Bacterial Two-component and Hetero-heptameric Pore-forming Cytolytic Toxins: Structures, Pore-forming Mechanism, and Organization of the Genes

Jun Kaneko and Yoshiyuki Kamio

Laboratory of Applied Microbiology, Department of Microbial Biotechnology, Division of Bioscience and Biotechnology for Future Bioindustry, Graduate School of Agricultural Science, Tohoku University, Tsutsumi-dori Amamiya-machi 1-1, Aoba-ku, Sendai 981-8555, Japan

Staphylococcal γ-hemolysin (Hlg), leukocidin (Luk), and Panton–Valentine leukocidin (PVL) are two-component and hetero-oligomeric pore-forming cytolytic toxins (or cytolsin), that were first identified in bacteria. No information on the existence of hetero-oligomeric pore-forming cytolytic toxins in bacteria except for staphylococcal strains is available so far. Hlg (Hlg1 of 34 kDa/Hlg2 of 32 kDa) effectively lyses erythrocytes from human and other mammalian species. Luk (LukF of 34 kDa/LukS of 33 kDa) is cytolytic toward human and rabbit polymorphonuclear leukocytes and rabbit erythrocytes, and PVL (LukF-PV of 34 kDa/LukS-PV of 33 kDa) reveals cytolytic activity with a high cell specificity to leukocytes. Hlg1 is identical to LukF and that the cell specificities of the cytolsins are determined by Hlg2 and LukS. Based on the primary and 3-dimensional structures of the toxin components, Luk, Hlg, and PVL are thought to form a family of proteins. In the first chapter of this article, we describe the molecular basis of the membrane pore-forming nature of Hlg, Luk, and PVL. We also describe a requirement of the phosphorylation of LukS and LukS-PV by protein kinase for their leukocytolytic activity besides their pore formation on human leukocytes.

Recently, the assembly mechanism of the LukF and Hlg2 monomers into pore-forming hetero-oligomers of Hlg on human erythrocyte membranes has been clarified for the first time by our study using a single-molecular fluorescence imaging technique. We estimated 11 sequential equilibrium constants for the assembly pathway which includes the beginning with membrane binding of monomers, proceeds through single pore oligomerization, and culminates in the formation of clusters of the pores. In the second chapter of this article, we refer to an assembly mechanism of LukF and Hlg2 on human erythrocytes as well as the roles of the membranes of the target cells in pore formation by Hlg.

The LukF, LukS, and Hlg2 proteins are derived from the Hlg locus (hlg), and have been found in 99% of clinical isolates of Staphylococcus aureus. In contrast, LukF-PV and LukS-PV are derived from the PVL locus (pvl) which is distinct from the hlg locus, and only a small percentage of clinically isolated S. aureus strains carries pvl. Recently, we discovered pvl on the genome of lysogenic bacteriophages, φPVL, and determined the entire gene of the phage. We also demonstrated the phage conversion of S. aureus leading to the production of PVL through the discovery of a PVL-carrying temperate phage, φSLT, from a clinical isolate of S. aureus. In the third chapter of this article, we discuss genetic analyses of the Hlg, Luk, and PVL genes. We also discuss the current status of knowledge of the genetic organization of PVL-converting phages in order to achieve an understanding of their molecular evolution.

Key words: staphylococcal two-component pore-forming cytolsins; structure; single molecular imaging; assembly mechanism; phage conversion

Staphylococcus aureus secretes six cytolytic toxins, α-hemolysin (Hla), β-hemolysin, γ-hemolysin (Hlg), δ-hemolysin, leukocidin (Luk), and Panton–Valentine leukocidin (PVL), in which Hlg, Luk, and PVL have the unique characteristic of being composed of two separate and water-soluble proteins. Hlg (Hlg1 of 34 kDa/Hlg2 of 32 kDa) effectively lyses erythrocytes from human and other mammalian species. Luk (LukF [34 kDa] and LukS [33 kDa]) is cytolytic toward human and rabbit polymorphonuclear leukocytes and rabbit erythrocytes (but not hemolytic toward human erythrocytes), and PVL (LukF-PV [34 kDa] and LukS-PV...
[33 kDa]) shows cytolytic activity with a high cell specificity to leukocytes. Studies by us and by other groups have shown that Hlg2 is identical with LukF and that the cell specificities of the cytolsins are determined by Hlg2 and LukS. Hence, Hlg1 is referred to as LulF hereinafter. Based on the primary and 3-dimensional (3-D) structures of the toxin components, Hlg, Luk, and PVL are thought to form a family of proteins. Class F proteins (LukF and LukF-PV) and class S proteins (Hlg2, LukS, and LukS-PV) are approximately 70% identical with each other, whereas between classes the identity is approximately 30%. Interestingly, the class F and class S components of Hlg and Luk have 20 to 30% identities with the single-component staphylococcal Hla, which has been studied intensively as a prototype of pore-forming cytolysin. The alignment of the single-component staphylococcal Hla, the water-soluble LukF and LukF-PV components of Luk and PVL respectively, have been resolved from X-ray analyses of their crystals. It was also found that the phosphorylation of LukS and LukS-PV of Luk and PVL respectively by protein kinase, besides their pore formations on the leukocytes, is required for the Luk- and PVL-specific leukocytolytic activities.

In the previous chapter, we referred to the molecular cloning of the genes coding for Hlg, Luk, and PVL, the molecular domains of the toxins which determine the cell specificities, and the modes of action of these cytolsins. For the past 6 years, since our previous article, these two-component cytotoxic toxins and Hla have also been proven to be pore-forming cytolysins, and the fine structures of Hla, the water-soluble LukF and LukF-PV components of Luk and PVL respectively, have been resolved from X-ray analyses of their crystals. It was also found that the phosphorylation of LukS and LukS-PV of Luk and PVL respectively by protein kinase, besides their pore formations on the leukocytes, is required for the Luk- and PVL-specific leukocytolytic activities.

In the first chapter of this article, we describe the molecular basis of the membrane pore formation of Hlg, Luk, and PVL. We also discuss the phosphorylation of LukS and LukS-PV and its correlation with leukocytolytic activity in Luk and PVL.

The assembly of large macromolecular complexes such as membrane pores is essential for cell function. A crucial problem of protein complex assembly is to understand the mechanisms of the assembly processes by elucidating information on the beginning, intermediate, and final stages. Heterogeneous populations of intermediate states, however, cannot readily be analyzed using ensemble-averaged data. By contrast, single-molecule imaging methods provide direct information about individual intermediate states. Recently, individual protein–protein interactions at very low concentrations have been observed in vitro under the total internal reflection fluorescence microscope (TIRF-microscope). At high concentrations of proteins, however, dimers cannot be distinguished from crowded monomers using this technique. Fluorescence resonance energy transfer (FRET) between single pairs of acceptor and donor fluorophores (single-FRET) has allowed observation of dimerization even at high concentrations because the acceptor emits fluorescence only if located within several nanometers of the donor. Oligomers consisting of more than two molecules are also of great interest in protein assembly although they have not been analyzed yet using single-molecule imaging. Pore-forming cytolysins of bacteria are excellent models for studying the nature of the assembly of oligomeric molecules on membranes because of the high stability of recombinant monomeric subunits in solution. In 2003, the assembly mechanism of the LukF and Hlg2 monomers into pore-forming hetero-oligomers of Hlg on the human erythrocyte membranes was first clarified by our study using a single-molecular fluorescence imaging technique. We estimated 11 sequential equilibrium constants for the assembly pathway, which includes the beginning with membrane binding of monomers, proceeds through single pore oligomerization, and culminates in the formation of clusters of pores. Our single molecule observation technique offered the ability to distinguish intermediates under physiologically relevant conditions. The FRET measurement for single and multiple molecules is effective in distinguishing oligomers from crowded monomers. Moreover, we developed a novel method to deduce the number of LukF (donor) and Hlg2 (acceptor) molecules in small oligomers, such as trimers and tetramers, by measuring the stepwise photobleaching of FRET and acceptor signals respectively. In the second chapter of this article, we discuss the assembly mechanism of LukF and Hlg2 on human erythrocytes and the roles of membranes of the target cells in pore formation by Hlg in order to understand their pore-forming nature.

The LukF, LukS, and Hlg2 proteins are derived from the Hlg gene locus (hlg), and have been found in 306 of 309 S. aureus clinical isolates. In contrast, LukF-PV and LukS-PV are derived from the PVL gene locus (pvl), which is distinct from the hlg locus, and only a small percentage of clinically isolated S. aureus strains carried pvl. It is well known that several bacterial virulence factors are carried on mobile genetic elements such as phages, plasmids, or transposons. Recently, we discovered the presence of pvl on the genome of lysogenic bacteriophages, φPVL. We also demonstrated the phage conversion of S. aureus leading to the production of PVL by the discovery of a PVL-carrying temperate phage, φSLT, from a clinical isolate of S. aureus. In the third chapter of this article, we discuss genetic analyses of the Hlg, Luk, and PVL genes. We also discuss the current status of knowledge of the genetic organization of the PVL-converting phages in order to understand their molecular evolution.

Chapter I. The Molecular Basis of Pore Formation by Hlg, Luk, and PVL on the Target Cell Membrane

1. The pore-forming nature of Hlg, Luk, and PVL on the target cell membrane

When we monitored the Hlg-induced hemolysis for single cells of human erythrocytes under a phase contrast microscope, it was observed that intact, disc-
Fig. 1. Alignment of the Amino Acid Sequences among Classes F and S Components of the Staphylococcal Two-component Cytolysins.

