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Direct transfer of membrane proteins from bacteria to planar bilayers for rapid screening by single-channel recording

Matthew A Holden[1], Lakmal Jayasinghe[1,2], Oliver Daltrop[1,2], Amy Mason[1] & Hagan Bayley[1]

Although the examination of membrane proteins in planar bilayers is a powerful methodology for evaluating their pharmacology and physiological roles, introducing membrane proteins into bilayers is often a difficult process[1]. Here, we use a mechanical probe to transfer membrane proteins directly from *Escherichia coli* expression colonies to artificial lipid bilayers. In this way, single-channel electrical recordings can be made from both of the major classes of membrane proteins, α-helix bundles and β barrels, which are represented respectively by a K^+ channel and a bacterial pore-forming toxin. Further, we examined the bicomponent toxin leukocidin (Luk), which is composed of LukF and LukS subunits. We mixed separate LukF- and LukS-expressing colonies and transferred the mixture to a planar bilayer, which generated functional Luk pores. By this means, we rapidly screened binary combinations of mutant Luk subunits for a specific function: the ability to bind a molecular adaptor. We suggest that direct transfer from cells to bilayers will be useful in several aspects of membrane proteomics and in the construction of sensor arrays.

Recently, we used probes tipped with agarose to deliver purified membrane proteins to lipid bilayers[2]. Although this means of mechanical delivery improved the efficiency of protein insertion into the membranes, laborious and expensive protein purification was necessary, even when we used protein expression by *in vitro* transcription and translation (IVTT) as a potential shortcut. Expression in bacteria, such as *E. coli*, produces recombinant protein as a far larger fraction of the total protein compared with IVTT, and it is less expensive; however, purifying proteins from bacteria is burdensome. Here, we present a method for the rapid analysis of membrane channels and pores in which sharp glass probes are used to deliver proteins directly from bacterial colonies to artificial membranes. The direct transfer of both α-helix bundle and β-barrel membrane proteins can be accomplished, and the functional properties of the transferred proteins can be examined at the single-molecule level.

In our approach, a glass probe with a tip of radius 5–50 μm is first touched to the surface of an *E. coli* colony expressing either an α-helix bundle channel protein or a β-barrel protein pore and is then brought into contact with a planar bilayer, resulting in the insertion of functional proteins (**Fig. 1a**,**b**). When transferring their protein cargo, the probes can penetrate hundreds of micrometers beyond the membrane plane without causing the bilayer to rupture (**Supplementary Fig. 1** and **Supplementary Video 1**). Channel or pore insertion is observed as a characteristic increase in ionic current during an applied potential. For example, we inserted a single pore of α-hemolysin (αHL) using this method (**Fig. 1c**). It is not necessary to break open the cells before probe transfer, and it is likely that enough protein is supplied by the fraction of bacteria in a colony that spontaneously lyse. Whether the protein is in a soluble fraction or in membrane fragments is not known and may differ from case to case. The tip of the probe can be cleaned after screening a colony by dragging it through fresh agar, and the cleaned tip does not produce

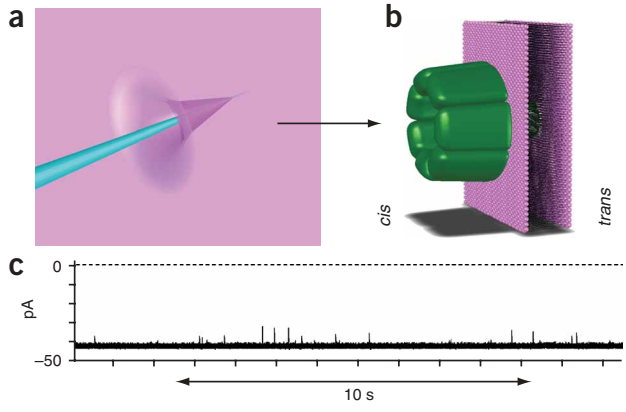

Figure 1 Screening membrane proteins expressed in bacteria. (**a**) A sharp glass tip (radius between 5–50 μm) is dipped into a bacterial colony expressing a membrane protein and driven into the bilayer from the *cis* side using a micromanipulator. (**b**) A single pore is transferred and the probe is retracted. (**c**) Recording of a wild-type αHL pore, transferred from bacteria with the probe, in 10 mM MOPS, 1 M KCl, pH 7.0 at a transmembrane potential of –50 mV. The dashed line indicates the current at 0 mV.

