × *g* at 5°C, and protoplasts were obtained by suspending the bacteria for 60 min at 0°C in 3 ml of 20 mM Tris-HCl [pH 8.0]–25% (wt/vol) sucrose containing 2 mg of lysozyme per ml (Boehringer, Mannheim, Germany). Crude extracts were obtained by a 3-min sonication procedure at 0°C. Soluble proteins were separated from cell debris by 10 min of centrifugation at 5,500 × *g* at 4°C. The latter proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE), as described earlier (8), and then transferred (26) onto Immobilon P membranes (Millipore). The F and S components of leucocidins from Luk-R or Luk-PV were identified by immunoblotting with LukF-PV and LukS-PV affinity-purified antibodies, respectively.

Affinity-purified LukF-R and LukS-R were obtained from *S. aureus* P83 crude supernatant by using affinity-purified LukF-PV and LukS-PV antibodies, respectively. Recombinant LukF-R and LukS-R were purified from *E. coli* periplasm (5) by a procedure similar to one described earlier (2), except that the Carboxymethyl trisacryl-M (IBF, Villeneuve-la-Garenne France) chromatography step was replaced by Alkylsuperose (Pharmacia, Uppsala, Sweden) chromatography with an ammonium sulfate gradient in potassium phosphate buffer.

Biological assays. The leukotoxicity to PMNs was tested as previously described (2). Bovine PMNs were prepared from blood collected into citric acid-citrate-dextrose solution by the method of Roth and Kaeberle (18). Finally, bovine PMNs were suspended at 2×10^7 cells per ml of Hanks balanced saline solution without Ca^{2+} or Mg^{2+} (Flow Laboratories, Irwine, Scotland). The latter concentration allowed the adsorption of about 10^5 cells from 10 μl of the preparation onto a 7-mm-diameter well formed in a Teflon layer covering a glass slide.

Erythrocytes from humans or cattle were washed three times and suspended in phosphate-buffered saline (PBS) to a final concentration of 1.5% (vol/vol). The samples to be tested for hemolytic activity were serially diluted twofold in 150 μl of PBS in round-bottom 96-well microtitration plates. Fifty microliters of erythrocyte suspension was then added to the wells, and the plates were incubated first for 60 min at 37°C and then for 18 h at 4°C. After centrifugation, hemolysis was assessed visually.

Nucleotide sequence accession number. Leucocidin R sequences were recorded in the EMBL data base under accession number X64389.

RESULTS

Cloning of *luk*S-R and *luk*F-R and expression of gene products. A 2.2-kb *Cla*I DNA fragment from total DNA of *S. aureus* P83 was inserted into *Acc*I-cleaved pUC19 (Fig. 1B). The crude extract of an *E. coli* recombinant clone hybridizing with the degenerated probe was examined by Western blotting (immunoblotting) with affinity-purified Luk-PV antibodies. It was also examined by determining its biological activities upon human PMNs after being combined with the F or S component from Luk-PV. This recombinant clone produced a protein antigenically related to the F component of Luk-PV, as observed by immunoprecipitation and Western blotting. The crude extract of this recombinant clone also induced a leukotoxic activity with human PMNs when combined with the S component of Luk-PV, whereas this crude extract alone did not show any leukotoxic activity. Sequencing of this DNA fragment (Fig. 2) demonstrated a complete open reading frame (ORF) whose translation in amino acids showed sequences homologous but not identical to those of the peptides previously determined for LukF-PV (Fig. 3). Therefore, the corresponding plasmid was called pUC-*luk*F-R. Upstream from the ORF corresponding to *luk*F-R, there was a truncated ORF whose amino acid