The number of elements given above the sequence corresponds to that of LukF 3-D structure in Fig. 2. Residues shaded in black are identical between two of the classes F and S, and Hla. Residues shaded in magenta and sky blue are identical in classes F and S respectively. Dashed lines indicate deleted amino acids. The 5-residue segment KRLAI and the R217 residue of Hlg2, and the 2-residue segment DI of LukS are pivotal regions for the hemolytic activity of Hlg towards human erythrocytes and for the hemolytic activity of LukS towards rabbit erythrocytes respectively (red boxes). The 4-residue segments, indicated by boxes Z and I, viz., KRST in LukS, RRTT in LukS-PV, and KKIS in LukS-I, in which the forth residue of each 4-residue segment is phosphorylated by protein kinase on the human PMNLs, are pivotal regions for their leukocytolytic activity towards human and rabbit PMNLs (blue boxes). The residue W275 of LukS is a binding site of GM1 (green box).
shaped erythrocytes became swollen, round-shaped cells with clear edges after incubation with LukF and Hlg2 for 10 min, and the swollen cells lysed thereafter. Since swelling of cells is generally caused by permeabilization of cell membranes, it was assumed that Hlg induced colloid osmotic lysis of human erythrocytes through pore formation. This assumption was supported by the following findings: (i) Hlg-induced hemolysis was prevented by extracellular nonselectolectrates (such as polyethylene glycols) with a diameters of >2.5 nm, suggesting that the toxin forms a hydrophilic pore with a functional diameter of approximately 2.5 nm. (ii) Electron microscopy of the negatively-stained, toxin-treated erythrocytes revealed that Hlg forms a ring-shaped structure, whose outer and inner diameters are approximately 7 and 3 nm respectively. Hence, the complex formation of Hlg on human erythrocytes was examined as follows: Cell-bound toxin was solubilized with SDS from erythrocyte membranes and then analyzed by SDS-polyacrylamide gel electrophoresis, followed by Western immunoblot using specific antiserum raised against LukF and Hlg2. The data indicated that Hlg forms a high-molecular-sized complex(es) of approximately 200 kDa, which contain LukF and Hlg2 at a molar ratio of 1:1 on the surface of human erythrocytes. It was also shown that the preceding binding of LukF is essential for complex formation as well as for Hlg2 binding under the physiologically relevant conditions. Recently, we found that LukF binds strongly to membranes with a binding constant (Kf) of $2.1 \times 10^{-4} \mu m^{-2}$ and a large number of binding sites (Rf) of $2.0 \times 10^4 \mu m^{-2}$, and that Hlg2 also binds to membranes with a lower binding constant (Kh = $1.2 \times 10^{-5} \mu m^{-2}$) combined with a similar number of binding sites (Rh, $1.8 \times 10^4 \mu m^{-2}$), indicating an approximately 18-fold decrease in the extent of binding of Hlg2 compared with that of LukF. We also found that Hlg2 binds approximately 4 times more than it did without LukF (as described in Chapter II). We also demonstrated that the membrane component(s), which are accessible to proteinase K, are required for complex formation of Hlg on human erythrocytes.

Membrane pore formation by Luk in the cell membranes of human PMNLs and rabbit erythrocytes was also examined and the following findings were evident: (i) Luk caused an efflux of potassium ions from rabbit erythrocytes and swelling of the cells before hemolysis. But ultimate lysis of the toxin-treated swollen erythrocytes did not occur when polyethylene glycols with hydrodynamic diameters of >2.1 nm were present in the extracellular space. (ii) Electron microscopy showed the presence of a ring-shaped structure with outer and inner diameters of 9 and 3 nm respectively on the Luk-treated human PMNLs and rabbit erythrocytes. (iii) Ring-shaped structures of the same dimensions were isolated from the target cells, and they contained LukS and LukF in a molar ratio of 1:1. (iv) A single ring-shaped toxin complex had a molecular size of approximately 200 kDa. These results indicated that LukS and LukF assemble into a ring-shaped oligomer of approximately 200 kDa on the target cells, forming a membrane pore with a functional diameter of approximately 2 nm.

Finck-Barbançon et al. have reported that PVL causes an influx of ethidium chloride, an impermeable ion-to-cell membrane, into human PMNLs. Stalli et al. reported that PVL may stimulate an intrinsic calcium channel(s) of human PMNLs, and the toxin may thereafter form membrane pores, leading to an influx of extracellular ethidium ions. Thus, it has been suggested that PVL forms membrane pores in the early stage of its leukocytolytic action. Recently, the 3-D structure of the water-soluble LukF-PV monomer was reported, and it was found that the 3-D structure of LukF-PV is closely similar to that of LukF (as described below).

2. Assembly of two-component Hlg into heteroheptameric transmembrane pores with alternate subunit arrangements in ratios of 3:4 and 4:3

The analyses of populations of LukF and Hlg2, and LukF and LukS molecules in Hlg and Luk respectively implied that the single pore complex of Hlg and Luk may be a hexamer consisting of three molecules, each of two components. Later, Ferreras et al. reported that Hlg and Luk assembled into oligomers on the phosphatidylcholine (PC)–cholesterol liposomes and that hexamerization of the toxins was best fitted to the kinetics of vesicle permeabilization by Hlg and Luk. Thereafter, Olson et al. resolved the crystal structures of the monomeric water-soluble forms of LukF and LukF-PV respectively. These studies showed that LukF and LukF-PV have almost identical structures with a fold of the membrane insertion domain and contain a core structure very similar to that of Hla (see section 3 of this chapter). On the basis of the crystal structure of the monomeric LukF, Olson et al. constructed a model of the heptameric pores of the two-component cytolsins using the Hla heptamer as a template, whereas Pedelaq et al. proposed a hexameric structure for the transmembrane pores. Miles et al. reported that LukF and LukS of Luk form an octameric transmembrane pore. This conclusion was based on the results obtained from gel shift electrophoresis and site-specific chemical modification during single-channel recording. Is the complex hexameric, heptameric, or octameric, then? Does each ring-shaped complex contain both LukF and Hlg2? What is the molecular arrangement(s) of LukF and Hlg2 in the pore complexes? The transmembrane pore complexes of Hlg were isolated from human erythrocyte membranes and analyzed electron microscopically and biochemically. High-resolution electron microscopic images of negatively stained pore complexes clearly revealed a heptameric structure. When adjacent monomers in the pore complexes were randomly cross-linked using glutaral-
dehyd, LukF–LukF, LukF–Hlg2, and Hlg2–Hlg2 dimers were detected in an approximate ratio of 1:12:1, suggesting that LukF and Hlg2 were alternately arranged in the pore complex in molar ratios of 3:4 and 4:3. Alternate arrangements of LukF and Hlg2 in molar ratios of 3:4 and 4:3 were also visualized under an electron microscope with pore complexes consisting of glutathione S–transferase fusion protein of LukF or Hlg2 and wild-type protein of Hlg2 or LukF respectively. Thus it was concluded that LukF and Hlg2 assemble in a stochastic manner to form alternate complexes with subunit stoichiometries of 3:4 and 4:3 (see Fig. 5C).34)

3. The structures of LukF and LukF-PV

(1) LukF structure.9) LukF has the shape of a prolate ellipsoid with dimensions of 72 Å × 34 Å × 25 Å (Fig. 2A). In LukF the amino latch and the pre-stem adopt dramatically different conformations when compared to the corresponding regions of an Hla protomer (Fig. 2B). Excluding these areas, the fold of LukF is identical to the fold of an Hla protomer. The residues that comprise the amino latch (E2-K16) adopt a β-strand conformation and extend the inner β-sheet of the β-sandwich by one strand in LukF (Fig. 2A). Measured in terms of solvent-accessible surface area, the binding amino latch to the LukF core buries 414 Å². In striking contrast to the Hla protomer, the glycine-rich region of LukF forms a three-strand antiparalleled β-sheet that packs against the inner β-sheet of the β-sandwich domain. The pre-stem β-sheet, which includes the seven disordered residues spanning strand 7* and strand 8 (Figs. 1 and 2A), has an α(βββ(β(β)α) fold. Hydrophobic residues predominate in the pre-stem interface with the β-sheet domain: there are 24 van der Walls interactions and the buried surface area is 852 Å², which is close to the 670 Å² buried by the Hla amino latch when bound at a similar site on an adjacent protomer in the heptamer structure. Direct contact between the pre-stem and the amino latch happens through a cluster of hydrophobic residues that includes V13, V17, Y117, and F119. In Hla, the residues that occupy the latter two positions are predicted to lie at the interfacial region of the lipid bilayer. The juxtaposition of Y117 and F119 between the pre-stem and the amino latch makes direct commu-

Fig. 2. Water-soluble LukF Monomer (A), Single Protomer of Hla (B), and Predicted LukF Protomer (C), Taken from the Hla Homo-heptamer and Hlg Hetero-heptamer, Respectively, and the Residues in LukF, Which Are Involved in Binding to PC (D) and an Unidentified Component(s) (E), on the Human Erythrocyte Membrane.

The pre-stem region of LukF makes hydrophobic contact with the inner sheet of the β-sandwich (Cap) domain. Hla protomer and LukF comparison is described in the text. Positions of amino acid residues for creating double-cysteine mutations in LukF, shown in (A) and (C). Ribbon representation of LukF monomer (A) and Hla protomer (B) featured with back-bond representation of amino acid residues mutated to cysteine: V13 (green), T118 (cobalt), T137 (red). The black ribbon represents the pre-stem/stem domain. The two double-cysteine mutants generated were V13C–T137C (Cap–Stem) and T118C–T137C (Stem–Stem). In panel D, the molecule marked in red represents DiC₃PC, which is bound to the cleft which is surrounded by three residues, W177, E192, and R198. Unlike native LukF preparation, which was shown in Fig. 1A, the N-terminus of the recombinant LukF preparation used in the experiments in references 23 and 37 was E residue. Therefore the V13, T118, and T137 residues shown in Fig. 1 correspond to the V12, T117, and T136 residues respectively in the figures in references 23 and 37.
neciation between these two key regions possible.

