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Existence of a new protein component with the same function as the LukF component of leukocidin or γ-hemolysin and its gene in *Staphylococcus aureus* P83

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Abstract Staphylococcal toxins, leukocidin and γ-hemolysin, consist of two protein components, i.e. LukF and LukS for leukocodin and HγI and HγII for γ-hemolysin. From a culture fluid of *Staphylococcus aureus* strain P83, a new protein component of leukocidin or γ-hemolysin which was designated as LukM was isolated. This component showed the same biological activity as that of LukF component for leukocidin or HγI component of γ-hemolysin in combination with LukS or HγII. However, the LukM component cross-reacted with the anti-LukS antibodies but not with the anti-LukF antibodies. On the basis of chemical analysis of the LukM component and the cloning and nucleotide sequencing of the *lukM* gene of *S. aureus* P83, we have demonstrated that LukM is an entirely new protein component of leukocidin or γ-hemolysin. The deduced amino acid sequence of LukM from the lukM gene showed 66.7% and 67% indentity to that of LukS and HγII, respectively. However, the amino acid sequence of LukM and LukF showed only 29% homology.

Key words: Leukocidin; γ-hemolysin; LukM component; LukF component; LukS component; HγII component; *Staphylococcus aureus*

1. Introduction

Staphylococcal toxins, leukocidin and γ-hemolysin, consist of two protein components, i.e. LukF and LukS for leukocidin and HγI and HγII for γ-hemolysin. By the chemical and immunological analyses of the four components and the cloning and nucleotide sequencing of the genes, we demonstrated that leukocidin and γ-hemolysin share LukF or HγI component in the two toxins, and the specificity of both toxins depends on the LukS and HγII component [1–3]. Supersac et al. also reported the nucleotide sequence of lukR gene for leukocidin, known as leukocidin R of *Staphylococcus aureus* P83 [4]. The deduced amino acid sequences of their recombinant LukF (LukF-R) and LukS (LukS-R) components are not identical but very close to that of our LukF and LukS, respectively [4]. Unlike our LukS and LukF, LukF-R in combination with LukS-R induced hemolysis of human erythrocytes as well as leukocytotoxic activity on human polynuclear leukocytes [4]. However, no information about intact LukF and LukS from *S. aureus* P83 is available, because their results came from the study only on the recombinant LukF and LukS in *Escherichia coli*.

The gross dissimilarity of the activity between leukocidin and leukocidin R raises the question whether or not there is any relationship between them. To answer this question, we started to obtain homogeneous preparations of the native LukF and LukS from S. aureus P83. While purifying the LukF and LukS from a culture fluid of *S. aureus* P83, we accidentally isolated a new protein component which was designated LukM component. In this study, by the chemical and biological analyses of the LukM and the identification of its gene from *S. aureus* P83, we demonstrate that the LukM is an entirely new protein component, having the same activity as LukF.

2. Materials and methods

2.1. Isolation and purification of LukM component

S. aureus P83 (ATCC 31890) was grown in 2.5% heart infusion broth (Difco) at 37°C for 24 h under aeration with O_2/CO_2 (80:20, v/v) and the culture supernatant was collected by centrifugation at 4°C. The leukocidin and γ-hemolysin components were purified according to the methods described previously [1]. Four protein peaks, designated I, II, III, and IV in order of elution, were obtained (Fig. 1). Components I, III, and IV were eluted at the positions where LukF, LukS, and HγII were usually eluted, respectively. In contrast, component II, which was designated as LukM, was eluted at a new position. These were separetely purified by rechromatograpy on the SP-5PW column (Tosoh, Tokyo), dialyzed against 30 mM sodium phosphate buffer, pH 6.5 and stored at −80°C until used. As will be described below, components I, III, and IV were identified as LukF, LukS, and HγII, respectively.

