

Staphylococcus aureus Bicomponent γ -Hemolysins, HlgA, HlgB, and HlgC, Can Form Mixed Pores Containing All Components

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Staphylococcal γ -hemolysins are bicomponent toxins forming a protein family with leucocidins and α -toxin. Two active toxins (AB and CB) can be formed combining one of the class-S components, HlgA or HlgC, with the class-F component HlgB. These two γ -hemolysins form pores with marked similarities to α -toxin in terms of conductance, nonlinearity of the current–voltage curve, and channel stability in the open state. AB and CB pores, however, are cation-selective, whereas α -toxin is anion-selective. γ -Hemolysins' pores are hetero-oligomers formed by three or four copies of each component (indicated as 3A3B and 3C3B or 4A4B and 4C4B). Point mutants located on a β -strand of the class-S component that forms part of the protomer–protomer contact region can prevent oligomer assembly. Interestingly, these mutants inhibit growth of pores formed not only by their natural components but also by nonstandard components. This lead to the hypothesis that mixed ABC pores could also be formed. By studying the conductance of pores, assembled in the presence of all three components (in different ratios), it was observed that the magnitudes expected for mixed pores were, indeed, present. We conclude that the γ -hemolysin/leucocidin bicomponent toxin family may form a larger than expected number of active toxins by cross-combining various S and F components.

INTRODUCTION

Staphylococcus aureus is one of the major human pathogens, causing diverse infections both in the community and during hospitalization. Among a large panel of virulence factors, it produces leucotoxins, among which, the γ -hemolysins (Hlg) and Panton–Valentine leucocidins (Luk-PV) share the peculiarity of being bicomponent. (See the abbreviations section at the end of the text for abbreviations used in this paper.) These toxins comprise two different but structurally related proteins belonging to class S (only LukS-PV, HlgA, and HlgC were studied here) and class F (only HlgB was studied). Individually secreted components are totally inactive.^{1,2} They act synergically to efficiently damage host defense cells after assembling into a membrane-bound oligomer. Leucotoxins target human polymorphonuclear cells, monocytes, macrophages, and red blood cells, but in many cases, they also interact with model membranes.³ Since the discovery of leucocidins by Panton and Valentine in 1932,⁴ the number of bicomponent toxins has grown to at least 13 members.³ Staphylococcal leucocidins and γ -hemo-

lysins actually form a single family but are included in the superfamily of β -barrel pore-forming toxins, which comprises the staphylococcal α -toxin.^{5,6} Sequence identity among different class-S components is around 63–75%, while a slightly lower identity is found when comparing class-S components with LukF-PV or HlgB. On the other hand, LukF-PV and HlgB are around 70% identical. Interestingly, α -toxin has around 26–30% sequence identity with class-F components and 20–25% with class-S components. It was demonstrated that, although the sequence alignment among α -toxin and leucotoxins is not strictly conserved, the 3D structure of each protomer is similar.⁷ Moreover, the solved X-ray structure of LukS, superimposed on that of LukF-PV, established that the class-S configuration resembles that of class F.⁸ This homogeneous structural organization and the comparable function displayed strongly suggest that these pore-forming toxins evolved from a common ancestor.⁸

A single locus encodes γ -hemolysins, and it comprises three genes. The first one (*hlgA*, encoding HlgA) constitutes a single open reading frame, whereas the other two (*hlgC* and *hlgB*, encoding HlgC and HlgB, respectively) are cotranscribed.⁹ Thus, the γ -hemolysin bicomponent toxins may display two bicomponent combinations (A + B and C + B). Since the V8 strain (ATCC 49775) produces both Luk-PVL and γ -hemolysins,¹⁰ the possibility exists of finding leucotoxic or hemolytic activity in mixed couples containing one leucocidin protein plus one γ -hemolysin protein. Indeed,

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it was found that the mixed couple HlgA + LukF-PV presented both toxic activities, whereas the couples HlgC + LukF-PV and LukS-PV + HlgB presented only leucotoxic properties.^{2,10} It was inferred that the S component is responsible for the selectivity of the couple,^{11,12} concluding that HlgA and HlgC are able to bind to both red and white cells, whereas LukS-PV-derived couples can attack only polymorphonuclear cells.