(2) **LukF-PV structure.** The 3-D structure of the water-soluble LukF-PV monomer was analysed and was found closely to resemble that of LukF.

4. Conformational change of LukF accompanying formation of the transmembrane pores in Hlg and Luk, on the target cells

(1) **Prediction of the conformational change of LukF by comparison of the 3-D structures of the LukF monomer and the Hla protomer.** Superposition of the LukF and Hla protomer structures reinforces the conclusion that the cores are very similar despite a sequence identity of only 31.7% for the mature polypeptides. The comparison also emphasizes the divergence in conformation at the amino latch and glycine-rich stem regions, as illustrated in Fig. 1 and Fig. 2, A and B. Fitting of individual domains demonstrated that the r.m.s deviation between Cα positions for the β-sandwich and rim are 2.1 Å and 2.4 Å respectively. To a first approximation, the β-sandwich and rim domains behave as rigid bodies that adopt different relative conformations in the monomer (LukF) and heptamer (Hla) due to small changes spread over a number of residues at the β-sandwich/rim domain juncture. Major conformational differences between LukF and the Hla protomer structures occur in the triangle region (see Fig. 2, A and B). In contrast to the diffuse nature of the conformational differences relating the β-sandwich and rim domains, there are larger differences in main chain phi and psi angles for 4 residues in the triangle region which allow the LukF pre-stem to fold against the inner surface of the β-sandwich domain. In the Hla protomer, these main chain dihedral angles differ by >90° and are associated with a triangle conformation in which the polypeptide chain extends from the protomer core. The LukF pre-stem occupies approximately the same site that the amino latch of a neighboring protomer occupies in the oligomeric toxin, based on the analogy with the Hla heptamer.

The point of conformational divergence for the amino latch region is localized to residue K16 in LukF and to N17 in the Hla protomer. Large differences in the phi and psi angles at these sites cause the polypeptide chain either to fold back on the β-sandwich core to interact with β-strand 1 (LukF), or to extend from the core to interact with a neighboring protomer (the Hla heptamer). There are large additional differences in the conformation of residues within the amino latches of LukF and Hla. For details, see reference 9.

(2) **Demonstration of structural change in the LukF stem-domain during conversion from pre-pore to pore, and imaging pore-formation events on human erythrocytes membranes:** The mechanisms involved in the assembly of Hlg and Luk as well as Hla are poorly defined, mainly because of the difficulty in obtaining structures of the early, intermediate, and final states. The mechanism of pore assembly for Hla has been studied extensively. The crystal structure of the heptameric pore of Hla has been determined, although the structure of the monomer is not yet available. Various methods have been used to identify intermediate pre-pore states in order to elucidate the mechanism of assembly of Hla. These include: (a) truncation mutagenesis combined with limited proteolysis, (b) single-cysteine scanning mutagenesis followed by labeling with environmentally sensitive fluorescent dyes, (c) design of stem-truncated Hla which spontaneously oligomerize into heptamers in aqueous solution, and (d) arrest and release of the pre-pore using a metal-regulated switch. Based on biochemical, biophysical, and genetic evidence, four sequential states in pore assembly have been defined as follows: the water-soluble monomer (1), the membrane-bound monomer (1*), the non-lytic heptameric pre-pore (7*), and the lytic heptameric pore (7). The 3-D structure of the heptameric pore is available, the crystal structure of water-soluble LukF has been elucidated (Fig. 2A), providing a basic model for water-soluble and membrane-bound monomers of the staphylococcal toxin family (Fig. 2B). The LukF monomer has a basic structure similar to that of the Hla protomer (Fig. 2A), a subunit of the staphylococcal Hla heptameric pore. The LukF monomer consists of them, and three functions have been ascribed to them: the rim domain is for membrane binding, the β-sandwich (Cap) domain is for oligomerization with Hlg2, and the pre-stem domain is for membrane insertion to lyse the cell. The major differences between LukF and Hla protomer are the structures of the N-terminal amino latch and the pre-stem domain. The LukF amino latch folds back onto the Cap domain core, whereas the Hla amino latch extends from the core to interact with a neighboring protomer. The LukF pre-stem domain folds back against the Cap domain, while in Hla, it makes a long excursion into the lipid bilayer. Although these differences suggest start and end points for a model of Hlg pore assembly, the details of pore oligomerization and formation by the two components are unclear and might differ from these processes in the formation of the homomeric Hla pore. There are several pieces of evidence which contribute to this interpretation. LukF, Hlg2, and Hla do not share more than 30% amino acid identity with each other. The extent of sequence identity in the transmembrane β-barrel is weakest and the pre-stem domain of LukF is shorter than that of the Hla protomer by about 10 amino acid residues. The N-terminal amino latch of LukF has no role in pore formation or cell lysis, indicating that structural changes in the LukF amino latch are not important in pore assembly. This is in contrast with the essential rearrangement of the amino latch of Hla to the lumen of the Cap domain for conversion of the pre-pore...
to the lytic pore.\textsuperscript{9,38,40} To elucidate the mechanism for the assembly of Hlg and the role of the LukF pre-stem domain, it is important to have information on each of the component proteins at the beginning, intermediate, and final stages. But because the reaction time is short, we have to search for methods to arrest intermediate states by temporally interrupting the reaction process step by step. The method that uses engineered single-disulfide bonds connecting functional domains to switch the reaction on and off appeared to be simple and effective for studying the assembly of oligomeric proteins where the monomeric structures at the beginning are known. In the toxin field, this method has recently been used for aerolysin\textsuperscript{40} and perfringolysin O\textsuperscript{47} to inhibit one step in the pore assembly pathway. Given our understanding of the structure of the LukF monomer representing an early state in the pore assembly process, we designed two double-cysteine mutants of LukF whereby the pre-stem domain is trapped by an internal covalent disulfide bond formation, so that several intermediates would be arrested and then released to proceed to pore formation on exposure to a reducing reagent. These two mutants in fact partially assemble into different types of pre-pores which then transit into fully functional pores on the addition of a reductant. These are important tools for studying the mechanism of Hlg pore assembly.

Both LukF and Hlg lack cysteine residues. We have created double-cysteine LukF mutants, in which single disulfide bonds connect either the pre-stem domain to the Cap domain (V15C–T137C, Cap–Stem), or two β-strands within the pre-stem domain (T118C–T137C, Stem–Stem) to control pore assembly of Hlg at intermediate stages (Fig. 2A and Fig. 3B, left). The disulfide-trapped mutants were inactive in erythocytolysis, but gained full hemolytic activity when the disulfide bonds were reduced (Fig. 2C and Fig. 3B, right). The disulfide bonds blocked neither the membrane binding nor the intermediate pre-pore oligomerization, but efficiently inhibited the transition from pre-pores to pores. The pre-pores of Cap–Stem were dissociated into monomers in 1% SDS. In contrast, the pre-pores of Stem–Stem were stable in SDS and had ring-shaped structures similar to those of wild-type LukF, as observed by transmission electron microscopy (see Fig. 4D in reference 37). The transition of both mutants from pre-pores to pores could even be achieved by reducing disulfide bonds at low temperature (2°C), whereas pre-pore oligomerization was effectively inhibited by low temperature. Finally, real-time transition of Stem–Cap (or Stem–Stem) from pre-pores to pores on ghost cells, visualized using a Ca\textsuperscript{2+}-sensitive fluorescent indicator (Rhod2), was shown by the sequential appearance of fluorescence spots, indicating pore-opening events (Fig. 3, B and C). Taken together, these data indicate that pre-pores are legitimate intermediates during Hlg pore assembly, and that conformational changes around residues T118 and T137 of the pre-stem domain are essential for pore formation, but not for membrane binding or pre-pore oligomerization. We propose a mechanism for Hlg pore assembly based on the demonstrated intermediates (Fig. 3D).

Recently, Kawate and Gouaux determined the essential residues of α-hemolysin, D108 and K154 for the conformational rearrangements from homo-heptameric pre-pore intermediate to pore, by creating a double cysteine mutant, D108C/K154C,\textsuperscript{48} according to our method for γ-hemolysin which was described above.\textsuperscript{37}

5. LukF surface properties and phospholipid binding sites for LukF binding to human erythrocytes

The surface of the pre-stem β-sheet facing the LukF core is primarily hydrophobic while the side directed toward the solvent is polar. Like Hla, the LukF rim domain contains many exposed aromatic residues. Located in a cleft lined by W177 and R198 is a binding site for phospholipid head groups (Fig. 2D).\textsuperscript{9} In the Hla heptamer there is a similar lipid binding site, viz., the W179 and R200 residues of Hla, and the latter residue is critical for binding Hla to an erythrocyte membrane.\textsuperscript{9}

As mentioned above, LukF is a common component in Luk and Hlg. But LukF-PV cannot substitute for LukF of Hlg, despite 73% identity and approximately 90% similarity in amino acid sequence and close similarity in 3-D structure between them.\textsuperscript{49} The correlation between the hemolytic and binding activities of LukF-PV to human erythrocytes in human erythrocytes was examined. The results clearly indicated that null hemolytic activity of LukF-PV with Hlg2 towards human erythrocytes is due to the failure of initial binding of LukF-PV to human erythrocytes. LukF-PV has a cleft which is formed by W176 and R197 for binding the phosphocholine moiety of PC.\textsuperscript{10} Indeed, LukF-PV binds to PC. Hence, it is concluded that failure of LukF-PV in binding to erythrocytes is not due to a failure to bind to PC on human erythrocytes.