2.2. Assay of leukocidin and γ-hemolysin activities

Leukocidin activity was determined by the method described previously [1]. For the determination of the γ-hemolysin activity, the LukF or LukM component (4 pmol) was incubated with the human red blood cells (1.5×10^8) in 300 μl of saline containing 20 mM sodium phosphate buffer (pH 6.8) at 34°C for 1 min. Then the HγII component (4 pmol) was added to the reaction mixture and incubated for 15 min at 34°C. After the reaction mixture was centrifuged at $7,000 \times g$ for 5 min, a supernatant was collected. The absorbance of the supernatant was measured at 541 nm.

2.3. N-terminal amino acid sequence of the intact LukM component and its peptide fragments by cyanogen bromide cleavage

The purified LukM component was cleaved by CNBr according to the method described by Matsudaira [5]. The LukM (100 μg) was dissolved in 1 ml of 70% formic acid containing 200 μg of CNBr, and was kept in the dark under argon gas at 24°C for 24 h. After lyophilization, the sample was applied to the SDS-PAGE using 15% gel. The CNBr-fragments were blotted on the PVDF membrane. After being staining with Ponceau S, CNBr-fragments on the membrane were directly analyzed for an N-terminal amino acid sequence. Intact I, III, and IV components were also analyzed for N-terminal amino acid sequences. The N-terminal sequence was determined by the method

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Abbreviations: LukM, M-component of leukocidin/γ-hemolysin; LukF, F-component of leukocidin/γ-hemolysin; LukS, S-component of leukocidin; HγII, HγII component of γ-hemolysin; PCR, polymerase chain reaction

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described previously [6] using a gas phase protein sequencer (model PSQ-1, Shimadzu, Kyoto) equipped with an on line amino acid analyzer (model RF-550, Shimadzu, Kyoto).

2.4. Amplification of a part of the LukM gene by PCR

The purified chromosomal DNA from *S. aureus* P83 was used as a template. According to the N-terminal amino acid sequence of intact LukM and its CNBr-fragments, oligonucleotide primers were synthesized. The PCR amplification was performed 35 cycles with *Taq* polymerase (Takara shuzo, Kyoto) using the following temperature profile, 94°C, 1 min; 55°C, 2 min; 72°C, 2 min. The amplified products were purified by agarose electrophoresis. After phosphorylation, the DNA fragment was inserted into *Sma*I site of pUC119 vector DNA, then was fransformed into *E. coli* DH5α.

2.5. Genomic cloning of the lukM from the chromosomal DNA of S. aureus P83

The purified chromosomal DNA from *S. aureus* P83 was double-digested with *Hin*dIII and *Eco*RI, and was applied to agarose gel electrophoresis. After southern blotting, the DNA was hybridized with the ^{32}P-labeled 500 bp-DNA fragment which was a part of the DNA of *lukM* obtained by PCR. About 4 kbp-fragments were hybridized. Accordingly, the fragments were extracted from the gel, and were inserted into the *Eco*RI–*Hin*dIII site of pUC118 plasmid vector DNA. After transformation of the recombinant DNA into *E. coli* DH5α, the recombinant containing the *lukM* gene was obtained by colony hybridization with the ^{32}P-labeled 500 bp-fragment.

2.6. Other analytical procedures

SDS-PAGE and Western immunoblot analyses were done by the methods described previously [1]. The amino acid analysis was performed by the method described previosuly [1].

The nucleotide sequence was determined by the methods described previously [2].

3. Results and discussion

3.1. Isolation of the LukM component

Attempting to purify LukF, LukS, and HγII from the culture fluid of *S. aureus* P83, we noticed that a new protein peak, designated as LukM was eluted from the HPLC column at the position between LukF and LukS (Fig. 1A). We therefore purified the protein as well as the LukF, LukS, and HγII components to gel electrophoretic homogeneity (Fig. 1B). Identity of the components I, III, and IV with LukF, LukS, and HγII, respectively, was confirmed by sequencing the N-terminal 40-residues (data not shown).