Therefore, a given *Staphylococcus aureus* strain produces at least two potent leucotoxins by the bias of the constant γ -hemolysin locus.¹³ They are produced at relatively low levels in vivo but occur early during the exponential growth of the bacteria, especially HlgA.¹⁴ Since it is unknown if HlgA and HlgC compete on the same cell sites alone, it is possible that both might interact at neighbor sites or that the presence of an F protein might be involved in their binding.

The dissociation constants of HlgB–HlgA and HlgB–HlgC are very similar,^{11,15,16} supporting the idea that leucotoxins may also create mixed pores constituted by the three components. The fact that this pathogen can produce from three to seven related proteins leading to binary or mixed leucotoxins may confer to the bacterium some advantages with respect to broad cell specificity. It also may confer a means to escape the specific humoral immune response and its primary activation and to promote cell lysis and a more rapid acquisition of nutrients for the bacterium.

We have previously described the properties of two mutated S components of the γ -hemolysins: HlgC T30D (C_{T30D}) and HlgA T28D (A_{T28D}).¹⁵ This position corresponds to T28 of LukS-PV and to H35 of α -toxin, as it results from the sequence alignment. This important position is located at a crevice formed by strand 6 and the loop between strands 9 and 10 of the neighboring protomers.¹⁷ A mutation of this residue in LukS-PV does not change the overall structure of the T28 mutant, but it is crucial in terms of activity.⁸ Accordingly, both C_{T30D} and A_{T28D} retained their ability to bind human polymorphonuclear leukocytes and rabbit red blood cells (RRBCs) but were unable to secondarily bind the F component (HlgB) and form the binary toxin. As a consequence, both couples were not only inactive but were also able to decrease the activity of the corresponding functional wild-type (wt) component. Such an inhibition was observed on cells and on model membranes (i.e., lipid vesicles). This excludes the case of exclusive competition with the S-component receptor. Inhibition could, instead, be clearly attributed to the ability of the mutants to incorporate into neo-forming heterologous oligomers containing wt S and F components and block the oligomeric pore achievement. In this way, the consumption of functional components into nonfunctional oligomers was produced, leading to the observed inhibition.

We have now investigated the interaction of these and similar mutants in more detail, with particular attention to their ability to inhibit other S components of the family. Point-mutated class-S components (unable to form functional oligomers) inhibit the activity of various couples on red blood cells and on model membranes. The idea of an interposition of mutants on oligomers, comprised of different class-S and class-F components, was confirmed by the analysis of the conductance of mixed pores which were assembled in the presence of all three components introduced in different ratios. The conclusion is that mixed pores formed by two

class-S components and HlgB could assemble on both biological and model membranes.

MATERIALS AND METHODS

Chemicals. The lipids used were phosphatidylcholine (PC) and 1,2-diphytanoyl-sn-glycerophosphocholine (DPhPC), purchased by Avanti Polar Lipids, and cholesterol (Cho) by Fluka, all more than 99% pure by thin-layer chromatography. Calcein, EDTA, and Sephadex were from Sigma; the Triton X-100 was from Merck. The preparation and purification of single leucotoxin components has been described previously.^{10,15} Lyophilized α -toxin was kindly supplied by Dr. Hungerer (Behring, Marburg, FRG) and used without further purification. α -Toxin mutants were a kind gift from Prof. Sucharit Bhakdi and have been described previously.¹⁸

Mutants. Mutants were constructed using the Quick-Change mutagenesis kit (Stratagene) and dedicated oligonucleotides as described previously.¹⁹ After removal of the GST tag with Precision Protease (Amersham-Pharmacia), the proteins were purified by affinity chromatography on glutathione-Sepharose 4B followed by cation-exchange fast-performance liquid chromatography.¹⁰ They were then controlled for homogeneity by radial gel immunoprecipitation and SDS-polyacrylamide gel electrophoresis before being stored at -80°C .