Twenty mutants, in which amino acids located at the bottom surface of the LukF rim domain were replaced by either A or T residue, were used to detect the putative residues that affect the hemolytic activity of LukF (in combination with Hlg2). Mutation at residues Y72, W177, E192, R198, F260, and Y261 (Fig. 2, D and E) showed a dramatic decrease in hemolytic activity, about 5–10% compared to that of the wild type. These five residues may be critical either to the membrane-binding or the pore assembly stage. Interestingly, among these residues, mutations at W177 and R198 preserved membrane-binding activity, while mutations at other points almost lose the binding activity (data not shown). Looking at the close-up view of the bottom of the rim domain, showing the model of interaction between W177, R198 and dipropionyl phosphatidylcholine (DiC\textsubscript{2}PC), which was proposed by Olson \textit{et al.} in 1999 (Fig. 2D),\textsuperscript{9} we suspect an interaction between these two residues with membrane components, which may contain a choline head, and might play a role in
regulating the pore assembly of Hlg. Hence we made a combined mutant of the two residues, W177T/R198T, in which W177 and R198 were all replaced by T residue, and characterized the properties of this mutant. We expected to find an effect of W177 and R198, not only on the membrane interaction, but also on sequential pore-assembly stages. Even at extremely high concentrations of mutant proteins of 5 mM applied to 1% human
erythrocytes (with the presence of Hlg2), hemolysis did not occur completely. The data indicate that two residues are pivotal for the hemolytic activity of LukF in combination with Hlg2.

To examine which stage of pore assembly is prevented by mutation at W177 and R198 in LukF, we tested for an early stage of pore assembly, viz., membrane binding of LukF. We applied a wide range of concentrations of double-cysteine mutants of LukF, from 5 to 160 nM, to a reaction mixture containing 1% human erythrocytes to ascertain the binding affinity of the mutants in comparison with that of the wild-type of LukF. The association constant ($K_a$) for membrane-binding of the W177T/R198T mutant was calculated to be $10^{-3}$ $\mu$m$^{-2}$, that is, about 20 times lower than that of wild-type LukF. This result showed that the W177T/R198T mutant can bind on the erythrocyte membranes, although the binding level is low. Notably, the finding is unlikely to the cysteine-point mutant of Hla at R200, corresponding to R198 of LukF, that has no binding activity to the erythrocyte membranes.

Therefore, it is concluded that PC-binding to W177 and R198 residues of LukF is important but not essential for stable binding of the LukF rim domain on the erythrocyte membranes, which is an early stage of pore formation of Hlg.

6. Assembly of the W177T/R198T mutant of LukF with Hlg2 into intermediate oligomers

As mentioned above, the W177T/R198T mutant failed to bind to DiC$_3$PC and showed a lack of hemolytic activity, but was able to bind onto erythrocyte membranes. It was examined if this mutant can undergo any further oligomerization stages and at what stage it stops. For detection of intermediate oligomers, the single-cysteine mutant W177T/R198T-S45C labeled with TMR and Hlg2 labeled with IC5 were used to observe the oligomerization of the mutant under a single-molecule fluorescent microscope (see next chapter). The data showed that the W177T/R198T mutant can assemble into abortive and nonfunctional pores which are unstable and cannot further oligomerize into clusters. It is possible that the mutant hexamer/heptamer is not a complete ring-structure or that its pre-stem domain could not be switched to insert through the membranes. The data obtained strongly suggest that the interaction between the rim domain through the W177 and R198 residues of LukF and a special binding site of the erythrocyte membranes is essential for pore formation. The W177 and R198 residues might be taken in consideration as the key residues for the functional binding of LukF to PC on the erythrocyte membrane.

7. Phosphorylation of LukS by a protein kinase that is crucial for the LukS-specific function of Luk on human PMNLs

As mentioned above, the W177T/R198T mutant failed to bind to DiC$_3$PC and showed a lack of hemolytic activity, but was able to bind onto erythrocyte membranes. It was examined if this mutant can undergo any further oligomerization stages and at what stage it stops. For detection of intermediate oligomers, the single-cysteine mutant W177T/R198T-S45C labeled with TMR and Hlg2 labeled with IC5 were used to observe the oligomerization of the mutant under a single-molecule fluorescent microscope (see next chapter). The data showed that the W177T/R198T mutant can assemble into abortive and nonfunctional pores which are unstable and cannot further oligomerize into clusters. It is possible that the mutant hexamer/heptamer is not a complete ring-structure or that its pre-stem domain could not be switched to insert through the membranes. The data obtained strongly suggest that the interaction between the rim domain through the W177 and R198 residues of LukF and a special binding site of the erythrocyte membranes is essential for pore formation. The W177 and R198 residues might be taken in consideration as the key residues for the functional binding of LukF to PC on the erythrocyte membrane.

The identity of the amino acid sequences between LukS of Luk and Hlg2 of Hlg is 72% (Fig. 1). The specificities of the two toxins towards the target cells raise the question of what region(s) of LukS or Hlg2 play a pivotal role in the LukS- and Hlg2-specific functions for Luk or Hlg activity. The result obtained from a series of chimeric genes (lukS/hlg2) and their expression in Escherichia coli showed that there is an essential region for the leukocytolytic function of Luk within the C-terminal 122-residue segment (between S164 and the C-terminus) of LukS. The 5-residue segment I242K243R244S245T246 (Box Z, Fig. 1), in which 4-residue KRST is identical with the recognition site (K/R-R/K-X-T/S) of protein phosphorylated by protein kinase A, was identified as a pivotal region of LukS responsible for the leukocytolytic function of Luk. The denatured LukS which was heated in boiling water for 10 min, but not the intact one, was strongly phosphorylated by $[^{32}P]ATP$ in the presence of protein kinase A in a cell-free system. But neither the mutants MLS-TY and MLS-TA, in which T246 was replaced by Y or A residue respectively, nor Hlg2 itself, in which the 5-residue segment of LukS was deleted, was phosphorylated by protein kinase A in the cell-free system. The Hlg2 mutant MHS-Z, in which the 5-residue segment was inserted at the position in which the segment is deleted in Hlg2, was phosphorylated by protein kinase A and conferred leukocytolytic activity cooperatively with LukF. The results obtained indicate that the 5-residue segment IKRST is the pivotal segment of LukS responsible for the LukS function of Luk. It was also found that LukS is phosphorylated by exogenous $^{32}$P-H$_3$PO$_4$ on human PMNLs and that the phosphorylation is prevented by $\gamma$-amino) ethyl]-5-isoquinolinesulfonamide (H-89), which is a selective inhibitor of protein kinase A with an inhibition constant of 0.048 $\mu$m. The IC$_{50}$ value of H-89 in the incorporation of $^{32}$P-H$_3$PO$_4$ into LukS was 0.05 $\mu$m, which coincided with its inhibition constant of 0.048 $\mu$m. These results indicate that LukS is phosphorylated by protein kinase A on human PMNLs. It is of interest to note that the cells which were exposed to 50 $\mu$m H-89 without Luk components showed no change in morphology. The addition of H-89 at concentrations over a range of 50 to 0.5 $\mu$m to the reaction mixture containing LukS and LukF allowed the cells to remain swollen but not lysed for at least 20 min as observed at 37°C. Hence, it is concluded that phosphorylation of LukS is crucial for the LukS-specific function of Luk. Although the mechanism of the lysis of human PMNLs by Luk remains to be elucidated, it is feasible that the modification of LukS to the phosphorylated version by protein kinase on human PMNLs after its initial binding to human PMNLs followed by the subsequent binding of LukF is essential for inducing cell lysis, because Luk in the presence of H-89 induced human PMNLs to become swollen but not to be lysed. There are two possibilities for the involvement of the phosphorylation of LukS in its leukocytolytic activity. One is the fragility of the membrane of human PMNLs caused by the induction of a lytic enzyme, such as an autolytic enzyme or...
phospholipase, which degrades the membrane. It is known that the binding of LukS to rabbit PMNLs induces an activation of phospholipase A₂ in the leukocytes.52) Taken together with our data, if any, the phosphorylated LukS-mediated signal transduction might be required for an activation of phospholipase A₂. The other possibility is that the membrane pores consisting of LukF and phosphorylated LukS on human PMNLs might regulate ions such as calcium current through the membrane. It is known that Luk induces an increase in free intracellular Ca²⁺ under the physiological conditions ([Ca²⁺] = 1 to 1.5 mM) in human PMNLs.53) Gouaux et al. have analysed the aligned sequences of Hla and Luk components in the context of Hla heptamer structure determined by crystallographical analysis. They suggested that even though the level of sequence identity among Hla and Luk components (LukS and LukF) is distant, the 3-D structures of the protomer are conserved.7) Based on this suggestion, we propose that the phosphorylated residue T246 of LukS is at the bottom of rim domain which spans the hydrophobic domain of the membrane bilayer of human PMNLs and that the T246 residue is phosphorylated by protein kinase A at the contact point of the inner leaflet of the membrane and cytoplasm of human PMNLs. As mentioned above, LukF and LukS of Luk assemble into a ring-shaped 200 kDa complex, which forms a membrane pore with a functional diameter of 2.1–2.4 nm. The MLS-TS, MLS-TA, and MLS-TY mutants of LukS perhaps form the pore cooperatively with LukF on the human PMNLs. From these findings, the leukocytolytic function from the complex formation which forms the membrane pore on the surface of human PMNLs can be distinguished. Interestingly, Hlg also assembles to form pores on human PMNLs. But no lysed cells were observed after incubation for more than 20 min, indicating that LukF and Hlg2 cooperatively caused only a swelling of the human PMNLs without lysis.