3.2. Leukocidin and γ-hemolysin activities of LukM in combination with LukS and HγII

A synergistic action of LukM and LukS caused leukotoxic activity. The concentration of LukM as well as LukF required for 100% leukocytolytic activity toward 10^6 human polymorphonuclear leukocytes in 10 min in the presence of LukS (1 μg/ml) was determined to be 1 μg/ml. Furthermore, LukM showed a γ-hemolysin activity in combination with HγII (Fig. 2, wells 1 and 5). Although the reason why the hemolysin activity of LukM was about 1/5 of that of LukF, in the presence of HγII is still to be elucidated, this finding indicates that

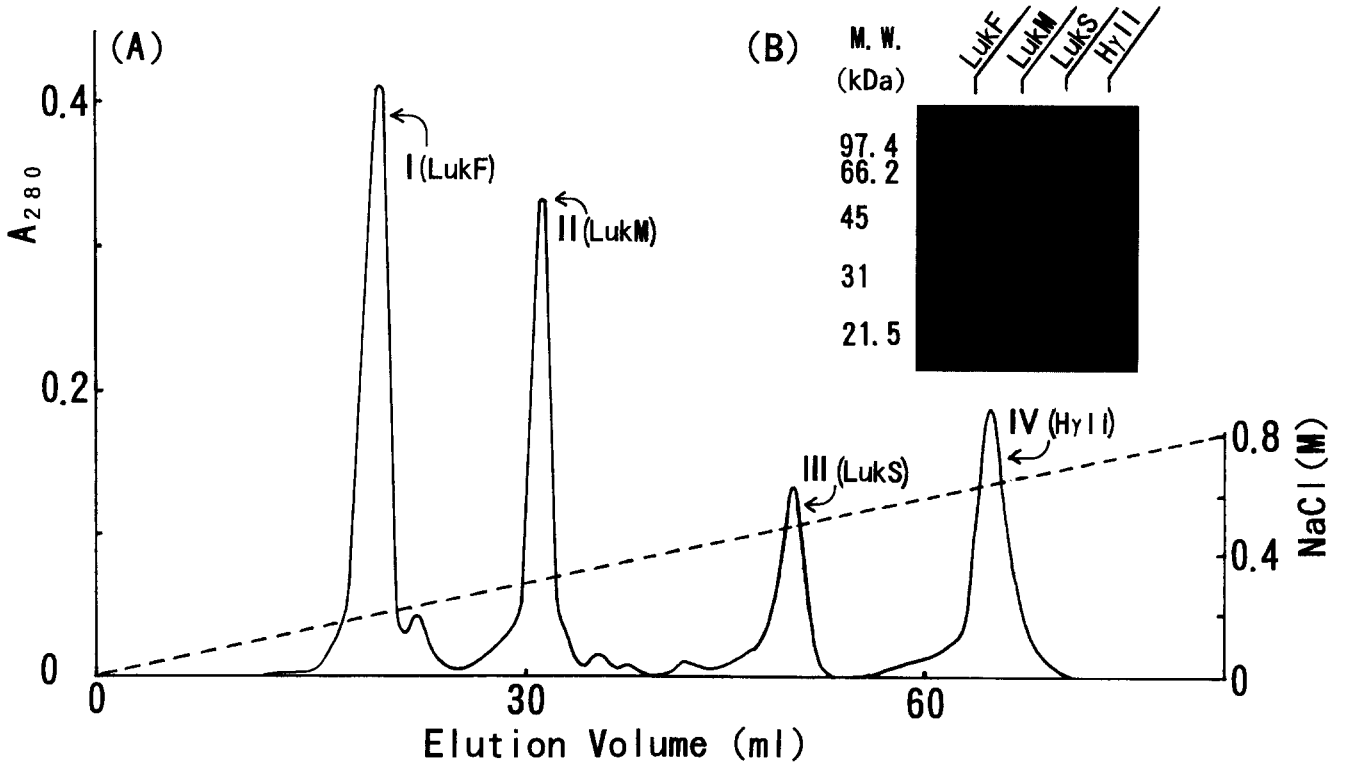

Fig. 1. Elution profile of leukocidin and γ-hemolysin components from the SP-5PW cation exchange column and SDS-PAGE analysis of the purified LukF, LukM, LukS, and HγII components. (A) Leukocidin and γ-hemolysin fractions eluted from a hydroxylapatite column were subjected to HPLC (TOSOH, Tokyo) epuipped with a TSK gel SP-5PW column. —, Absorbance at 280 nm; - - -, NaCl concentration. (B) The gel was stained with Coomassie brilliant blue R-250. Molecular mass standards used were phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa) and soybean trypsin inhibitor (21.5 kDa).