Determination of the Hemolytic Activity. RRBCs were prepared from fresh venous blood collected in 6 mM EDTA and washed thrice (10 min centrifugation at 700g, room temperature) in 30 mM Tris-HCl, 100 mM NaCl, and 1 mM EDTA at pH 7.0 (hereafter, buffer A).

The time course of hemolysis was followed photometrically at 650 nm, in a 96-well microplate, as described previously.²⁰ In each well, mutant toxins were 2-fold serially diluted and added to the protein couple (the two components at equimolar concentrations, if not otherwise stated) in a final volume of 100 μL of buffer A. The reaction was started by adding 100 μL of RRBC, at a final concentration of 0.13% (v/v), which corresponds to an initial A₆₅₀ value of 0.1. The microplate was stirred and read every 8 s for 45 min. The extent of hemolysis was calculated as follows:

$$\% \text{ hemolysis} = 100(A_i - A_f)/(A_i - A_w)$$

where A_i and A_f are the absorbances at the beginning and end of the reaction and A_w is that obtained after hypotonical lysis with pure water.

Permeabilization of Lipid Vesicles. Large unilamellar vesicles (LUV) comprised of PC/Cho (1:1, molar ratio) were prepared by extrusion through polycarbonate filters (carrying 100 nm holes) of a multilamellar liposome preparation. Liposomes, as a 3 mg lipid/ml suspension, were prepared in 80 mM calcein, neutralized with NaOH. The untrapped dye was removed by washing on a Sephadex G-50 equilibrated with buffer A. The permeabilizing activity of the toxins on the vesicles was evaluated by measuring the release of calcein.²¹

Single-component mutants were 2-fold serially diluted in buffer A supplemented with a fixed amount of the bicomponent toxin. Finally, an aliquot of washed LUV (at a final lipid concentration of 10 μM in 200 μL) was added to each well. The time course of calcein release was recorded by the increase in fluorescence due to the dequenching of the