*Staphylococcus intermedius*, which is a coagulase-positive veterinary pathogen, secretes Luk-I, which consists of Luk-S-I and Luk-F-I.54) Luk-I shows strong leukocytolytic activity for human PMNLs. It also has slight hemolytic activity on a rabbit erythrocytes. Dermatonecrotic activity was also observed on rabbit skin model. From the deduced amino acid sequence of Luk-S-I, a 4-residue sequence, K135K136I137S138, at the root of the stem region was found to be identical with that of the phosphorylated segment of a protein phosphorylated by protein kinase A.55) A mutant of Luk-S-I (MLSI-SA), in which the S138 residue was replaced by an alanine residue, forms a ring-shaped complex with Luk-F-I on rabbit erythrocytes and human PMNLs membrane. But MLSI-SA shows no leukocytolytic activity with Luk-F-I. Luk-S-I was phosphorylated by protein kinase A in the presence of [γ-³²P] ATP in the cell-free system, but MLSI-SA was not phosphorylated. H-89 showed 50% inhibition of the Luk-I-induced cell lysis at 0.5 nM. Hence, it is concluded that phosphorylation of the S138 residue in the 4-residue segment of Luk-S-I is important for leukocytolysis of human PMNLs.55) Figure 4 shows 3-D structure models of LukS or LukS-I based on the 3-D structures of LukF, LukF-PV, and Hla. In this model, the 3-D locations of KKIS in LukS-I, KRST in LukS, and RRTT in LukS-PV in a ring-shaped complex are similar on the target cell membrane. Accordingly, it is reasonable to suppose that LukS-I is phosphorylated by the same protein kinase A as LukS and LukS-PV. The location of the 4-residue segment in LukS and LukS-I might be important for its interaction with protein kinase in the

---

**Fig. 4.** Three-dimensional Positions of the Phosphorylation Sites in LukS-I or/and LukS.

The 3-D structures of monomer (A) and protomer (B) of LukS-I or/and LukS were computed based on the data of 3-D and primary structures of LukF, LukF-PV, and Hla. The positions of the 4-residue segments, KKIS and KRST in LukS-I and LukS, were indicated by alphabets I and Z in circles respectively.
target cells. If the oligomeric state of Hla on the target cells is applicable to Luk-I and Luk, the phosphorylated residues of Luk-S-I are located at the root of the stem region that spans the hydrophobic domain of the membrane bilayer of human PMNLs and can easily interact with protein kinase A, which might be localized on the target cell membrane. On the other hand, the T246 residue in the KRST segment of Luk-S is located at the bottom of the rim domain and might be in contact with the surface of the target cells. The evidence, that the H-89 concentration for 50% inhibition of leukocytolytic activity of Luk-I was 100 times lower than that of Luk, can be explained by the location of the 4-residue segment between them. The phosphorylation intensity of the the 4-residue segment of the native Luk-S-I at the S138 residue in the cell-free system was less than 1/50 of that of the boiled Luk-S-I by protein kinase A in the presence of ATP. The low and null phosphorylation of the native Luk-S-I and Luk-S, respectively, might be due to the conformational difference in the pre-stem domain for Luk-S-I and the rim domain for Luk-S between native and boiled components. The conformational changes by boiling might cause the phosphorylation sites that are masked, to be exposed on the surface where they are easily accessible to protein kinase.

Chapter II. The Assembly Mechanism of Hlg on the Human Erythrocyte Membrane

Luk-F and Hlg2 are secreted out of staphylococcal cells as water-soluble proteins and assemble in a stochastic manner to form alternate hetero-heptameric pores with subunit stoichiometries of 3:4 and 4:3 on human erythrocyte membranes. The crystal structure of Luk-F monomer in solution and the pore structures observed by electron microscopy suggest beginning and ending stages of pore assembly. Nevertheless, an Hlg2 structure is not yet available, and little information about intermediates has been reported. Ensemble methods such as equilibrium analytical ultracentrifugation have been used to measure concentration averages of assumed monomers, tetramers, and octamers during cooperative association of an oligomeric DNA-binding protein. But the oligomers are not well separated and the concentrations of monomers required are high (μM). Transmission electron microscopy at the level of single molecules cannot allow for the observation of the oligomerization process from monomers to tetramers due to limited spatial resolution. Using the powerful single-FRET method, hetero-oligomeric toxins consisting of two distinct components are more advantageous to study than homo-oligomeric toxins, including Hla, because the individual components can be treated as specific fluorescence donors and acceptors respectively. Recently, we directly observed the assembly of single Luk-F and Hlg2 monomers into pore-forming oligomers on human erythrocyte membranes under a TIRF-microscope. Since Luk-F and Hlg2 lack cysteine residues, single-cysteine mutants of Luk-F (S46C) and Hlg2 (K222C), in which introduced cysteine residues are located on top of the respective Cap domains, were created (Fig. 5A), because the distance between S46 of Luk-F and K222 of Hlg2 is estimated to be approximately 3.0 nm in the heptamer that is suitable for FRET measurement. The Luk-F-TMR and Hlg2-IC5 derivatives were synthesized by incubation of Luk-F(S46C) and Hlg2(K222C) with TMR-maleimide and IC5-maleimide respectively. Hemolytic activity of the labeled mutants was the same as that of wild-type proteins, indicating that fluorophore labeling of the mutants did not inhibit membrane binding and pore oligomerization. We detected sequential stages beginning with the binding of monomers to the membranes, then the assembly of dimers, small oligomers and single pores, and finally the formation of clusters of pores, and measured their equilibrium constants, as described in the following.

1. Cooperative binding of Hlg2 in the presence of Luk-F

We verified binding constants (KF, KH) for Luk-F-TMR and Hlg2-IC5 respectively, and the number of binding sites per μm² of erythrocyte membranes (RF, RH) for individual components. Luk-F bound strongly to membranes with a KF of 2.1 × 10⁻⁴ μm⁻² and a large number of binding sites (RF) of 2.0 × 10⁴ μm⁻². By contrast, Hlg2 bound to membranes with a lower binding constant (KH, 1.2 × 10⁻⁵ μm⁻²) combined with a similar number of binding sites (RH, 1.8 × 10⁴ μm⁻²), indicating a 15-fold decrease in the extent of binding of Hlg2 compared with that of Luk-F, Hlg2 bound approximately 4 times more than it did without Luk-F. Combining all these data, it was demonstrated that both Luk-F and Hlg2 can bind spontaneously to erythrocyte membranes, but with different binding constants, and that the binding of Hlg2 was enhanced by Luk-F. The higher KF for Luk-F supports a previous suggestion regarding stable interactions between aromatic amino acids of the rim domain of Luk-F and the choline moiety of PC on the erythrocyte membranes. The lower KH for Hlg2 suggests different properties of putative binding sites for Hlg2 or different membrane-binding surfaces compared with those of Luk-F.

2. Individual monomers and dimers on the membranes

We visualized the assembly of Luk-F-TMR and Hlg2-IC5 on the membranes, at equilibrium, under a TIRF-microscope. At very low concentrations of the two proteins, at which hemolysis did not occur, only a few punctate FRET-IC5 spots, ranging from 0 to 10, were observed on each cell. The equilibrium association constant for dimerization KF-H was estimated, based on the concentrations of monomers ([F] = 3.8 and [H] = 6.9 μm⁻²) and dimers ([F-H] = 0.026 μm⁻²) on the cell membranes (number of cells = 37), to be 0.0010 ±
At the dimerization stage, stepwise photobleaching of single-FRET in the presence of LukF-TMR and Hlg2-IC5 on the membranes proved the existence of hetero-dimer \( F \cdot H \). But the lack of observable FRET of the couples LukF-TMR and LukF-IC5 and Hlg2-TMR and Hlg2-IC5 proves that there is no possibility of \( F \cdot F \) and \( H \cdot H \) homo-dimer formation. The hetero-dimer \( F \cdot H \) formed not in solution but on the membranes, for no FRET was observed in solution. The specific formation of hetero-dimer on the membranes is perhaps promoted by two-dimensional side-by-side collisions between LukF and Hlg2, as confirmed by negative FRET evidence using LukF(S34C)-TMR and Hlg2(Q29C)-IC5, the point mutants at interaction surfaces lacking oligomerization ability.

3. Tetramerization: the dimer-dimer interaction

In the assembly of LukF and Hlg2 into dimers and other small oligomers, most of the individual FRET intensities were equal to or twice the value of the single-FRET intensities measured. \( m \) and \( n \) in \( F \cdot H \) were deduced from the number of steps observed during photobleaching of FRET-IC5 and IC5 signals respectively. A tetramer could be formed by two pathways: (i) tetramerization of two dimers \( [F \cdot H + F \cdot H \rightarrow (F \cdot H)_2] \), or (ii) step-by-step oligomerization of monomers \( [F \cdot H + F \rightarrow F_2 \cdot H_1 + H \rightarrow (F \cdot H)_2 ; \text{or } F \cdot H + H \rightarrow F_1 \cdot H_2 + F \rightarrow (F \cdot H)_2] \). To test which is the main pathway, we measured the association constants for each stage from the concentrations of oligomeric intermediates \( (K_{F_2 \cdot H_1}, K_{F_\cdot H2}, \text{and } K_{(F \cdot H)_2}) \). The association constants for dimer-dimer tetramerization, \( K_{(F \cdot H)_2} = 3.8 \mu m^{-2} \), were >30 times those for the step-by-step processes (thin arrow), \( K_{F_2 \cdot H_1} = 0.081 \mu m^{-2} \), and \( K_{F_\cdot H2} = 0.12 \mu m^{-2} \). This result can be interpreted to mean that the step-by-step pathway is of far less significance than the dimer-dimer pathway (Fig. 5B).