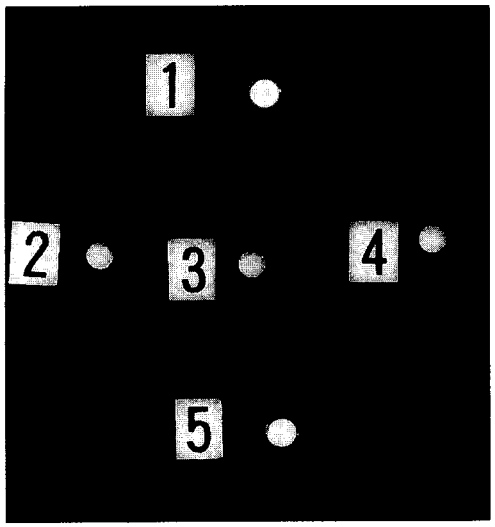

Fig. 2. γ-Hemolysin synergism on human blood agarose plate. 1, LukF + HγII; 2, LukF; 3, HγII; 4, LukM; 5, LukM + HγII. The concentration of each component was adjusted to 100 ng/μl in PBS (pH 7.2). The amount of each component applied to the well was 120 ng. The plate was incubated at 37°C for 14 h.

the LukM component has the same physiological property as that of the LukF component. No phospholipase A and C activity was detected in the LukM preparation (data not shown).

3.3. Characterization of the LukM component

The molecular mass of LukM (32 kDa) was smaller than that of LukF (34 kDa). Although physiological property of LukM was the same as LukF, LukM was positive to anti-LukS antibody, whereas this cross-reacted neither anti-LukF nor anti-HγII antibodies. Furthermore, the N-terminal 47-residue sequence of LukM determined in this study was 70% identical with that of LukS [4,7] but only 17% homology was found between those of LukM and LukF [4,8]. The isoelectric point of LukM was determined to be 9.4 by the method described previously [8].

3.4. Molecular cloning and nucleotide sequence of the lukM from S. aureus *P83*

According to the N-terminal amino acid sequence of intact LukM and its CNBr-fragments, C (17 kDa) and D (5 kDa), the oligo-nucleotides primers were synthesized (Fig. 3). In combination with primers I and III, about 250 bp-fragment was amplified from the chromosomal DNA of *S. aureus* P83 by PCR. Also, in combination with primers II and IV, a DNA fragment with about 500 bp was amplified. By DNA sequence of the two fragments, it was confirmed that the 250 bp- and 500 bp-fragments had a DNA sequences corresponding to the amino acid sequence of determined N-terminal 47-residue of