[1]Department of Chemistry, University of Oxford, Chemistry Research Laboratory, Mansfield Road, Oxford OX1 3TA, UK. [2]These authors contributed equally to this work. Correspondence should be addressed to M.A.H. (matthew.holden@chem.ox.ac.uk).

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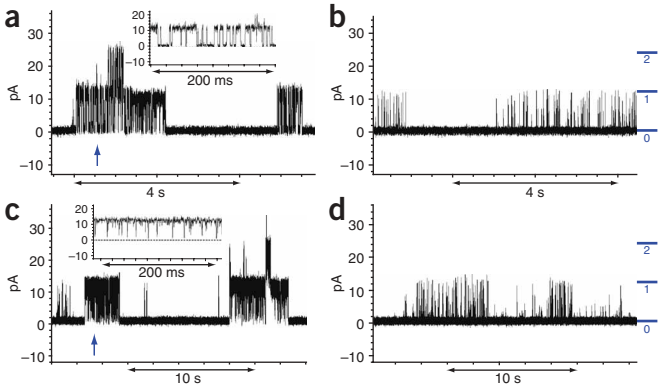

Figure 2 Transfer of K^+ channels from bacteria to a planar bilayer using the probe technique. (**a**) Recording of wild-type KcsA channels transferred from bacteria with the probe. An expanded 200-ms segment of the trace, starting at the arrow, is shown in the inset. The solutions were 10 mM HEPES, 200 mM KCl, pH 7.0 in the *cis* chamber and 10 mM succinic acid, 200 mM KCl, pH 4.0 in the *trans* chamber. The transmembrane potential was +100 mV. (**b**) Upon the addition of 1 mM Ba^{2+} (as $BaCl_2$) to the *trans* chamber, wild-type KcsA was blocked. (**c**) Recording of A108T KcsA channels under the same conditions as wild-type KcsA, with a 200-ms segment expanded in the inset. (**d**) A108T KcsA channels were also blocked using 1 mM Ba^{2+} (*trans*). Current levels 0, 1 and 2 indicate the states when all channels were closed, one channel was open or two channels were open, respectively.

channel or pore activity. Because very little material is dispersed into solution from the tips, it is not necessary to clean the bilayer recording cell after recording from channels or pores before moving to the next sample. Instead, the system is reset by breaking and reforming the bilayer. These manipulations considerably improve the speed at which colonies can be screened.

We first showed that this approach works for both major structural classes of membrane proteins: the α-helix bundle and the β barrel. The α-helix bundle was represented by the tetrameric potassium channel KcsA[3,4]. Wild-type KcsA channels transferred from *E. coli* to a planar bilayer opened in short bursts at +100 mV and had characteristic unitary conductance values of 112 ± 8 pS (n = 3) at +100 mV (**Fig. 2a**) and 54 ± 5 pS (n = 3) at –100 mV (10 mM HEPES, 200 mM KCl, pH 7.0 in the *cis* chamber; 10 mM succinic acid, 200 mM KCl, pH 4.0 in the *trans* chamber). These values were similar to previously reported values of 56 pS at +200 mV and 31 pS at –200 mV in buffers containing half the concentration of KCl used in our experiments (10 mM HEPES, 100 mM KCl, pH 7.0 in the *cis* chamber; 10 mM succinic acid, 100 mM KCl, pH 4.0 in the *trans* chamber)[4]. The intracellular (*trans*) portion of KcsA must be below pH ~5 for KcsA gating to be observed[4]. Although it is likely that KcsA channels that are transferred to planar bilayers using the probe adopt both orientations in the bilayer, only the channels with the intracellular domain facing the pH 4.0 buffer are able to gate. The asymmetric unitary conductances observed in our experiments are in agreement with previous reports, which show that KcsA weakly rectifies ionic current and does so with a higher conductance at positive potential[4]. Finally, the KcsA channels were blocked from the *trans* side by the use of 1.0 mM Ba^{2+} (**Fig. 2b**)[5]. We found that burst durations and conductance values were similar before and after channel blockage, but the time that KcsA was open during a burst was greatly reduced. This was manifested as groups of current spikes (**Fig. 2b**).