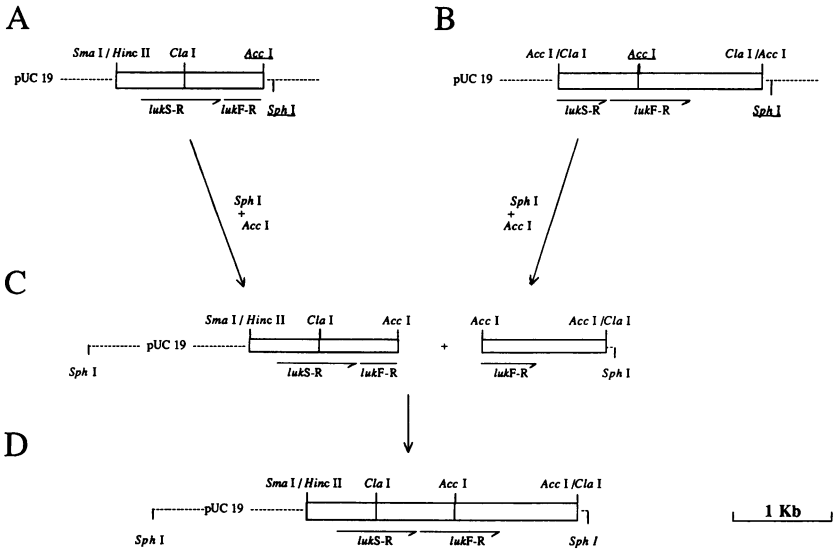

FIG. 1. Cloning of *luk*S-R and *luk*F-R. (A) After the library was screened with the oligonucleotide probe, a 1,537-bp *Hin*cII-*Acc*I DNA fragment inserted in pUC19 containing *luk*S-R (indicated by a horizontal arrow) and a part of *luk*F-R was selected. (B) After screening with the same probe, a 2,099-bp *Cla*I DNA fragment inserted in pUC19 containing *luk*F-R and a fragment of *luk*S-R (indicated by horizontal arrows) was selected. (C) In order to obtain both *luk*S-R and *luk*F-R in the same DNA insert (D), the *Acc*I-*Sph*I DNA fragment bearing *luk*F-R was ligated to *Acc*I-*Sph*I-linearized pUC-*luk*S-R containing *luk*S-R and the 5′ end of *luk*F-R.

translation showed a sequence (MDVTHAIKRST) homologous to that (MDVTHATRRDT) of a previously sequenced peptide (obtained after CNBr hydrolysis of the protein) of LukS-PV. This suggested that this new sequence was part of LukS-R, owing to the immunological relationship between LukS-PV and LukS-R. This observation led us to clone in *E. coli* a 1,537-bp *Hin*cII-*Acc*I DNA fragment containing the whole gene of the S component and the 5′ end of *luk*F-R (Fig. 1A). The corresponding crude extract of recombinant *E. coli* contained a protein recognized by LukS-PV antibodies which was also able to produce a biological activity with human PMNs, but only when it was associated with LukF-PV or recombinant LukF-R. The corresponding plasmid was called pUC-*luk*S-R. A third plasmid, called pUC-*luk*(S + F)-R, was constructed (Fig. 1D) by the ligation of a 1,286-bp *Acc*I-*Sph*I DNA fragment from pUC-*luk*F-R to *Acc*I–*Sph*I-linearized pUC-*luk*S-R (Fig. 1C and D). *E. coli* transformed by pUC-*luk*(S + F)-R expressed proteins antigenically related to the S and F components of Luk-PV (Fig. 4).

***S. aureus* P83 encodes only one leukotoxin acting by means of two components.** Total DNA from *S. aureus* P83 was hybridized at 60°C to the random-labeled *Hin*cII-*Acc*I 1,537-bp DNA fragment encoding *luk*S-R and the 5′ end of *luk*F-R and washed twice under the same conditions as those described in Materials and Methods. P83 total DNA showed a single, 4.2-kb *Hin*cII DNA fragment (Fig. 5A, lane A) and a single, 1.6-kb *Acc*I-*Hin*cII DNA fragment. Single DNA fragments were also detected with *Acc*I, *Bam*HI, *Hin*dIII, and *Bam*HI-*Hin*dIII restriction hydrolyses (Fig. 5A, lanes B, C, D, and E, respectively), indicating that there should not be other genes closely related to leucocidin in strain P83. CsCl-ethidium bromide centrifugation never revealed the presence of plasmids in this strain, which is susceptible to numerous antibiotics, including penicillin G. Additionally, the *luk*S-R- and *luk*F-R-specific probe hybridized (Fig. 5B) with an approximately 300-kb single DNA fragment obtained after *Sma*I restriction hydrolysis (Fig. 5B, lane D) and pulsed-field gel electrophoresis of total DNA from *S. aureus* P83 (lane B). This DNA fragment should be constitutive of the chromosome only, since we demonstrated earlier (14) that plasmids which are generally less than 40 kb in *S. aureus* were lost during DNA preparation for pulsed-field gel electrophoresis (PFGE) analysis.

Nucleotide sequence analysis. Sequencing of the 2,813-bp *Hin*cII-*Cla*I insert (Fig. 2) of pUC-*luk*(S + F)-R evidenced three ORFs. The first ORF of 948 nucleotides (*luk*S-R) spanned from positions 221 to 1168, started with an ATG codon, and ended with a TGA stop codon. Upstream from this sequence, a ribosome binding site (RBS) consensus sequence (GAAAGTG) was found at positions 208 to 214, and promoter consensus sequences were located at positions 179 to 184 (TTTAAT) and at positions 163 to 168 (TAACGTC).

The second ORF (*luk*F-R) of 978 nucleotides extended from position 1170 to position 2147, started with an ATG codon, and ended with a TAA stop codon. An RBS consensus sequence (GAAAGGA) was found at positions 1153 to 1159. The latter sequence might suggest that *luk*S-R and *luk*F-R are derived from two separate ancestral genes. No promoter region was found in the upstream vicinity of *luk*F-R. An inverted-repeat sequence consisting of a stem-loop with a 12-bp arm appeared at positions 2153 to 2192. It is probably used as a transcription terminator for *luk*(S + F)-R mRNAs.