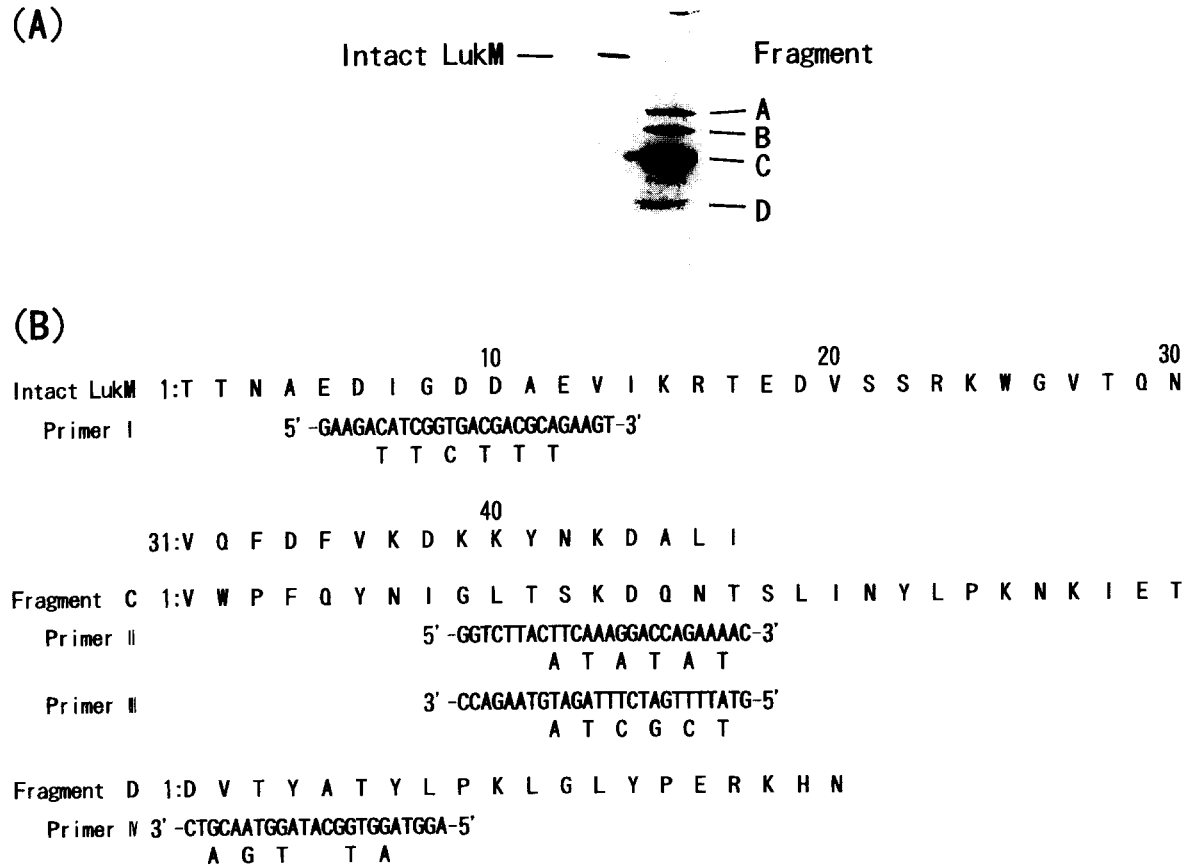

Fig. 3. SDS-PAGE analysis of the peptide fragments by CNBr cleavage of the purified LukM component (A) and the N-terminal amino acid sequences of the intact LukM component and its CNBr-fragments, C and D (B). The oligonucleotide primers, I, II, III, and IV expected from the N-terminal amino acid sequence of the intact LukM, the fragments C and D are also shown in this figure.