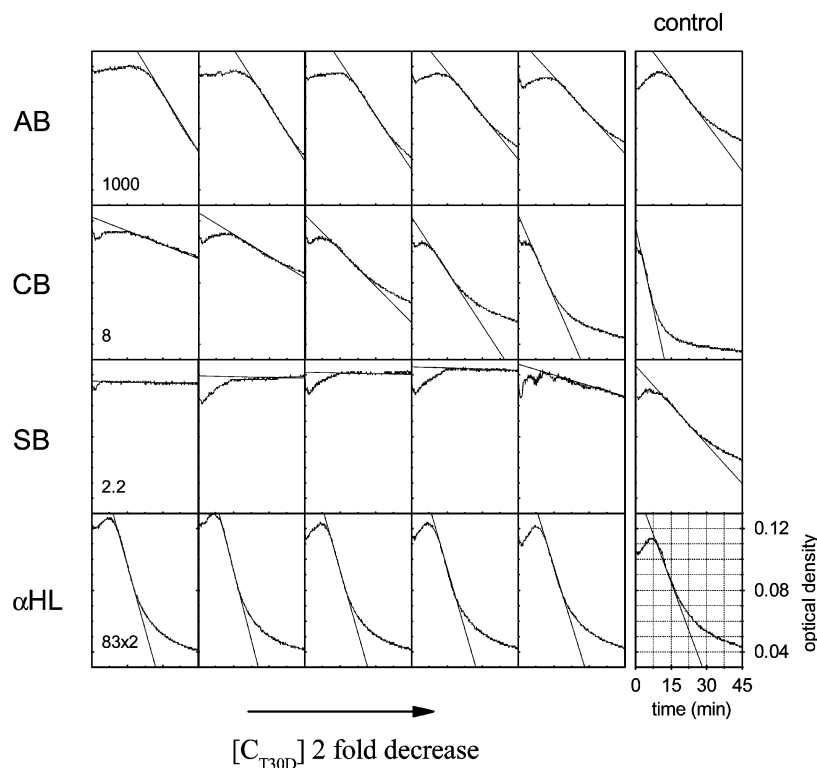


Figure 1. Inhibitory effect of HlgC T30D on four hemolytic toxins. The hemolysis kinetic of RRBC exposed to the action of three bicomponent toxins and α -toxin (as indicated next to each row) was measured with a microplate reader in the presence of different amounts of C_{T30D} . The time course of hemolysis was followed turbidimetrically at 650 nm. The amount of toxin was constant along each row; the inhibitory C_{T30D} was, instead, diluted in 2-fold steps from well to well, starting from a concentration of $0.3 \mu\text{M}$ in the first column. The molar ratio $C_{T30D}/(\text{S component})$ in the first column is given at the bottom, inside the first well. The RRBC concentration was 0.13% (v/v). Scales are the same for each well and are indicated on the bottom right panel. The last column is a control in the absence of an inhibitor. The straight lines, superimposed over the experimental curves, are maximum slopes. The control column represents the toxin activity in the absence of C_{T30D} .

released dye, which dilutes in the external medium. Toxin-induced permeabilization was calculated as

$$R\% = 100(F_f - F_i)/(F_m - F_i)$$

where F_i is the initial fluorescence before adding the toxins, F_f is the steady-state value at the end of the kinetic (45 min), and F_m is the maximal value after the addition of 1 mM Triton X-100. The spontaneous release of calcein was negligible.

Planar Lipid Membranes. The electrical properties of the pores were studied on planar lipid membranes (PLM) formed by the apposition of two monolayers of DPhPC spread from 5 mg/mL lipid solution in *n*-hexane. Toxins were added on one side only (called *cis*, while the *trans* side was used as a reference) to stable, preformed bilayers in nanomolar concentrations and at constant applied voltage (+40 mV). All experiments were performed in 10 mM Hepes, 100 mM KCl, and 0.1 mM EDTA at pH 7 at room temperature, as already described.²² Single pore opening events were recorded as discrete steps of currents by using a patch-clamp amplifier (3900A of Dagan Corporation) equipped with the 3910 expander module for PLM application. Membrane current was stored on a VCR-PCM recorder (PCM-701ES from Sony) after lowpass filtering the signal at 1 kHz. To manually analyze data, signals were filtered at 0.1 kHz.

RESULTS AND DISCUSSION

Competition in Hemolytic Activity. The ability of C_{T30D} to inhibit the hemolytic action of wt components was

examined with respect to RRBC hemolysis induced by different leucotoxins (Figures 1 and 2b). A clear competition with the couple HlgC–HlgB (Figure 1, second row) was observed in terms of all the significative parameters related to hemolysis, that is, a lower extent (Figure 2b), smaller maximal rate, and longer delay before onset. Interestingly, an even stronger inhibition was observed with the couple LukS–HlgB (third row), a nonstandard couple which we have shown to be hemolytic also.^{10,20} Furthermore, a certain competition, essentially in terms of increasing the lag time before the beginning of the hemolysis, was also observed with HlgA–HlgB (first row). No inhibition whatsoever was seen with the parent staphylococcal toxin α -toxin (last row). The dose dependence of toxin inhibition by C_{T30D} was examined in more detail in Figure 2b, in terms of the percentage of hemolysis. The cross-competition between C_{T30D} and other S components of the binary toxins (e.g., HlgA and LukS) is intriguing. It may indicate a simple competition for a common receptor (or acceptor) shared on RRBC membranes, but it may also suggest the possibility that C_{T30D} coassembled into S–B (or A–B) oligomers is blocking (or at least retarding) their growth. Therefore, the possibility of cross-competition was further investigated, examining the effects of A_{T28D} , LukS–PV T28D (S_{T28D}), and α -toxin H35R (αHL_{H35R}) on various hemolytic toxins (Figure 2 and Table 1).