A third ORF of 492 nucleotides was found extending from position 2690 to position 2199 in the DNA strand opposite to that encoding leucocidin R. This gene started with an ATG codon and ended with a TAA stop codon (Fig. 2). The proposed consensus sequences are an RBS (GAAGC) at positions 2704 to 2700 and promoter regions at positions 2718 to 2714 (TTAGA) and 2736 to 2731 (AATCAT). This gene would use, as a transcription terminator, the same inverted-repeat sequence (located at positions 2192 to 2153) as *luk*(S + F)-R. The corresponding ORF encoded a 163-amino-acid protein called protein 3. From this primary structure, 101

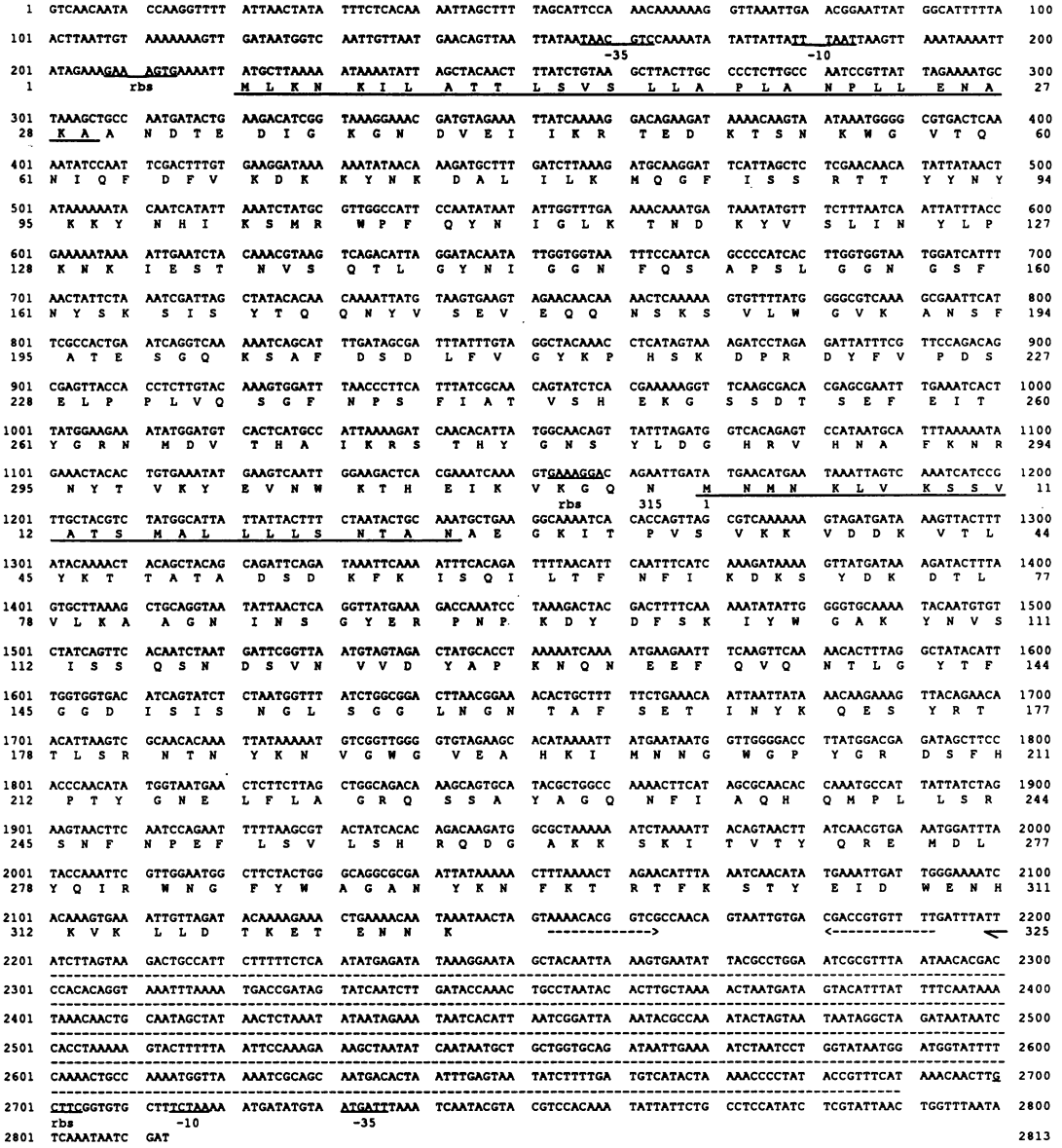

FIG. 2. Nucleotide sequence of *luk*S-R followed by that of *luk*F-R and peptide sequences of LukS-R and LukF-R from *S. aureus* P83. The stop codon of *luk*S-R is located (positions 221 to 1168) 1 base upstream from the ATG codon of *luk*F-R (positions 1170 to 2147). Signal peptides are indicated as underlined peptide sequences. Putative consensus sequences (i.e., RBSs) and promoter sequences are also underlined. An inverted-repeat sequence (positions 2153 to 2192) is indicated by arrows and is probably used as a transcription terminator of both *luk*-R and a third ORF. This third ORF (positions 2690 to 2199) is encoded by the strand opposite to that encoding *luk*-R, and it as well as its own consensus sequences is also underlined.

amino acids (62%) could be aligned (Fig. 6) with those of the sequence of the expression product of *bioX*, a gene of the biotin operon (22), from *Bacillus sphaericus*. Moreover, in this alignment, 58 amino acids (36%) were strictly identical to those of BioX, and 43 positions (26%) were conservative substitutions of residues of the same amino acid family.

Transcription analysis. In order to determine whether LukS-R and LukF-R are translated from one or two mRNAs, a Northern blot analysis was performed with *S. aureus* P83 total RNA (Fig. 7). The *Hin*cII-*Acc*I DNA fragment from pUC-*luk*(S + F)-R was labeled by random priming (10) and used as a probe for both *luk*S-R and *luk*F-R mRNAs. A single mRNA was detected; it was about 2,000 to 2,100 bases long, as determined from the migration of *E. coli* 16S and 23S rRNAs. The signal obtained was also located between the positions of the *S. aureus* 