To confirm that we were indeed observing KcsA, we examined the behavior of a mutant form of the protein. We repeated the above experiments for the A108T KcsA mutant, which displayed characteristic unitary conductance values of 123 ± 6 pS (n = 4) at +100 mV (**Fig. 2c**) and 37 ± 3 pS (n = 4) at –100 mV under the same buffer conditions as the wild-type KcsA[6]. The gating behavior of the wild-type KcsA can be distinguished from that of the A108T mutant (**Fig. 2a,c**). The A108T channels were also blocked with 1 mM Ba^{2+} from the *trans* side (**Fig. 2d**). The two proteins have similar conductances at positive potential, although the A108T mutant has about 68% the conductance of the wild type at negative potential.

It was important to demonstrate that the potassium channels we observed were indeed the overexpressed proteins and not endogenous *E. coli* channels. We tested JM109 (DE3) colonies (n = 11) that contained an empty expression vector using the probe method under the same conditions used to investigate wild-type KcsA. Although rare observations of endogenous pores or channels were made, the gating

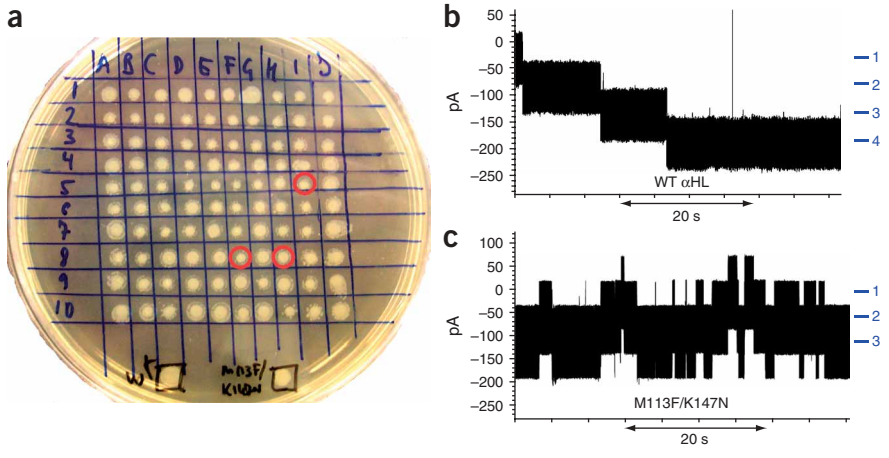

Figure 3 Screening colonies for the M113F/K147N αHL mutant in a wild-type (WT) αHL background with the probe technique. Control colonies of the WT and mutant proteins were grown at the bottom of the plate and were used to verify the electrical properties of the respective pores. To maintain a blind study, the plate preparer did not reveal the number of mutant colonies to the screener. Both the *cis* and *trans* chambers contained 10 mM MOPS, 1 M KCl, pH 7.0, and the bilayer was held at –50 mV. Recording was performed with 90 μM βCD in the *cis* chamber; βCD bound to the mutant pores but not the WT pores. (**a**) Three mutant colonies were found, as indicated by the red circles, which correspond to the number and location of the mutant colonies spotted by the preparer of the plate. (**b**) Typical signal from WT pores; four pores inserted during this trace. The average current levels after each pore insertion are marked with blue lines. (**c**) Typical signal from M113F/K147N pores. In this example, two pores inserted before the start of the recording, and no additional pores inserted during the recording. The average current level varied among two pores blocked by βCD (1), one open and one blocked pore (2) and two open pores (3). For convenience, the lid of the Faraday box (see Methods) was left open during recording. Although the signal is superimposed onto the resulting ambient electrical noise, the wild-type and M113F/K147N current signatures are distinct.