We observed that A_{T28D} was able to decrease the percentage of hemolysis induced not only by the couple HlgA–HlgB but also, even though to a much lower degree, by the

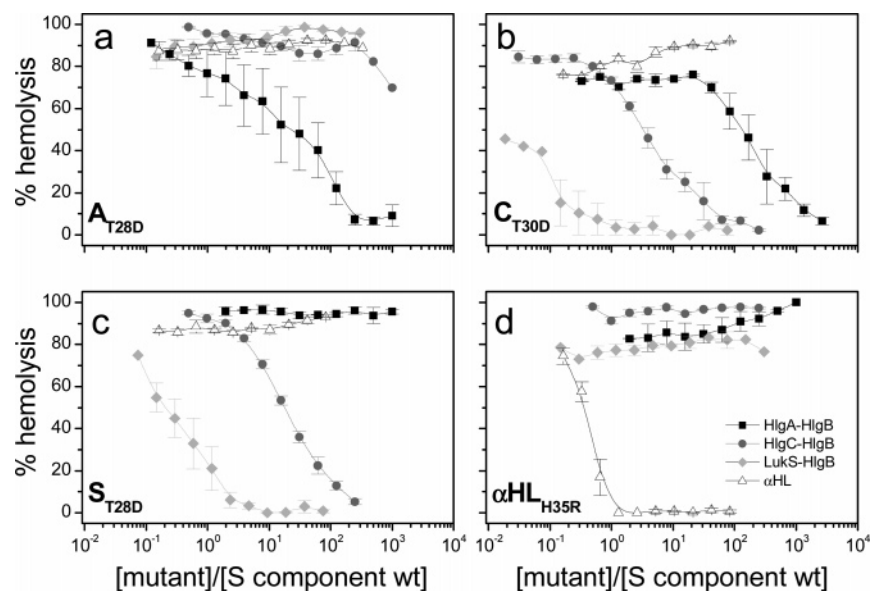


Figure 2. Dose-dependence cross-inhibition of the hemolytic activity of three leucotoxins and α -toxin. Percent of hemolysis was determined as described in the Materials and Methods. Experiments were as in Figure 1, except that all four inhibitory mutants were tested and reported one per panel. Experiments were performed on RRBC. Different symbols and gray levels have been used for different couples, as indicated. Points are mean \pm standard error of the mean (SEM) of three to four independent experiments. The concentrations (in nM) of the wt S component were as follows: 0.3–0.45, 1.2, 4.0, and 3.6 for AB, CB, SB, and α -toxin, respectively. In the absence of the any inhibitory mutant, these concentrations caused a percentage of hemolysis between 80 and 90%.

Table 1. Summary of the Cross-Inhibitory Effect on Hemolysis^a

inhibitor	toxin							
	A–B		C–B		S–B		α	
	R_{50}	c_{50} /nM	R_{50}	c_{50} /nM	R_{50}	c_{50} /nM	R_{50}	c_{50} /nM
A _{T28D}	46 \pm 28	15 \pm 8	1000 ^b	1200 ^b	— ^c	—	—	—
C _{T30D}	250 \pm 160	74 \pm 9	4.8 \pm 0.9	45 \pm 18	0.12 \pm 0.09	11 \pm 11	—	—
S _{T28D}	—	—	17 \pm 2	21 \pm 3	0.4 \pm 0.2	2.9 \pm 1.0	—	—
α HL _{H35R}	—	—	—	—	—	—	0.43 \pm 0.04	1.5 \pm 0.2

^a R_{50} : ratio of mutant over wt S component (or α -toxin) providing a 50% reduction in the percentage of hemolysis. Reported is mean \pm SEM (three to four experiments). c_{50} : concentration of mutant providing a 50% reduction in the percentage of hemolysis (mean \pm SEM of three to four experiments). ^b Highest concentration tested, providing 30% of inhibition. ^c Dash means no inhibitory effect.