16S and 23S rRNAs, whose respective lengths are not precisely known. This mRNA size was in agreement with the lengths of both *luk*S-R and *luk*F-R, including part of the promoter sequence of *luk*S-R. This observation strongly suggested that *luk*S-R and *luk*F-R are tandemly transcribed in a single mRNA. At the translation level, the *luk*-R mRNA is translated into two separate proteins, since the beginning of *luk*F-R is separated from *luk*S-R by a stop codon. Using immunoblots on crude extracts from recombinant *E. coli* or *S. aureus* P83, we never

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LukF-R      MNMNKLVKSSVATSMALLLLSNTANAEGKITPVSVKKVDDKVTLYKTTATADSDKFKISQILTFNFI 67
LukF (16)   MKMNKLVKSSVATSMALLLLSGTANAEGKITPVSVKKVDDKVTLYKTTATADSDKFKISQILTFNFI 67
Hlg B         MNKLVKSSVATSMALLLLSGTANAEGKITPVSVKKVDDKVTLYKTTATADSDKFKISQILTFNFI 65
LukF-PV                              AQH ITPVSEKKVDDK

LukF-R      KDKSYDKDTLVLKAAGNINSGYERPNPKDYDFSKIYWGAKYNVSISSQSNDSVNVVDYAPKNQNEEF 134
LukF (16)   KDKSYDKDTLVLKATGNINSGFVKPNPNDYDFSKLYWGAKYNVSISSQSNDSVNAVDYAPKNQNEEF 134
Hlg B       KDKSYDKDTLVLKATGNINSGFVKPNPNDYDFSKLYWGAKYNVSISSQSNDSVNVVDYAPKNQNEEF 132
LukF-PV

LukF-R      QVQNTLGYTFGGDISISNGLSGGLNGNTAFSETINYKQESYRTTLSRNTNYKNVGWGVEAHKIMNNG 201
LukF (16)   QVQNTLGYTFGGDISISNGLSGGLNGNTAFSETINYKQESYR-TLSRNTNYKNVGWGVEAHKIM-NG 199
Hlg B       QVQNTLGYTFGGDISISNGLSGGLNGNTAFSETINYKQESYRTTLSRNTNYKNVGWGVEAHKIMNNG 199
LukF-PV

LukF-R      WGPYGRDSFHPTYGNELFLAGRQSSAYAGQNFIAQHQMPLLSRSNFNPEFLSVLSHRQDGAKKSKIT 268
LukF (16)   WGPYGRDSFHPTYGNELFLAGRQSSAYAGQNFIAQHQMPLLSRSNFNPRFLSVLSHRQDAAKKSKIT 266
Hlg B       WGPYGRDSFHPTYGNELFLAGRQSSAYAGQNFIAQHQMPLLSRSNFNPEFLSVLSHRQDGAKKSKIT 266
LukF-PV                                          MPVLSGD FDPEFIGVVLSRKE

LukF-R      VTYQREMDLYQIRWNGFYWAGANYKNFKTRTFKSTYEIDWENHKVKLLDTKETENNK 325
LukF (16)   VTYQREMDLYQIRWNGFYWAGANYKNFKTRTFKSTYEIDWENHKVKLLDTKETENN  323
Hlg B       VTYQREMDLYQIRWNGFYWAGANYKNFKTRTFKSTYEIDWENHKVKLLDTKETENN  322
LukF-PV
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FIG. 3. Alignment of the peptide sequence of LukF-R to those of the F component of the Rahman et al. leucocidin (16), the B component of gamma-hemolysin (3), and the known peptides of LukF-PV.

detected a protein having the length of a possible precursor for both LukS-R and LukF-R.