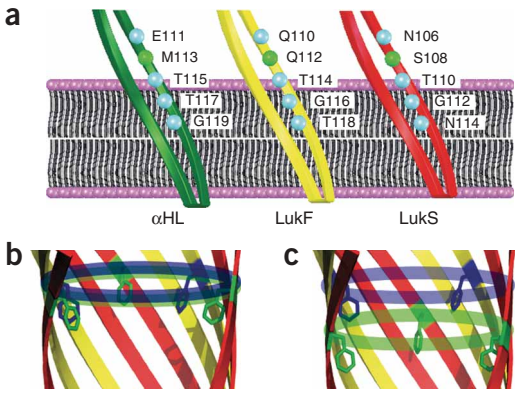

Figure 4 Concept for the Luk screen. (**a**) Proposed alignment of the β strands in the αHL, LukF and LukS pores as deduced from sequence homology and the crystal structures of the αHL heptamer and the LukF and LukS monomers. Because the M113F mutant of αHL binds βCD and TRIMEB with longer dwell times than the wild type, we speculated that similar modifications could be made in the β barrel of Luk to enhance TRIMEG binding (see the text). (**b**) Depending on their position, the mutated residues might align to form a single ring of eight phenylalanines. (**c**) Alternatively, they might be offset to form two separate rings of four phenylalanines each. We hypothesized that a single ring of phenylalanine residues would bind TRIMEG with higher dwell times than offset residues. By mixing LukF and LukS colonies expressing the WT and mutant subunits shown in **a** (each position was individually changed to phenylalanine), we were able to screen 35 Luk combinations for TRIMEG binding by the probe technique.

behavior and conductances were distinctly different from either wild-type or A108T KcsA. Further, these native conducting components were not blocked by Ba^{2+} ions.

The β barrel was represented by αHL, which forms a heptameric pore featuring a 14-stranded transmembrane barrel. Wild-type αHL transferred from *E. coli* to a planar bilayer yielded an open pore with a stable conductance (880 pS in 10 mM MOPS, 1 M KCl, pH 7.0) similar to that of purified αHL (775 pS in 5 mM HEPES, 1 M KCl, pH 7.4)[7] (**Fig. 1c**). Engineered β-barrel pores have been extensively characterized in our laboratory[8] and therefore were chosen as a model system to further demonstrate the power of the approach, including the ability to screen a library with a channel blocker.

As a first demonstration of rapid screening, we prepared an agar plate with 100 αHL-expressing colonies (**Fig. 3a**). We grew two cultures of *E. coli* JM109 (DE3), one expressing wild-type αHL and the other expressing the αHL mutant M113F/K147N, separately in liquid medium and subsequently spotted the cultures in a 10 ×10 grid on the plate such that 95–99 colonies expressed wild-type αHL and 1–5 colonies expressed the αHL mutant M113F/K147N. The two types of colonies were indistinguishable, and the exact number of mutant colonies was not revealed to the plate screener. We also grew an identified colony expressing each type of αHL; we used these colonies as references for the two binding behaviors (**Fig. 3a**). The two types of αHL form pores of similar unitary conductance. However, only the mutant αHL pore binds the molecular adaptor β-cyclodextrin (βCD, **1**) from the *cis* side of the bilayer[9]. We screened the plate using the probe method, with 90 μM βCD in the *cis* chamber. Wild-type αHL produced a stepwise increase in current for each pore that inserted into the bilayer (**Fig. 3b**). However, the M113F/K147N mutant bound the βCD, giving a characteristic attenuation of the current (**Fig. 3c**). We completed the screening of all 100 colonies in 5 h, and the three mutant-expressing colonies found at the marked positions matched those subsequently revealed by the plate preparer (**Fig. 3a**). This experiment demonstrated rapid screening for rare instances of specific function within a background of wild-type pores.

After demonstrating that wild-type and mutant αHL can be reliably and directly transferred from bacterial colonies and assayed, we determined whether the technique could be used to screen a library of pores for mutants that bind an open-pore blocker. We chose the Luk pore as a model system, as it shares many characteristics with αHL[10,11]. However, unlike αHL, Luk is composed of two different subunits: four LukF and four LukS subunits are arranged in alternating fashion around a central axis to form an octameric pore[12,13]. We first tested LukF (HlgB)– and LukS (HlgC)–expressing bacterial colonies separately with the probe method. As expected, neither pores of pure LukF nor pores of pure LukS were observed. We then scraped separate colonies expressing wild-type LukF and wild-type LukS from agar plates, placed them together on a fresh agar surface and mixed them thoroughly with a scalpel blade. Notably, we found that a glass probe could be dipped into the bacterial mixture and engaged with the bilayer to produce wild-type Luk pores. Thus, we were able to assemble fully functional bicomponent membrane pores from separate components without purification.