Membrane-bound trimers and tetramers were observed for the first time. The abundance of dimers compared to trimers, together with the observation of similar concentrations of trimers and tetramers, even when the membrane-bound LukF monomer concentrations are ~10–35 times those of Hlg2 monomers, prove that monomers have a tendency to oligomerize into the dimer.
dimers and tetramers, rather than into trimers. Hence, dimer-dimer interaction is the major pathway for formation of tetramers (Fig. 5B). It is possible that dimerization triggers structural changes at the interaction sides of their subunits for further cooperative tetramerization.

4. Assembly into a single pore: a cooperative step

Various intermediates from dimers to larger oligomers were obtained at intermediate concentrations. Individual FRET intensities were ~1, ~2, and ~3 times the single-FRET intensity of F·H. The multi-FRET efficiencies of representative spots was also ~90%, indicating a close distance between LukF and Hlg2 components in larger oligomers.

The histogram of FRET intensities was fitted as the sum of three Gaussian distribution curves. The spots at intensity ~3 were more abundant than those at ~2, and ~4. Since the ratio of initial concentrations of F:H applied to the cells is 1:10, the population of spots at intensity ~4 presenting heptamers of 4F:3H was low. The possibility of octameric formation of 4F:4H also appeared minor. Assuming that the area under each Gaussian curve is proportional to the number of oligomers, F·H, (F·H)2, and F3·H3,4, we estimated sequential association constants K(F·H)2 and KF3·H3·4 to be 3.1 and 37 μm−2 respectively. Since single Hlg pores have been reported as hexamers and/or heptamers,28,34,57) KF3·H3·4 was designated the association constant for single a pore, Kp. Obviously, Kp was more than 10 times higher than K(F·H)2, suggesting that LukF tends to assemble with Hlg2 into hexameric and/or heptameric pores F3·H3·4 rather than into tetramers (F·H)2.

Recent reports by Comai et al., and by us, suggest respectively that 6 and 7 subunits of LukF and Hlg2 make up a single pore.28,34,57) In another report, Miles et al. have proposed an octameric structure for the staphylococcal leukocidin pore of LukF and LukS formed on lipid bilayers, based on the results obtained from gel shift electrophoresis and site-specific chemical modification during single-channel recording, but not by direct imaging.26) In view of this, we assume that the 3 × single FRET spots, which could be resolved as heptamers (F4·H3 and F3·H4), represent single pores. Cell lysis started occurring at the protein concentrations applied, confirming that functional pores were formed, although we have not been able to correlate the 3 × single-FRET spots with the direct imaging of the pore opening (see Fig. 4 in reference 23). Actually, in modeling the pathway of pore assembly (Fig. 5A), if the pore is assumed to be composed of 7 subunits, the further calculation of association constants and final fitting of intermediate populations (see Fig. 6A in reference 23) are not affected because the populations of groups and clusters of pores were simply deduced based on the value of 3 × single FRET. In this study, the F·H dimers, (F·H)2 tetramers, and F3·H4/F4·H3 single pores presented major microscopic intermediates in single pore oligomerization (Fig. 5C). The closed, ring-shaped structure of a pore would be very stable to minimize free energy at surface regions. At the pore formation step in Fig. 5A, we depict the transmembrane domains (red lines) inserted into lipid bilayers because previous studies have reported that the transmembrane domain of LukF inserts into membranes to form a barrel-shaped channel on conversion from a pre-pore to a functional hemolytic pore (see Fig. 3).37)

![Strain Diagram](image_url)

Fig. 6. Staphylococcal Genes Coding for a Family of Two-component Cytolysins.

The genes encoding classes F and S components of the staphylococcal two-component cytolsins are illustrated for seven S. aureus and one S. intermedius strain. Flags and hair pins represent the transcription initiation and termination positions on the genes respectively.
5. Assembly into clusters of pores

At higher concentrations (~15–100% hemolysis), the number of pores incorporated into each FRET spot was deduced from the FRET intensity of the spot divided by 3 times the single-FRET intensity. The association constants of single pores into two (K_{2p}), three (K_{3p}), and four pores (K_{4p}) were 1.1, 2.7, and 3.4 μm^{-2} respectively. Those increasing values indicate that single pores tend to assemble into 3-pore or 4-pore clusters rather than into 2-pore clusters.

When LukF-TMR and Hlg2-IC5 were increased over the concentrations that start causing 100% hemolysis, the power of the green excitation was reduced to 2.5%. Very highly intense domains of multi-molecular FRET, clusters of pores, were observed. We plotted a distribution of clusters with various numbers of pores on the membranes. Hlg pores have a greater tendency to condense into clusters as the concentration of toxin is increased, and may assemble into ≥3 pores because K_{3p} and K_{4p} were ~3 times higher than K_{2p}. This result is supported by the previous data showing that large clusters of pores are observed at higher concentrations of proteins (1 μM) under a transmission electron microscope. Since clusters of pores are also observed in many other toxins, even in lipid vesicles. We suppose that the aggregation of pores is not only driven by the distribution of their receptors on the membranes, but also by noncovalent linkages between amino acids located at the outer surfaces of the pores. One advantage of pore aggregation might be the switching of the equilibrium balance of single pore assembly toward association, thereby increasing the total number of pores per cell. The other advantage would be that large clusters of pores on the membranes weaken the cell membranes and enhance cell bursting. Cell membranes were recently found to be prominently disrupted in areas surrounding the clusters, as observed under TEM (Sugawara et al., unpublished data).

Other toxins from pathogenic bacteria, such as aerolysin, streptolysin, and perfringolysin O, which form homo-oligomeric pores on human target cell membranes, may also cooperatively assemble in a mechanism similar to that of Hlg. Outside of the toxin field, there are many kinds of ring-shaped membrane channels and pumps made up of multiple subunits. Selective, cooperative interactions between distinct subunits may establish structures, distributions, and functions of these membrane proteins. Notably, cooperative assembly is also fundamental to the linear polymerization of actin filaments and microtubules, wherein the nucleation mechanism is similar to cooperative single pore oligomerization. In conclusion, the three cooperative stages: dimer–dimer interaction, single pore assembly, and clusteringization of pores, substantially enhance the efficiency of assembly of oligomeric pores. These results are the first application of single molecule fluorescence microscopy to the observation of cooperative oligomeric pore assembly for a bacterial toxin on target cell membranes. The detection of heterogeneous oligomeric populations, based on single and multi-FRET analysis, is applicable to the study of individual protein interactions in living cells using conjugates fused with fluorescent proteins including CFP, GFP, and YFP. In general, single-molecule observations and statistical analysis of populations of intermediates will be useful for understanding how single molecules are brought together into macromolecular complexes in cells.

Chapter III. Genetic Organization of the Staphylococcal Two-component and Pore-forming Cytolysin Genes

1. Characteristics and genetic organization of Hlg, Luk, and PVL family genes

(1) hlg and luk: The genes coding for LukF and LukS components were first cloned and sequenced by our group in 1991 and 1992, and were designated lukF and lukS respectively. These genes are located in one cluster. LukF and LukS cooperatively lyse rabbit erythrocytes (but not human erythrocytes) as well as human and rabbit PMNLs. The Hlg genes are in a gene cluster composed of the hlgA, hlgB, and hlgC genes (Fig. 6). Of the three hlg genes of S. aureus Smith 5R, hlgB, and hlgC were found to have 97% homology with lukF and lukS respectively, which have been previously cloned by our group from S. aureus RIMD 310925. Two genes encoding LukR (lukS-R and lukF-R) were identified on the chromosome of strain P83 (ATCC 31890) (Fig. 6). LukF-R and LukS-R are considered to correspond to HlgB and HlgC respectively, due to 97% identity in their amino acid sequences. LukR can be distinguished from our Luk and Hlg of the Smith 5R strain, because LukR can lyse human erythrocytes as well as PMNLs. The Hlg/Luk cluster was found in the more than 99% of S. aureus isolates. DNA sequencing analysis of the genome of the five S. aureus strains N315, Mu50, 2MW, NCTC8325, and COL suggests that the Hlg/Luk cluster is on the stable region of each bacterial chromosome.

(2) Panton–Valentine leukocidin genes (pvl): It was known that only a small percentage of S. aureus strains have a Second locus consisting of the LukS-PV–LukF-PV cluster, which is distinct from the Hlg/Luk locus (the First locus) (Fig. 6). In France, PVL-producing strains are isolated from 2% of clinical isolates of S. aureus, whereas Luk/Hlg is produced by almost all (≥99%) S. aureus clinical isolates. However, most cutaneous necrotic lesions, such as furuncles or primary abscesses, and severe necrotic skin infections as well as severe necrotic hemorrhagic pneumonia (all community-acquired) are associated with PVL-positive S. aureus strains. PVL has been believed to be an important virulence factor in these S. aureus infections, because of its high cytolytic specificity against human PMNLs and macrophages. The PVL genes are found on the genomes
of prophages, which we isolated.\textsuperscript{25–27} (3) lukM/lukF-PV(P83).\textsuperscript{71,72} The lukM and lukF-PV(P83) were identified in the strain P83 chromosome which was originally isolated from an infected bovine udder. The lukM and lukF-PV(P83) form pvl-like operon and are on the genome of prophage pPV83-pro.\textsuperscript{73} Recently, the importance of LukM/LukF-PV(P83) as a pathogens of mastitis has been reported.\textsuperscript{74} In the report, LukF-PV(P83) is called LukF-PV. The PMNLs of ruminants, especially bovine PMNLs, are highly sensitive to LukM/LukF-PV(P83). One hundred-twenty-eight strains of \textit{S. aureus} isolated from the infected mammary glands of ruminants were tested for the presence of LukM/LukF-PV(P83) and LukED. The LukM/LukF-PV(P83)-positive strains were found in 35% of cases of cow origin, 68% of cases of goat origin, and 97% of cases of ewe origin, while all strains had genes for Hlg/Luk and LukED.