Luk-R analysis. The first ORF encodes LukS-R, which is a 315-amino-acid protein. The calculated molecular mass of LukS-R is 35,721 Da, with a calculated pI of 9.37. This component is secreted by *S. aureus*, and a signal peptide is probably cleaved, as suggested by comparison of the translated ORF with the sequence of the N-terminal extremity of LukS-R previously affinity purified from strain P83 crude supernatant. The putative signal sequence of 29 residues contained N-terminal positively charged amino acids followed by hydrophobic amino acids. This signal peptide structure is in accordance with those of the commonly encountered signal sequences obtained for other bacterial

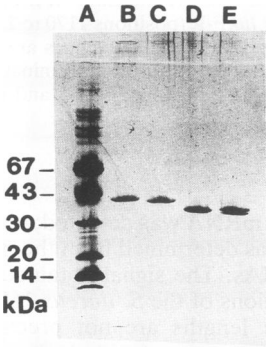

FIG. 4. LukF-R and LukS-R purified from periplasm of recombinant *E. coli*. Lanes: A, molecular weight markers consisting of α-lactalbumin (14.4 kDa), soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), and phosphorylase *b* (94 kDa); B, purified LukF-PV (38 kDa); C, purified recombinant LukF-R (37 kDa); D, purified LukS-PV (32 kDa); E, purified recombinant LukS-R (35 and 32 kDa).

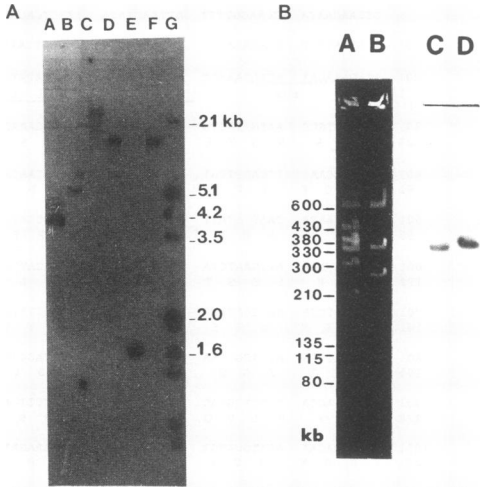

FIG. 5. Evidence that *S. aureus* P83 encodes only one leukocidin. (A) Total DNAs from *S. aureus* P83 (lanes A to F) were hybridized with a random-labeled *Hin*cII-*Acc*I DNA fragment encoding LukS-R and the first 123 residues of LukF-R. Lanes: A, *Hin*cII fragment; B, *Acc*I fragment; C, *Bam*HI fragment; D, *Hin*dIII fragment; E, *Acc*I-*Hin*cII fragment; F, *Bam*HI-*Hin*cII fragment; G, 5′-labeled bacteriophage lambda DNA digested with *Bst*EII. (B) After *Sma*I restriction, chromosomal DNAs from randomly selected MRSA (lanes A and C) and from *S. aureus* P83 (lanes B and D) were analyzed by PFGE. Lanes: A, *Sma*I DNA fragments of the MRSA strain (described previously as PFGE fingerprint 1 [14]) used as a DNA ladder; B, *Sma*I DNA fragments from *S. aureus* P83; C and D, hybridization of *Sma*I DNA fragments from the MRSA strain and from *S. aureus* P83 with the *Hin*cII-*Acc*I probe (specific for *luk*S-R and *luk*F-R).

exoproteins. The mature protein, therefore, would contain 286 amino acids, for a calculated molecular mass of 32,689 Da and a calculated pI of 9.27. The LukS-R peptidic sequence does not reveal any cysteine residue and contains 4 tryptophan residues. There are 37 strongly basic residues (lysine and arginine) and 29 acidic residues (aspartic and glutamic acids). The basic and acidic amino acids are more widely distributed in the extremities of the protein. Among the 9 proline residues, 6 are encountered in a stretch of 26 residues (amino acids 214 to 239).