We then examined a library of Luk pores to evaluate their interaction with a cyclodextrin. We have previously shown that αHL binds βCD near the top of the transmembrane β barrel, where the cyclodextrin acts both as a partial pore blocker and as an 'adapter' that can bind organic molecules, which block additional current. Further, certain amino acid residues in the β barrel of αHL can be mutated to enhance the binding of βCD (both αHL and βCD have seven-fold axial symmetry)[9,14–16]. For example, when Met113 is mutated to phenylalanine, the duration of binding of βCD to the pore is ~3 × 10^4 times longer than to the wild-type αHL pore[16]. Moreover, the permethylated form of βCD, TRIMEB, binds to the αHL mutant M113F/K147N ~4 times longer than βCD (unpublished data). Based on these findings, we hypothesized that similar modifications could be made in the β barrel of Luk such that the mutant

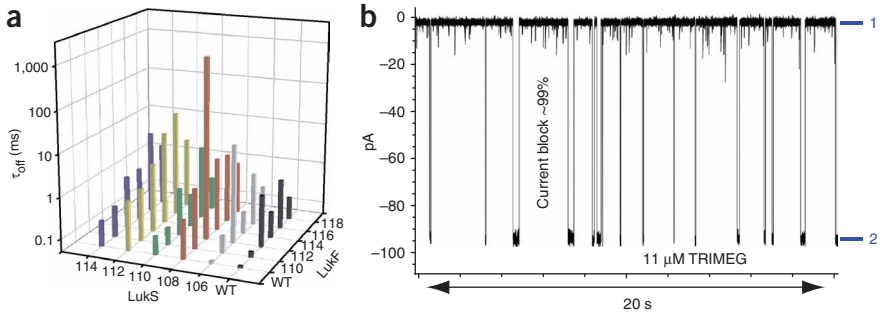

Figure 5 Rapid screening of 35 Luk mutants for binding of TRIMEG. All trials were performed in 10 mM MOPS, 1 M KCl, pH 7.0 at a potential of –40 mV. 110 μM TRIMEG was added to the *trans* chamber. The average residence time of the TRIMEG bound to the pore is τ_{off}. (**a**) The mean values of τ_{off} ($n = 3$ for each mutant) plotted against the LukF and LukS combination of each Luk pore. Note that the *z* axis is a log scale. (**b**) Single-pore recording of LukF Q112F/LukS S108F in the presence of 11 μM TRIMEG. Current level '1' represents the pore blocked by TRIMEG and level '2' the open pore.

proteins would bind permethylated γCD, TRIMEG (**2**) (both Luk and TRIMEG have eight-fold axial symmetry).

Based on sequence homology, the crystal structure of the αHL heptamer and the crystal structures of the LukF (HlgB)[17], LukF-PV[18] and LukS-PV[19] monomers, we modeled the alignment of amino acids in the β strands of the barrel for αHL, LukF and LukS (**Fig. 4a**)[12]. We made mutations that replaced amino acids having inward-projecting side chains at various positions along the β strands of LukF and LukS with phenylalanine. Inside the hetero-octameric β barrel, the mutated residues might align to form either a ring of eight phenylalanines (**Fig. 4b**) or two separated rings of four phenylalanines (**Fig. 4c**). By analogy with M113F αHL, we hypothesized that a single ring of eight aligned phenylalanine residues (for example, LukF Q110F/LukS N106F, LukF Q112F/LukS S108F, LukF T114F/LukS T110F and so on) would create a form of Luk that binds TRIMEG.