(4) lukE/lukD (lukED): In 1998, a new member of the staphylococcal two-component toxin family genes lukED and their gene products LukE and LukD were characterized.\textsuperscript{75} LukE is as effective as PVL for dermonecrosis in the rabbit skin model, but has no hemolytic or weak leukocytolytic activity. Recently, LukED variant (LukEDv), which consists of LukEv and LukDv with a strong leukocytolytic activity, was identified from \textit{S. aureus} strain ATCC 27733.\textsuperscript{76} The LukEv and LukDv components are very similar to LukE and LukD with 91 and 94% identity in amino acid sequences respectively. Interestingly, some parts of LukEv and LukDv are identical to LukS-PV and LukF-PV respectively, in their amino acid sequences (Fig. 1). It was reported that LukEDv shows leukocytotoxic and weak hemolytic activity against rabbit PMNLs and erythrocytes. It is not clear if leukocytotoxic activity means leukocytolytic activity in the report of reference 75. The sequencing of the whole genome of strains N315 and Mu50 revealed that lukEDv is on the pathogenicity island in the chromosome of these strains.\textsuperscript{62–64} Neither LukED nor LukEDv has the K/R/R/K-X-T/S segment, which is phosphorylated by protein kinase and essential for leukocytolytic activity in Luk and PVL.

(5) luk-I:\textsuperscript{74} The luk-I gene cluster, which consists of lukF-I and lukS-I, was identified in the chromosome of \textit{S. intermedius}. The gene products, LukF-I and LukS-I, cooperatively lyse human PMNLs. Luk-I also has a slight hemolytic activity on rabbit erythrocytes. Dermatonecrotic activity was also observed in the rabbit skin model. LukS-I has the KKIS segment in the root of the stem region (see the chapter I).

2. Genomic structure of PVL-carrying phages

(1) Exploration of PVL-carrying phages: Vijver et al. have reported evidence of lysogenic conversion in \textit{S. aureus} by a group A phage leading to an increase of “leukocidin” production.\textsuperscript{77} But it is not clear which leukocidin was concerned, Luk or PVL, because the assay system used could not distinguish PVL from Luk, and the direct evidence for a phage conversion for “PVL genes” was not available. In 1997, the gene cluster for PVL-like toxin components, lukM and lukF-PV(P83), in the strain P83 (ATCC 31890) and their flanking regions were sequenced, and a part of orf which encodes the possible phage lytic enzyme was found just upstream of lukM and lukF-PV(P83).\textsuperscript{72} We isolated a phage pPV with a hexagonal morphology (55–60 nm), which contains an approximately 40 kb double strand DNA genome carrying PVL genes, from a lysate of mitomycin C-treated \textit{S. aureus} V8.\textsuperscript{25} After sequencing of the flanking region of pvl, genes encoding a possible lytic enzyme and integrase, which are derived from the phage, were found upstream and downstream of pvl respectively. These findings show the presence of PVL-carrying prophage and a horizontal transmission of the PVL gene cluster via the bacteriophage. But no tail structure was observed in the phage particles nor could any \textit{S. aureus} strain tested be infected by the phage particles. Since pPV was suggested to be defective phages, other PVL-carrying phage(s) having maintenance of their infectious ability were desired to obtain evidence of phage conversion leading to production of PVL. In 2001, a novel temperate phage, φSLT (Staphylococcal Leukocytolytic Toxin), which converted PVL-negative strains of \textit{S. aureus} into PVL producers by infection, was isolated, and the complete nucleotide sequence of the φSLT genome was analysed by us.\textsuperscript{27} Sequencing of two \textit{XboI} fragments of 1893 bp and 1773 bp, which were hybridized with the pvl probe, showed that the C-terminal coding part of the lyt, lukS-PV, lukF-PV, and attP sites, and the C-terminal coding part of int of this phage are identical to those of φPV. But the restriction patterns of the 42-kbp genome of φSLT are distinct from those of the φPV genome.\textsuperscript{27} In contrast to the V8 strain, we obtained no phage particle carrying pvl regardless of treatment of \textit{S. aureus} P83 with mitomycin C. To examine the presence of prophage containing pvl and the defective induction mechanism(s), we determined the entire nucleotide sequence of 46 kbp of the flanking region of lukM–lukF-PV, and identified a novel prophage genome which we designated φPV83-pro. It was found that the lukM–lukF-PV cluster is located 2.1 kb upstream of the attR (originally named attL of p11) site and that two kinds of insertion sequences (ISs) are integrated into the phage genome (Fig. 7).\textsuperscript{73,78} Details are described bellow.

(2) Genomic structure of PVL-carrying phages:

(i) φPV: The complete nucleotide sequence of the φPV genome was determined for the first staphylococcal bacteriophage (Fig. 7).\textsuperscript{26} The linear double-stranded DNA of the φPV genome comprises 41,402 bp with 3’-staggered cohesive ends (cos) of 9 bases and contains 62 orfs (41,421b with att core sequences of 29 bases). The genome of φPV is separated into the lysogeny, replication, packaging, head, tail, and lysis regions. The pvl genes are located near the att site, and the genes
of the lytic enzyme (lyt) (peptidoglycan hydrolase), the [lukS-PV–lukF-PV] cluster, and the phage-bacterial junction (attR) are arranged in that order. φPVL is integrated into the 29 bp-attB-core sequence in the C-terminal region of an ORF encoding an unknown protein comprising 725 amino acid residues. Capsid and portal proteins were identified from the analysis of the N-terminal sequence of protein bands, which were isolated from φPVL particles. The N-terminal 116 amino acid segment, which has a highly coiled structure of pre-capsid protein, might be removed during the phage head maturation by protease that is a product of the gene, which is located between the portal protein gene and the capsid gene. This feature is the same as that established in the capsid maturation in coliphage HK97.89,90)

(ii) φSLT:27) φSLT has an elongated head of about 100 nm × 50 nm and a flexible tail 400 nm long, that is quite different from φPVL, which has an isometric hexagonal head of about 60 nm diameter. The linear double-stranded φSLT genome comprises 42,942 bp and contains 62 ORFs (Fig. 7). Both the attL and the attR sites on lysogen of φSLT are identical to those of φPVL, φPSL, and φPV, integrated into the same 29 bp-attB-core sequence of the host chromosomal DNA. The cos site of φSLT is made of 3′ extended DNA with 10 complementary bases with a high GC content. Only the 6.4-kbp region containing lysis cassette, PVL genes, attP, integrase, and orf204 of φSLT is identical to that of φPVL, but other regions are different from the corresponding ones of φPVL.

The N-terminal sequences of 8 protein bands on the SDS-PAGE of φSLT were determined, and an N-terminal 113 amino acid of capsid protein (ORF387) was found to be processed by a possible protease encoded by orf257 by the same system in the head maturation of HK97. Recently, a novel structural protein ORF636 with 66 kDa of φSLT, which contains two 97-amino acid sequence repeats with the sequence GW in its C-terminal region, was identified as an essential structural protein for the adhesion of φSLT to S. aureus through staphylococcal lipoteichoic acid.81) (iii) φPV83-pro:73) The precise genome size of

Fig. 7. Genetic Organization and Comparison of the Genomes of φPVL, φ13, and φPV83-pro, Which Belong to the φPVL/φ13 Group, and φSLT, φSa2mW, φ12, and φCOL, Which Belong to the φSLT Group.

Schematic representation of genomes of the phages, which belong to the φPVL/φ13 and φSLT groups with their putative orfs and features, were compared among the phages in each group. Each genome is linearized at the att sites (boxes in both sites), and the cos site is indicated by an oval. Gray shading indicates the identical regions in nucleotide sequence among phages in the two groups. For each phage, the upper and lower boxes represent right-to-left and left-to-right transcription units respectively. Junctions (A, B, C, and D) are indicated by arrow heads under the panel of φPV83-pro. In φPV83-pro, the number in parenthesis represents the number of bp of whole DNA excluding the total number of bps of ISS17 and ISS22. For further explanation, see the text.
φPV83-pro is 45,636 bp with an att core sequence of 10 base pairs. Sixty-four orfs including the lukM–lukF–PV(P83) and orfs-62-63 clusters were identified. Unlike φPVL, the φPV83-pro genome has two unidentified orfs62 and 63, which may be co-transcribed, between lukF–PV(P83) and the att site (Fig. 7). In contrast to lukM–lukF–PV(P83), the G + C content of the orfs62– orfs63 operon was clearly lower (27.3%) than that of other regions of the φPV83-pro and the host chromosome (34%). This suggests that this operon may originate in the genome of bacteria with low G + C content which infected the ancestor phage of φPV83-pro. The φPV83-pro genome consists of the attachment sites of φ11, genes encoding the packaging and morphogenesis region of φPVL, and the region from other phage(s) which occupied about 40% of φPV83-pro. Two kinds of insertion sequences (ISs) of 1070 bp (ISSA1) and 1946 bp (ISSA2) were integrated into the att site and orf in the tail region respectively. Insertion of ISSA1 into the att site and one base-missing of the structure gene of the integrase of φPV83-pro appeared to reject the excision of prophage despite mitomycin C treatment of the strain P83.