The second ORF encoded LukF-R, a 325-amino-acid protein. This protein was translated from the same mRNA as LukS-R, and a possible second RBS was found within the 3′ end of *luk*S-R. The native protein was supposed to be cut after N-25 to give the secreted toxin, since the sequenced

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                  10        20        30        40        50        60
Prot 3    1   MKRYRGFSMTSKDITQISVIAAILTILAVLKIPSIIPGLDFQLSAPAALLILAFFGIKKY
                |: |||::| :: : :  |||::||| :|||||:|::|:| || |:|
BioX      1       MRKFSFYDLAQISLLACLIIVTGMFKIPTGIPGSEFQLSAPIAVAIAAVFGFKRY

                  70        80        90        100       110       120
Prot 3   60   FLGGLLSSLLLLVFGVFNPINVIISIIFRVIAIAVVYLLKINVLSLVLASVLGSLVSRLI
              ||:|:: ||:| : |:   :|| |||||: ::::: ||  ::  ||:|: :|  | ||:
BioX     64   FLAGIIASLILFLLGIHSILNVEISIIFRLTVGLIIVLLGTSIPVLVVAGPIGTMVARLG

                  130       140       150       160
Prot 3   120  LSVILNLPVWVVLLNAIPGVIFTLIVAIPLYLILRKRMAVLLR  163
              |  | |  : : |||| : |:: : |:  |      ::
BioX     124  LAFTLGTPFLPLFVLAIPGMVITAVSVYPITKMLYAINKKVAGDHHVRNVL  166
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FIG. 6. Peptide sequence alignment of protein 3 (Prot 3) to BioX, a product of the biotin operon from *B. sphaericus*. Vertical lines indicate amino acids homologies, and double dots indicate conservative substitutions of residues.

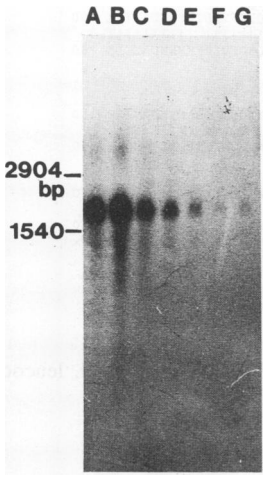

FIG. 7. Evidence that *luk*S-R and *luk*F-R are transcribed by only one mRNA. Lanes: A to G, total RNAs from 2.5×10^7, 1.25×10^7, 6×10^6, 3×10^6, 1.5×10^6, 8×10^5, and 4×10^5 bacteria, respectively, from *S. aureus* P83 hybridized with a 1,522-bp random-labeled *Hin*cII-*Acc*I DNA fragment specific for both *luk*S-R and *luk*F-R. The length of the detected mRNA (about 2,100 bases) was estimated by comparing the mobilities of the 1,540-base 16S and the 2,904-base 23S rRNAs from *E. coli*.

N-terminal extremity of the affinity-purified LukF-R was AEGKITPVSVKKVDD. The 25-amino-acid signal peptide so defined is constituted of 2 basic residues in the N-terminal part followed by 8 hydrophobic residues in the last 15 amino acids. This is also in agreement with commonly encountered signal peptide structures for exoproteins of *S. aureus* and other bacteria. The calculated molecular masses were 36,838 Da for the native and 34,203 Da for the mature LukF-R, with calculated pI of 9.3 and 9.2, respectively. The mature protein did not contain any cysteine residue and had 44 basic amino acids and 31 acidic residues. It also contained 127 polar and 98 hydrophobic amino acids. Five of the 6 tryptophan residues were distributed within the last 130 amino acids. The total charge was calculated to be 7.9 at pH 7.0. A lot of charged residues were encountered in the C-terminal extremity of LukF-R.

Comparison between purified Luk-PV and recombinant Luk-R components. As shown in Fig. 4, the purified recombinant LukF-R (lane C) appeared as a single band in SDS-PAGE. Its apparent molecular mass was estimated to be 36 kDa, which was a bit lower than that estimated for LukF-PV (38 kDa) (Fig. 4, lane B). This molecular mass value for LukF-R was between those calculated for the native and the mature proteins (36.8 and 34.2 kDa, respectively). On the basis of this sole SDS-PAGE observation, we have no evidence that LukF-R matured or was glycosylated in *E. coli*. This toxin component was recognized by affinity-purified LukF-PV antibodies in immunoblot experiments (data not shown) and seemed to have the same biological activities as the corresponding exoprotein secreted by *S. aureus* P83. For purified recombinant LukS-R (Fig. 4, lane E), two proteins appeared by SDS-PAGE (Fig. 4) and were also evidenced by immunoblot experiments (data not shown). The protein with the lowest molecular mass (32 kDa) (Fig. 4, lane E) migrated as purified LukS-PV (lane D), and the hypothesis that it was the mature protein was formulated. It was presumed that the second protein, whose molecular mass was approximately 34 kDa, was the native form. These experimentally determined molecular masses for LukS-R were in good accordance with those calculated for native and mature LukS-R from the *luk*S-R sequence.