In our previous work, we prepared mutant Luks by expressing individual monomers of LukF and LukS by IVTT. We assembled the monomers on rabbit red blood cell membranes to obtain octamers, then purified octamers by gel electrophoresis and examined them by planar bilayer recording[12]. Here, we used the far more rapid probe procedure to obtain functional Luk pores from mixed colonies. We examined three individual pores for each of 35 new combinations of the two subunits and then averaged the kinetic data (**Fig. 5a**). Based on our model, there are five combinations of LukF and LukS that have a single ring of eight phenylalanine residues. Although we expected these Luk mutants to bind TRIMEG longer than the other 30 mutant combinations, the distribution of τ_{off} values did not follow this trend (**Fig. 5a**). However, one of these combinations, LukF Q112F/LukS S108F, bound TRIMEG (**Fig. 5b**) 100 times longer than any other combination and 10^4 times longer than the wild-type Luk (for a current trace of wild-type Luk in the presence of TRIMEG, see **Supplementary Fig. 2** online.) These data suggest that the amino acids at positions 113, 112 and 108 share similar locations and orientations in αHL, LukF and LukS, respectively. It is possible that the phenylalanine side chains were disordered or not correctly oriented in the other four mutant pairs. Though the collection of this comprehensive set of data would have been laborious by alternative approaches, the transfer of protein from mixed Luk colonies with the glass probe enabled efficient screening and led to the discovery of the LukF Q112F/LukS S108F mutant. This mutant is being further explored as a stochastic sensor element.

Our experiments demonstrate a highly reproducible approach for the direct insertion of channels and pores into planar bilayers. We have shown the utility of this approach for both major classes of membrane protein, α-helix bundles and β barrels, which were represented respectively by a K^+ channel and a bacterial pore-forming toxin. The proteins investigated in these experiments were highly overexpressed (**Supplementary Fig. 3** online), and it is likely that the probe picked up proteins from bacteria that had spontaneously lysed. In the case of the Luk experiments, the protein in the colonies was probably monomeric, as the functional pores were hetero-octamers[12,13]. Observations of endogenous *E. coli* proteins were infrequent. Further, it was not possible to obtain the characteristic KcsA, αHL or Luk behavior from colonies that did not overexpress these proteins. The transfer of overexpressed proteins from bacterial colonies to planar bilayers is inefficient, and therefore it is reasonable to expect that the insertion of endogenous membrane proteins occurs rarely. Thus, although inefficient transfer of proteins would in many circumstances be undesirable, it is ideally suited for screening by single-channel recording.

The transfer of membrane proteins directly from bacterial colonies to lipid bilayers has several advantages over previous methods. The expense of IVTT and the labor of plasmid preparation or DNA amplification followed by protein assembly and purification are avoided. Moreover, bacterial colonies can serve as protein storage systems: we obtained pores from colonies for at least 3 months when they were stored at 4 °C. The probe is also faster than traditional techniques for several reasons. It is not necessary to clean the recording cell between measurements, because very little protein is introduced into solution from the probe tip. In addition, the probe surface can be cleaned and reused. The screening approach yields specific information about the properties of channels and pores at the single-molecule level in a few minutes per colony. For example, we screened 100 bacterial colonies expressing αHL and were able to find rare mutants based on the binding of βCD. This screen, which took 5 h to complete, would have taken weeks by standard methodology. Finally, as hetero-oligomeric proteins can be assembled from components grown separately, we were able to investigate 35 Luk mutants simply by mixing the colonies together. By this means, we obtained a quantitative estimate of blocker binding for each subunit combination and discovered a new candidate for protein-based stochastic sensing. Such an extensive survey would have been far slower and more costly by alternative approaches. The automation of our approach will allow high-throughput investigations of channels and pores that will be invaluable for proteomics and drug discovery and will aid the development of stochastic sensor arrays.

METHODS

Mutants and screening. Both the wild-type and mutant KcsA proteins[4,6] and the wild-type and mutant αHL proteins were expressed using the pT7-SC1 vector[20]. The vectors containing wild-type αHL and the mutant αHL-M113F/K147N were used in previous studies[7,9]. We altered LukF (*hlgB* gene product)[7] and LukS (*hlgC* gene product)[7] by PCR mutagenesis and ligation-free *in vivo* recombination[21] in the pT7-SC1 expression vector, and we verified the mutant genes by DNA sequencing. *E. coli* JM109 (DE3) (Promega) was used for protein expression. LB agar plates contained ampicillin (100 μg ml^{-1}).