couple HlgC–HlgB (Figure 2a and Table 1). On the other hand, it did not change the amount of hemolysis induced by the couple LukS–HlgB or by α -toxin. S_{T28D}, instead, was strongly inhibitory for the couples LukS–HlgB and HlgC–HlgB, but it was ineffective on HlgA–HlgB and α -toxin (Figure 2c). Finally, α HL_{H35R} was a powerful inhibitor of its native protein, α -toxin, but it did not give any indication of cross-competition either with HlgA–HlgB, HlgC–HlgB, or LukS–HlgB (Figure 2d). The results were similar if the maximal rate of hemolysis or the lag time was, instead, examined (data not shown).

To investigate whether, for the observed cross-competitions, a common receptor is required, we decided to perform similar experiments on pure lipid model systems.

Competition in Permeabilization of Lipid Vesicles. The ability of the mutants to reduce the permeabilization of LUV ensued by different bicomponent toxins and α -toxin was, therefore, examined. LUV was prepared with a mixture of PC/Cho in a 1:1 molar proportion, which is a composition very sensitive to toxin action.^{15,20}

The investigation was limited to HlgA–HlgB, HlgC–HlgB, and α -toxin since these are the only toxins able to induce the release of calcein from the liposomes (at least up

to the maximum concentration of 10 μ g/mL per component tested).²⁰ A_{T28D} inhibited the permeabilization of all three toxins studied, despite the fact that different molar ratios of mutant over wt toxin were needed (Figure 3a). The least efficient competition, albeit clearly present, was with α -toxin. C_{T30D} gave a similar pattern of competition and cross-competition (Figure 3b). α HL_{H35R} was able to inhibit LUV permeabilization of the parent hemolysins (Figure 3c). Since there is no endogenous receptor on LUV, the inhibition, observed on both LUV and RRBC, cannot be ascribed to a competition between wt and mutated S components for a pre-existing receptor. This finding reinforces the idea that cross-interaction of the S component with growing oligomers may indeed occur.

Cross-Interaction in the Channels Formed in Planar Lipid Membranes. Toxins able to release calcein from liposomes, that is, HlgA–HlgB, HlgC–HlgB, and α -toxin, are also able to form channels in PLM.^{6,22–24} The pores formed by γ -hemolysins and α -toxin show marked similarities at least in terms of the shape of the current–voltage curve (slightly nonlinear) and channel stability in the open state.²² However, under the same conditions, the conductance of the pore at +40 mV was relatively different: 90 pS