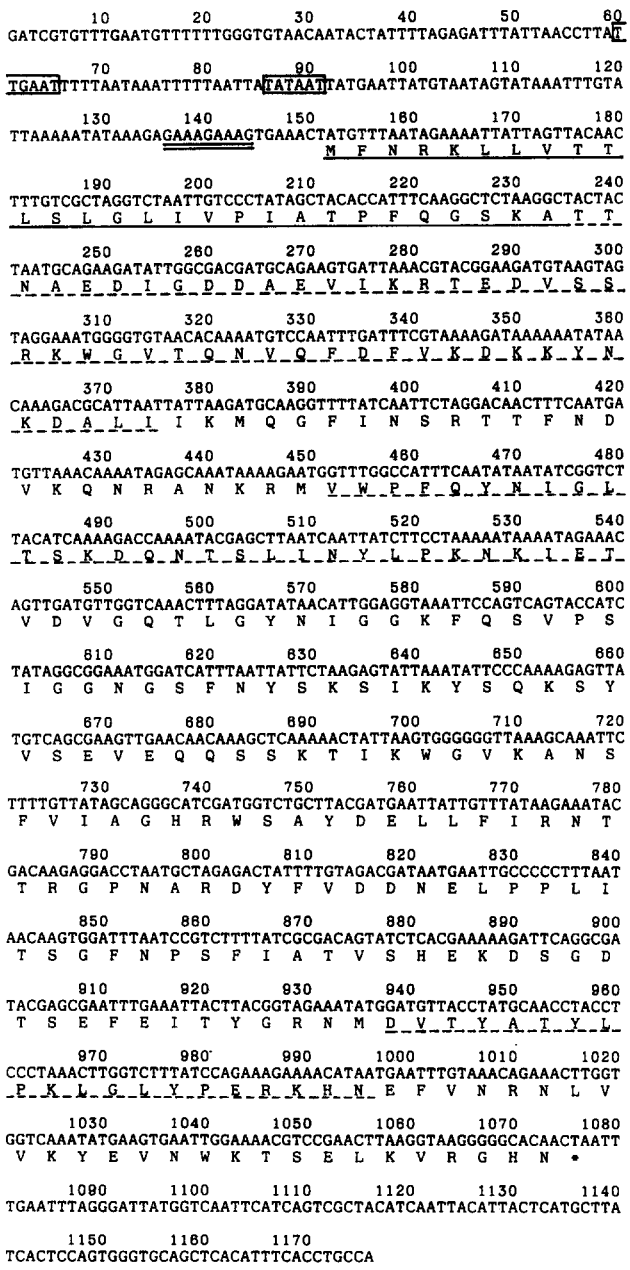

Fig. 4. Nucleotide sequence containing the *lukM* gene from *S. aureus* P83 and the deduced amino acid sequence. The strand shown is from the 5′ to 3′ direction. Amino acid sequences analyzed chemically, putative ribosomal binding sites, signal peptide sequence, promoter sequence are indicated by broken lines, double underlines, single underlines, and boxes, respectively.

intact LukM and N-terminal 30-residue of the fragment C, respectively. Therefore, we used the 500 bp-fragment as a probe for the genomic cloning of the *lukM* gene from *S. aureus* P83.

The 4.0 kbp *Hin*dIII–*Eco*RI-fragment of the chromosomal DNA from *S. aureus* P83 was strongly hybridized (data not shown). Accordingly, we cloned this fragment into the *Eco*RI–*Hin*dIII site of plasmid pUCl18 vector DNA and transformed into *E. coli* DH5α. This plasmid was designated as pMWK1. About 2.2 kbp *Sau*3A–*Sau*3A-fragment in this plasmid contained the lukM gene (data not shown). Therfore, we have determined the nucleotide sequence of the fragment containing the lukM and its 5′-and 3′-flanking regions. The determined nucleotide sequence of the fragment was comprising 1,176 bp as shown in Fig. 4. Within this sequence, we can identify an open reading frame which begins with ATG codon at position 152 and terminates with TAA at position 1,076. The open reading frame encoded protein of 309 amino acids with a calculated molecular mass of 35,006. The N-terminal 47, 30, and 20 amino acid residues of the intact LukM and its CNBr-fragments, C and D were identical to the deduced 47 amino acid residues from 29th N-terminal amino acid residues to 75th one, from 101th to 130th, and from 263th to 282th, respectively (Fig. 4).

On the basis of the N-terminal amino acid sequence of the secreted LukM component (Fig. 4), the position of Ala^{28} - Thr^{29} of the pre-matured LukM component might be processed by signal peptidase to secrete as a matured protein. No cystein residue was detected from the deduced amino acid sequence of LukM and chemical analysis of secreted LukM (data not shown). The total amino acid composition of matured LukM deduced from the nucleotide sequence (from bases 236 to 1,075) almost corresponded with that the purified LukM (data not shown).

3.5. *Amino acid homology study*

Comparison of the peptide sequence (Fig. 5) of matured LukM with that of the LukS, LukF, and Hγll from *S. aureus* RIMD 310925 [2] evidenced 66.7%, 29.0% and 67.0% homology in the entire amino acid sequence.

3.6. *The nucleotide sequence of 5′- and 3′-flanking regions*

The following observations were made concerning the DNA sequence of 5′- flanking region of *lukM*. (i) Typical Shine–Dalgarno sequence (GAAAG) [9] at both 16 base pair and 11 base pair upstream from the initiation codon for LukM component were found. (ii) The sequence TATAAT at −61 to −66 bp from the translation initiation codon of the gene assumed to be −10, shared the homology of the bases of the *E. coli* sequence with a conserved initial TA and the final T as Pribnow box [10]. Another sequence TTGAAT at positions −87 to −92 bp from the initiation codon and 21 bp from the −10 sequence was presumed to be a −35 promoter sequence. A typical transcription termination codon was not found between the stop codon of the lukM gene and the last codon sequenced.