TABLE 1. Toxic activities of components from Luk-PV and Luk-R[a]

Component	Sp act (U/mg) with the following type of granulocyte		Presence (+) or absence (−) of hemolysis with the following type of erythrocyte		
	Human	Bovine	Human	Bovine	Rabbit
LukF-PV	750	<750	−	−	−
LukS-PV	<800	<750	−	−	−
LukF-R	<860	<860	−	−	−
LukS-R	<800	<800	−	−	−
LukF-PV + LukS-PV	7×10^7	$<10^4$	−	−	−
LukF-PV + LukS-R	2.38×10^7	7.94×10^4	−	−	−
LukF-R + LukS-PV	2×10^7	2×10^6	−	−	−
LukF-R + LukS-R	2.58×10^6	7.94×10^6	+	+	+

[a] Components were taken both alone and in all (F + S) combinations. Associations of the latter are 1/1 molar ratios.

Biological activities. Toxins in homologous and heterologous F + S combinations were tested with leukocytes and erythrocytes (Table 1). With human PMNs, Luk-PV was about 27 times more active (7×10^7 U/mg of each component) than Luk-R (2.6×10^6 U/mg of each component). Heterologous combinations (LukF-R + LukS-PV) and (LukF-PV + LukS-R) showed intermediate biological activities with human PMNs compared with those of homologous F + S combinations of Luk-PV or Luk-R. This indicated that each component of Luk-PV potentiated the killing of human PMNs when associated with either component of Luk-R.

Conversely, when tested with bovine PMNs, Luk-PV did not produce any significant leukotoxicity, whereas Luk-R had a similar activity with these bovine granulocytes and human granulocytes, monocytes, and macrophages. Moreover, the combination of heterologous components brought to light a more complex situation concerning the bovine granulocytes: the heterologous combination (LukF-R + LukS-PV) showed activity only four times less than that of Luk-R, whereas the biological activity of the heterologous (LukF-PV + LukS-R) association was 100 times less than that of Luk-R.

As for hemolysis, the experiments showed that Luk-PV and heterologous F + S combinations were not effective in lysing erythrocytes. Only Luk-R induced hemolysis of human, bovine, and rabbit erythrocytes.

DISCUSSION

We report here the sequence of so-called staphylococcal leucocidin R. The genes encoding the two components (LukS-R and LukF-R) of this toxin were cloned on a 2,813-bp DNA fragment. We demonstrated that a single mRNA, corresponding to the length of the two genes, was transcribed in *S. aureus* P83. In accordance with this observation, possible promoter consensus sequences were found only upstream from *luk*S-R, and an inverted-repeat sequence was found at the end of *luk*F-R, suggesting that the two ORFs could be cotranscribed by staphylococcal RNA polymerase. Therefore, the leucocidin R genes constitute an operon. The translation of *luk*F-R mRNA may occur by two procedures. In the first, after the release of the 50S ribosomal subunit induced by the stop codon of *luk*S-R, the 30S ribosomal subunit still remains attached to the mRNA and

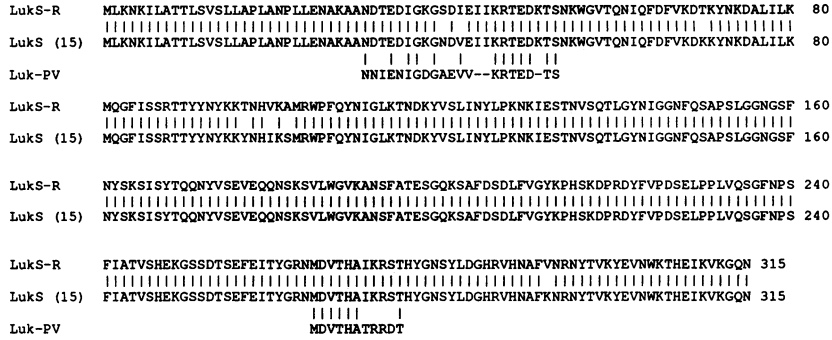

FIG. 8. Alignment of the peptide sequence of LukS-R to those of the S component of the Rahman et al. leucocidin (15) and some sequenced peptides of LukS-PV.

can progress from the stop codon of *luk*S-R to a downstream ATG codon. Thus, the same 30S ribosomal subunit should translate both LukS-R and LukF-R. If the first ATG codon of the second ORF encountered (located 1 base after the stop codon of *luk*S-R) is translated, cooperation with the RBS located 10 bases upstream is likely to occur. If the first translated codon is only the third codon of the *luk*F-R ORF, translation remains possible, but cooperation with the RBS should disappear because of the 17-nucleotide distance between the RBS and that ATG. The second procedure for LukF-R translation could involve the release of the 30S ribosomal subunit from the mRNA. In this case, the RBS located upstream from *luk*F-R would be necessary for the initiation of translation, and only the first ATG of the *luk*F-R ORF should be used as the initiating codon because of its optimal distance to the RBS. For these reasons, the first ATG codon of the *luk*F-R ORF is likely to be the first codon to be translated because of its markedly more favorable situation.