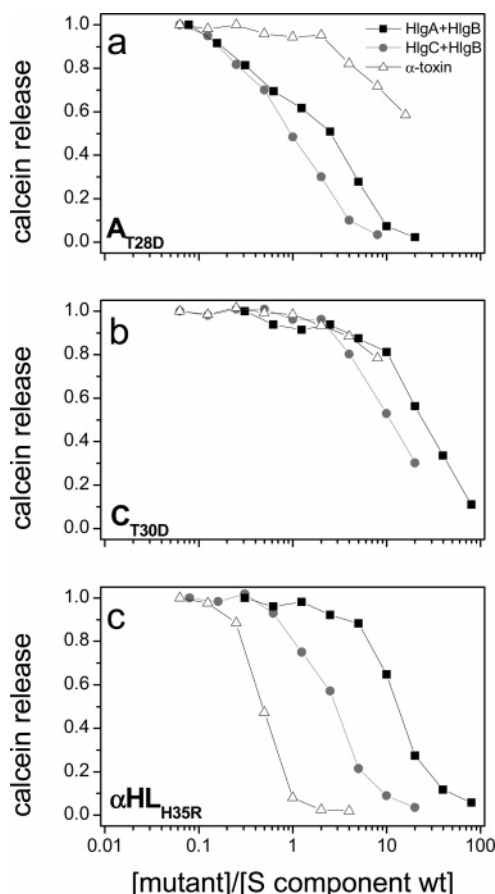


Figure 3. Dose-dependence cross-inhibition of the permeabilizing activity of three leukotoxins and α -toxin on liposomes. Normalized calcein release was determined as described in the Materials and Methods. Experiments were performed on LUV prepared with a mixture of PC/Cho in a 1:1 molar proportion. Different symbols and gray levels have been used for different couples, as indicated. Points are mean \pm SEM of three to four independent experiments. The concentrations (in nM) of the wt S component were as follows: 10–50, 40–160, and 260–300 for AB, CB, and α -toxin, respectively. In the absence of the any inhibitory mutant, these concentrations caused a calcein release between 60 and 80%.

α -toxin,²⁵ 115 pS AB, and 190 pS CB, as shown in Figure 4c (AB = 1:1) and Figure 4h (CB = 1:1). These results are in agreement with refs 22 and 24.

The high structural similarity^{7,8} between bicomponent toxins and α -toxin allows us to postulate that they form similar β -barrel pores. The heptameric oligomer of α -toxin was proved to be a reasonable model for the γ -hemolysin pore,^{22,24} confirming that the structures of both pores are nearly the same. However, some obvious differences do exist, the first being that both components (S and F) should be present in the γ -hemolysin pores. The stoichiometry of the single γ -hemolysin oligomer is still debated. It has been proposed to be a hexamer,^{20,22,26} a heptamer,²⁷ or an octamer,²⁸ all agreeing with an equimolar (1:1) presence of each component on average. Note that, in the case of heptamers, the presence of pores with 4:3 and 3:4 S and F single components should be taken into consideration.

In this paper, we address the question of the stoichiometry of the pore. Since the B component is common between AB and CB channels, the difference in single channel conductance should be attributed merely to the influence of the S component (either A or C), see also ref 22. It also implies

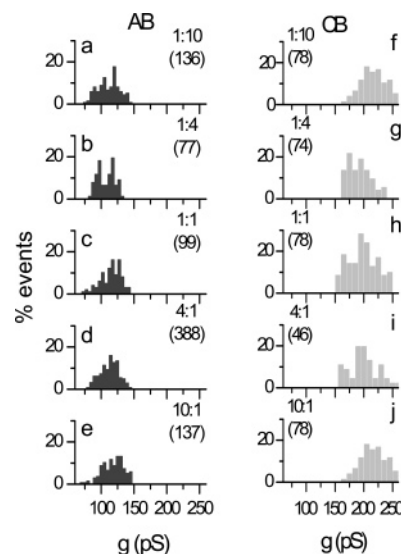


Figure 4. Single channel conductances of γ -hemolysins at different stoichiometric ratios of the single components. Single channel conductances are reported in cumulative probability histograms for both AB (left column) and CB (right) γ -hemolysins. The stoichiometric ratio between the constituting single components varied from 1:10–10:1 of class S and F, respectively. The number of events considered is reported in parentheses. The buffer was 100 mM NaCl, 20 mM Hepes, and 0.1 mM EDTA at pH 7.0. The toxins (\approx 2nM) were added to the cis side only, where a constant voltage (+40 mV) was applied. The membrane was composed of DPhPC. The cumulative histograms of all the compositions tested give a conductance of 113 ± 22 pS for the AB channel and 206 ± 51 pS for the couple CB.

that the contribution of B to the conductance cannot be equal to both that of A and C. (Otherwise, these would also be equal to each other, and all pores would have the same conductance.) More importantly, it seems evident that A and C represent the “low” and “high” conductance phenotypes. This observation prompted us to check for the stoichiometry of the channel, for example, if the 1:1 contribution of each component derives from an average distribution of heterogeneous pores containing, for example, five A and one B or one A and five B. This putative heterogeneous channel with an excess of the A component should have a lower conductance than the 1:1 channel and even lower than that of the 1:5 pore. What we observed, instead, is that the conductance of the pore does not depend on the relative ratio of A/B or C/B used, at least within the range of 10:1–1:10 (Figure 4). This result clearly confirms the hypothesis that the 1:1 stoichiometry is fixed for each single pore. Even though we are not able to discriminate between a hexamer and an octamer, our data clearly show that the heptamer could not be representative of an active pore.