From the results reported here, it was demonstrated that the LukM component is a new component which has chemical and immunological properties similar to that of the LukS component of leukocidin and has physiological properties same as that of the LukF of the two-component toxin, leukocidin or γ-hemolysin. Our sequence data will be helpful for the detail study of the role of the LukM protein in the toxic mechanism of leukocidin and γ-hemolysin.

3.7. *Distribution of the lukM gene among various* S. aureus *strains*

So far the distribution of the *lukM* gene among *S. aureus* P83, RIMD 310925, 5R Smith, and CN 4696 have been investigated by Southern blot hybridization and PCR, only P83 strain which was used in this study possesed the lukM (data not shown).

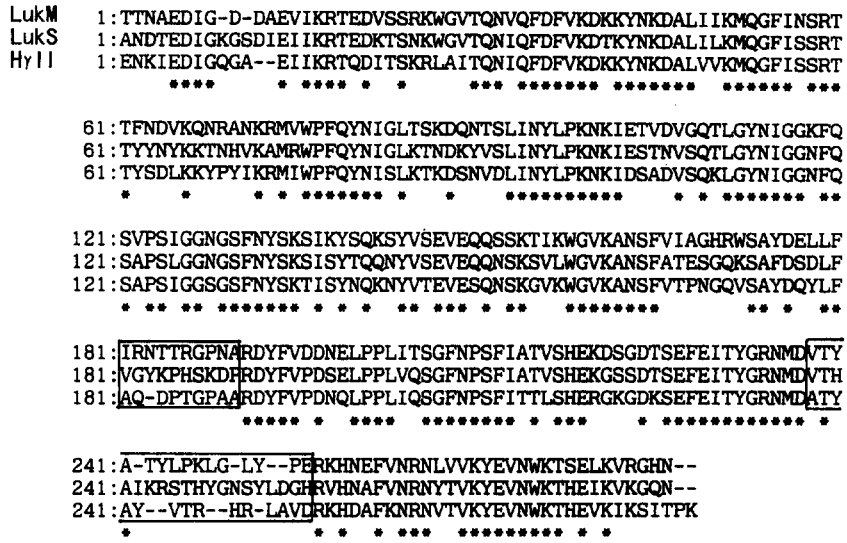

Fig. 5. Comparison of the deduced amino acid sequence of LukM component with that of LukS and Hγ II. The sequences were compared using the GCG program GAP uning weight of 4. Identical residues are indicated by stars. The gross differences between them are indicated by boxes.

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References

[1] Kamio, Y., Rahman, A., Nariya, H., Ozawa, T. and Izaki, K. (1993) FEBS Lett. 321, 15–18.
[2] Rahman, A., Izaki, K. and Kamio, Y. (1993) Biosci. Biotech. Biochem. 57, 1234–1236.
[3] Nariya, H., Izaki, K. and Kamio, Y. (1993) FEBS Lett. 329, 219–222.
[4] Supersac, G., Prevost, G. and Piemont, Y. (1993) Infect. Immunol. 61, 580–587.
[5] Matsudaira, P.T. (1989) A practical guide to protein and peptidepurification for microsequencing, Academic Press Inc., New York, pp. 49–69.
[6] Muramoto, K., Nokihara, K., Ueda, A. and Kamiya, H. (1994) Biosci. Biotech. Biochem. 58, 300–304.
[7] Rahman, A., Izaki, K., Kato, I. and Kamio, Y. (1991) Biochem. Biophys. Res. Res. Commun. 181, 138–144
[8] Rahman, A., Nariya, H., Izaki, K., Kato, I. and Kamio, Y. (1992)Biochem. Biophys. Res. Commun. 184, 640–646.
[9] Shine, J. and Dalgarno, L. (1975) Nature 254, 34–38.
[10] Rosenberg, M. and Court, D. (1979) Ann. Rev. Genet. 13, 319–353.