(3) Attachment sites of PVL carrying phages on the S. aureus chromosome: The flanking region of the attB site for the φPVL in the host genome was analyzed.²⁶ The ORF725, which was divided by PVL lysogenization, was composed of 2,175 bp which encodes a polypeptide which consists of 725 amino acid residues. It should be noted that the reading frame for the C-terminal 60 residues of the polypeptide was shifted into the frame of φPVL, when φPVL genomic DNA integrated into the attB site of the ATCC 49775 chromosome. As a result, it was found that the C-terminal 60-residue segment LNTQAHLRDQM-[48 amino acid residues]-carboxyl terminus was replaced by the 12-residue segment SRKALTPESSVL. A search of protein data bases did not reveal any protein with significant homology to this one. Therefore no information about the negative lysogenic conversions caused by the integration of φPVL into the gene for ORF725 is available. φSLT is also integrated into the C-terminus with the same 29 bp–attB-core sequence as φPVL. But the deduced amino acid sequence of the N-terminal portion of the corresponding ORF was different from that of ATCC49775. The genomic sequencing of several strains of S. aureus indicated a polymorphism of the ORF containing the attB for φPVL/φSLT. In contrast, the attB for φPV83-pro was the same as for φ11, and these phages were integrated into the 9-bp–attB-core sequence in the intergenic region between purA and meta.⁷³,⁷⁸,⁸²

3. Molecular evolution of PVL-carrying phages

(1) Genomic classification of the reported staphylococcal temperate phages:

Bacteriophages of S. aureus have been used as the phage typing of human pathogenic S. aureus strains.⁸³,⁸⁴ In the International Typing Set of Staphylococcal Phages, 23 reported temperate phages have been classified on the basis of lytic activity, morphology, and serological properties.⁵⁵ Among them, phages with isometric head and short tail of up to 200 nm, isometric head and long tail of more than 200 nm, and elongated head and long tail belong to serological groups B, F, and A respectively. Restriction mapping data and Southern analysis of the phage genomes with the specific DNA probes support this classification at the genomic level. Phages which belong to groups A and F have cohesive ends, whereas the phages of serotype B do not.⁵⁵

Canchaya et al. classified the sequenced Staphylococcal Siphiviridae phages or prophages into five groups on the basis of similarity of genomic structure by the dot plot matrix method.⁸⁶ This classification is linked to the head and tail genes of phages. In this article, we propose the naming of the following five groups:

(i) The φPVL/φ13 group: This group includes φPVL, φPV83-pro, prophage φSa3mw,⁵⁵,⁶⁶ which carries seg2, sek2, sep, and sak, and a double conversion phage φ13 belonging to serotype F, which is characterized by positive and negative conversions of staphylokinase and β-hemolysin respectively.⁸⁵,⁷⁷,⁸⁸ Phages in this group have cohesive ends, φ13 has an isometric head the same as φPVL in size. Both share the morphogenesis region in their genomes.²⁶,⁸⁹

(ii) The φN315 group: This group includes φN315 and φMu50A, which share about half of the morphogenesis region with that of φ13.⁶²–⁶⁴

(iii) The φSLT group: φSLT and prophages φSa2mw,⁵⁵,⁶⁶,⁷² φ12,²⁶,⁸⁹ and φCOL⁵⁷ belong to this group. The PVL-carrying prophage φSa2mw was found in the genomic sequencing data from S. aureus strain 2MW, a typical community-acquired MRSA strain. Prophages φ12 and φCOL were found on the chromosomes of S. aureus strains NCTC 8325 and COL respectively. The identity of the nucleotide sequences containing head and tail morphogenesis regions among the 4 phages strongly suggested that φSa2mw, φ12, and φCOL have identical morphology (Fig. 7). φSLT, a cos site phage with elongated head and long tail has the same characters as typical staphylococcal serogroup A phages.²⁶,⁸⁵

The following two groups include pac-site phages.

(iv) The φ11 group: This group includes phage φ11 and prophage φMu50B. Phage φ11 is a serotype B phage with isometric head and short tail.²⁸,⁹⁰

(v) The φETA group: Only exfoliative toxin A-converted pac-site phage φETA⁹¹ which has a similar morphology to φ11, belongs to this group.⁸⁶

There are few similarities in entire nucleotide sequence between pac-site phages and our 3 PVL-converting phages. No information on the presence of the PVL-carrying phage which belongs to the pac-site phage group is available. The table shows a summary of characterizations of staphylococcal temperate phages
and prophages which belong to the five groups.

(2) Chimeric and mosaic structures of the genomes from the PVL-carrying phages:

Multiple alignment among PVL-carrying phages and other related phages revealed the chimeric and mosaic structures of these phage genomes (Fig. 7). These structural differences of phage genomes are due mainly to the DNA recombination events proposed by Bostein as a “modular theory” for the horizontal evolution of the phage genome. Module means the exchangeable genetic unit among phages which consist of functional related genes.

As described above, PVL-carrying phages are classified into two major groups, viz., groups \(\phi PVL/\phi 13\) and \(\phi SLT\). Typical chimeric structure is observed in \(\phi PVL/\phi 83\)-pro, which belongs to the \(\phi PVL/\phi 13\) group. This prophage has an \(att\) site and an integrase gene of the pac-site phage, \(\phi 11\), while its packaging and morphogenesis modules are from the cos-site phage, \(\phi PVL\). These dynamic recombination events occur at the conserved region between functional modules, that is, the junction among the three phages (Fig. 7). Junction A consists of a segment of about 90-bp at the intergenic region of the integrase gene and the \(orf\) which is located at the left end of the lysogeny module. Junction B comprises a region of about 120-bp containing the \(rinB\) gene. Within the packaging and morphogenesis gene clusters between junctions B and C, no modular exchanges were observed. But insertions and deletions of \(orfs\) were observed in these modules. The region containing \(att\) and integrase, which are involved in the site-specific recombination between viral and bacterial genomes, exists between junctions D and A. Exchange of this region among staphylococcal phages is also observed in \(\phi COL\), with \(att\) sequences derived from \(\phi LS4a\), which belongs to the \(\phi SLT/\phi 12\) group. The PVL genes were located between the lysis module and the \(attR\) site as an independent transcribed unit which transcribed by its own promoter, and prophages \(\phi PVL\), \(\phi SLT\), and \(\phi Sa2mw\) share the 6.4-kb region consisting of the lysis module-pvl-\(attP\) site-integrase gene (Fig. 7). Recently, we analysed the entire genomes of 30 PVL-carrying phages from clinical isolates and found that all the phages examined had the 6.4-kb region. The data suggest that the 6.4-kb region might function as a PVL module for horizontal exchange across the PVL-carrying prophages in different groups (Fig. 8A). When compared with the genome between \(\phi SLT\) and \(\phi Sa2mw\), the region between the \(cos\) site and the integrase gene, which contains the genes for packaging, head and tail morphogenesis, lysis modules, and PVL, is identical. But the lysogeny and DNA replication/transcription regulation modules are different from each other (Fig. 7). Taken together with the modular theory, these findings lead to the following two possible mechanisms concerning the evolution of \(\phi SLT\) and \(\phi Sa2mw\): (a) the PVL module has been exchanged among the two phages, or (b) both \(\phi SLT\) and \(\phi Sa2mw\) are derived from the same ancestor.
phage which acquired PVL genes by an exchange(s) of the lysogeny and DNA replication/transcription regulation modules (Fig. 8B). Recently, it was found that φSa3mw in the strain 2MW carries two enterotoxin genes, sek2 and seg2, between the genes for cI repressor and integrase (Fig. 8C).65,66) DNA sequencing analysis of a related φSa3ms shows that transcription of seg2 and sek2 initiate from upstream of the cI promoter.95) Although all the sequenced PVL-carrying phages have no extra toxin genes in their genomes, they are capable of becoming multiple conversion phage not only with PVL genes but also with other toxin genes through exchange of modules between two phages (Fig. 8C).

Acknowledgments

We are grateful to the Editor-in-Chief of this journal, Dr. Isomaro Yamaguchi of the University of Tokyo, for enabling us to write this article. Our works cited in this article were supported in part by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (05304028, 06670280, 07660089, 08307004, 08660086, 09460042, 11694191, 11460034, 13460034, and 15380054 to Y. K.; and 09760064, 11760050, 13660075, 15580055 to J. K.), the Naito Foundation, the Yamada Science Foundation, the Takeda Science Foundation, the Uehara Memorial Foundation, the Intelligent Cosmos Academic Foundation, and the Japanisch–Deutsches Kulturinstitute Foundation. We thank all of our co-workers, past and present, for their experimental as well as their conceptual contributions.

References


62) Kuroda, M., Ohta, T., Uchiyama, I., Baba, T., Yuzawa,

64) Mu50 website; http://w3.grt.kyushu-u.ac.jp/VRSA/


66) 2MW website; http://www.bio.nite.go.jp:8080/dogan/

67) 8325 website; http://www.genome.ou.edu/staph.html


71) Chooriti, W., Kaneko, J., Muramoto, K., and Kamio, Y. 1995. "Existence of a new protein component with the same function as the LukF component of leukocidin or g-hemolysin from Staphylococcus aureus ATCC 49775 are encoded by distinct genetic loci and have different biological activities." Infect. Immun., 63, 4121–4129.


94) Chiba, J., personal communication.