Using single channel conductance as a sensitive parameter for investigating the pore architecture, we decided to further investigate the existence of the mixed-pore type. We demonstrated that point mutants located on a β -strand of the class-S component that forms part of the protomer–protomer contact region can prevent, with differing specificity, the oligomer assembly of parent bicomponent leukotoxins. Furthermore, HlgA mutants that inhibit the growth of AB pores can inhibit the formation of CB pores and vice versa. This led us to the hypothesis that mixed ABC pores could possibly be formed. By studying the conductance of pores assembled in the presence of all three components (in

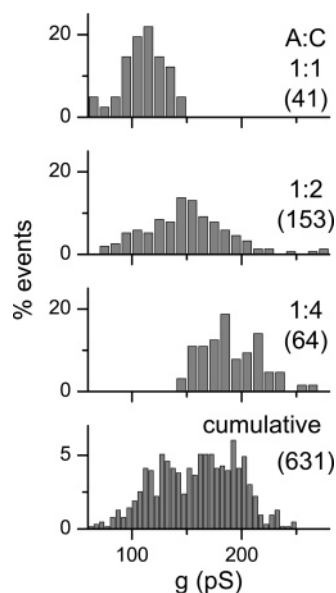


Figure 5. Single channel conductance of the mixed pores. Cumulative histograms of the single channel conductance of γ -hemolysin pores in which all three components A, B, and C are present in the same experiment. Reported are three examples in which the ratio between the S components A and C was varied between 1:1 and 1:4. The number of events considered is reported in parentheses. Other experimental details are as in Figure 4. A shift toward higher conductances is evident as the C component is more represented within a single conducting unit. A cumulative histogram of many similar experiments is reported in the bottom panel; the presence of different peaks related to the mixed pore is evident.

different ratios), we have, indeed, observed that the magnitudes of single channel conductance depend on the ratio between the A and C components present (Figure 5). In fact, we noticed a progressive increase of the pore conductance from 115 pS (pure AB pores) to 190 pS (pure CB pores). This modulation of single-channel conductance based on the amount of A and C evidences the presence of nonstandard three-component pores comprised of two class-S components and HlgB.

CONCLUSIONS

We selected four components, three point-mutated class-S components (HlgA T28D, HlgC T30D, and LukS T28D) and one α -toxin mutant α HL_{H35R}, which are unable to form functional oligomers, and exploited their possible cross interactions. We showed that they can inhibit the activity of various couples on red blood cells and on model membranes. In particular, comparing the competition tests performed on RRBC and LUV, we concluded that different components of class-F and class-S leucotoxins can interact. Moreover, one receptor is not indispensable. By using model membranes, we demonstrated that leucotoxins not only form equimolar pores but possibly mixed pores. This leads to the conclusion that the γ -hemolysin/leucocidin bicomponent toxin family can form a larger than expected number of active toxins by cross-combining various S and F components, with a potential for a nonstandard, mixed pore. Through the availability of more rapidly formed pores, *S. aureus* increases its power to defend itself against the host's immune system while also increasing its ability to acquire nutrients.

ABBREVIATIONS

PC, phosphatidylcholine; Cho, cholesterol; LUV, large unilamellar vesicles; DPhPC, 1,2 diphytanoyl-sn-glycero-phosphocholine; RRBC, rabbit red blood cells; Luk-PVL, Pantone–Valentine leucocidin; Triton X-100, octylphenoxy polyethoxy ethanol; PLM: planar lipid membranes; A_{T28D}, HlgA T28D; C_{T30D}, HlgC T30D; S_{T28D}, LukS-PV T28D; α HL_{H35R}, α -toxin H35R; A, HlgA; B, HlgB; C, HlgC.

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