However, as observed with the first of the two steps of the cloning procedure, *luk*F-R alone could be expressed from pUC-*luk*F-R in *E. coli*, although the promoter of the *luk*-R operon was lacking. The major hypothesis which can explain this observation is that transcription of *luk*F-R in *E. coli* is mediated by the promoter of β-galactosidase from the plasmid pUC19.

The hybridization of a specific probe of *luk*S-R and *luk*F-R (*Hin*cII-*Acc*I DNA fragment) with P83 total DNA as a single band, even under low-stringency conditions, indicated that *S. aureus* P83 likely encodes only one leucocidin. This leucocidin appears to be chromosomally encoded for three reasons: (i) preparation of *S. aureus* plasmids by a CsCl gradient does not show any extrachromosomal genetic element, (ii) the two genes encoding leucocidin R are located in front of another ORF whose expression product has significant homologies with a chromosomally encoded protein constitutive of the biotin operon from *B. sphaericus* (22), and (iii) in PFGE experiments, hybridization with the specific probe of *luk*S-R and *luk*F-R evidenced a single, 300-kb chromosomal *Sma*I DNA fragment (Fig. 5B) in strain P83.

The cloned genes encoded the two components of leucocidin R, since their expression products induced the cell death of bovine and human PMNs, according to previous reports (20). Moreover, the biological activities of these proteins were neutralized by Luk-PV antibodies. However, the cell specificities of Luk-R and Luk-PV were different. Luk-PV is toxic to human PMNs but not to bovine PMNs or erythrocytes. In contrast, the purified recombinant Luk-R had 27-fold less specific activity with human PMNs than that of Luk-PV but also induced lysis of bovine granulocytes. LukF-R seemed to be responsible for bovine leukocyte cell specificity when combined with LukS-PV, whereas for human granulocytes, LukF-PV or LukS-PV was able to increase the damage of glass-adsorbed granulocytes when combined with LukS-R or LukF-R, respectively. Furthermore, only Luk-R was responsible for hemolysis of bovine, human, or rabbit erythrocytes. The hemolytic activity of Luk-R was not due to contaminating hemolysins since recombinant Luk-R was used in these experiments and since crude extract of pUC19-transformed *E. coli* was not hemolytic. This hemolytic property had not yet been documented (9, 19, 20), most likely because this toxin had never been purified to homogeneity. Therefore, so-called leucocidin R appeared to share many properties with the staphylococcal gamma-hemolysin. This was confirmed by peptidic sequence alignments (Fig. 3) of LukF-R to (i) the F component of the Rahman et al. (16) leucocidin, (ii) the known sequence (3) of the B component from gamma-hemolysin (HlgB), and (iii) the sequences of peptides from LukF-PV obtained after cyanogen bromide hydrolysis. All four of these sequences appeared to be closely related. Moreover, we also observed a randomly selected, methicillin-resistant staphylococcal strain which possessed a single set of genes hybridizing with the *luk*S-R–*luk*F-R probe (Fig. 5B). Cloning of *luk*-PV is now in progress in our laboratory, and data already available for LukF-PV and LukS-PV indicate that the sequence for Panton-Valentine leucocidin diverges the most from these sequences. Moreover, it was shown by SDS-PAGE (Fig. 4) that LukF-PV had a molecular weight higher than that of LukF-R, HlgB, or the F component of the Rahman et al. (16) leucocidin. LukF-R differed from HlgB by only 10 amino acids and from the F component of a leucocidin from an MRSA strain by 13 residues. Furthermore, the latter protein was different from HlgB by only 7 amino acids. Comparison of a peptidic sequence (Fig. 8) of LukS-R with that of the S component of another leucocidin (15) from an MRSA strain (15) evidenced only 7 differing amino acids. However, the hemolytic ability of the leucocidin (15) from the MRSA strain was not reported.

These observations clearly demonstrate that staphylococcal gamma-hemolysins and leucocidins constitute a single family of toxins acting by means of two separate protein components to damage the membranes of PMNs and macrophages or those of PMNs, macrophages, and erythrocytes. Therefore, the study of staphylococcal leucocidins (or gamma-toxins) is required in order to assess the structural

polymorphism of this family of toxins, as well as the spectrum of sensitive cells for each of these toxins. The name for these toxins should also be modified to take into account the fact that they act on cell membranes by means of two separate and synergistic components. We propose to call them synergohymenotropic toxins, because they act by the cooperation of two components (synergy) and because they are directed (tropism) against cell membranes (hymen). As Luk-PV-producing strains are highly associated with furuncles and primitive cutaneous abcesses (1), the association of the other staphylococcal two-component toxins with particular pathological features should also be determined.

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