Bilayer recording. We carried out single-channel recordings by using folded bilayer membranes as previously described[22]. The apparatus consisted of a 25-μm-thick Teflon septum glued between two Delrin chambers. We treated each face of the septum with 10% (v/v) hexadecane in pentane and filled both chambers with 1.0 ml of a buffered salt solution (see below). 1,2-Diphytanoyl-*sn*-glycero-3-phosphocholine (Avanti, 8 μl of 10 mg ml^{-1} in pentane) was added to the surface of each chamber and the pentane was allowed to evaporate to leave behind lipid monolayers. Lowering and raising the liquid level in the chamber below and above the aperture caused lipid bilayers to be formed as needed. Recordings of wild-type and mutant KcsA proteins were carried out in 10 mM HEPES and 200 mM KCl at pH 7.0 for the *cis* chamber and in 10 mM succinic acid and 200 mM KCl at pH 4.0 for the *trans* chamber. Both buffers were titrated with KOH. Recordings of the αHL and Luk pores were carried out in 10 mM MOPS and 1.0 M KCl titrated to pH 7.0 with KOH. The electrical current was detected with two Ag/AgCl electrodes, amplified with a patch-clamp amplifier (Axopatch 200 B, Axon Instruments), filtered with a low-pass Bessel filter (80 dB per decade) with a corner frequency of 2 kHz and then digitized with a DigiData 1320 A/D converter (Axon Instruments) at a sampling frequency of 5 kHz. In all experiments, the *cis* chamber was at ground. A positive current represented the movement of cations from *trans* to *cis* or of anions from *cis* to *trans*. The bilayer recording platform, chambers, amplifying headstage and micromanipulator were enclosed in a metal box that served as a Faraday cage to shield against ambient electrical noise.

Probe fabrication and operation. We pulled solid glass rods of 1 mm diameter (Sutter Instrument Co.) using a Narishige PC-10 capillary puller to form sharp tips with a radius between 1 and 10 μm. Although it was possible to perform probe transfers using very sharp tips, most assays were performed with flame-treated tips (radius 5–50 μm), which were more robust. The probe was fastened

to the end of a clear plastic arm and positioned with a NMN-21 manual micromanipulator (Narishige). The tip was viewed through a Leica stereomicroscope during positioning. It was possible to push the glass tip directly into the bilayer (engagement) and withdraw the tip (disengagement) repeatedly without rupturing it (see **Supplementary Video 1**). In all experiments, the probe was placed into the *cis* chamber.

Capacitance measurements during probe engagement. The capacitance of the bilayer is proportional to the area of the thinnest part of the film. When the probe was engaged, the capacitance increased sharply (**Supplementary Fig. 1**). The capacitance decreased when the probe was withdrawn. This could be repeated several times without bilayer rupture.

Insertion of protein pores from bacterial colonies. In experiments involving bacteria expressing KcsA or αHL, we used colonies as grown on agar plates; we did not lyse or chemically treat them. The glass probe (while fixed in the arm) was inserted into a colony by hand and withdrawn. This procedure was used with both fresh colonies and colonies up to 3 months old. The bacterium-laden tip was immediately fixed to the micromanipulator, lowered into the *cis* chamber of the recording cell and engaged with the bilayer. Although probe engagement with the bilayer did not interfere with single-channel recording, the probe was usually disengaged during data acquisition. The time needed for single or multiple channels or pores to insert varied. The transfer of KcsA from colonies was the least consistent. The lag time between engaging the bacteria on the probe with the bilayer and the observation of KcsA activity ranged between seconds and tens of minutes. Typically, the probe was engaged once for ~0.5 s and withdrawn. If KcsA activity was not observed within 30 min after engagement, the bilayer was broken with an electrical pulse and re-formed, and a fresh tip laden with KcsA-expressing bacteria was engaged with the bilayer. By contrast, αHL-expressing colonies (wild type or mutant) were potent, meaning that even a brief engagement (~0.5 s) of the tip with the bilayer caused multiple pores to insert. To record single pores from these colonies, bilayers were broken and re-formed, and the probe was re-engaged. This process was repeated until the amount of pore-forming protein on the tip surface was reduced enough to allow the insertion of single pores. Probe engagement and withdrawal and bilayer re-formation could be repeated at least five times per minute. However, typically only two to four iterations were needed to obtain a single pore.

In the case of Luk, we scraped together *E. coli* JM109 (DE3) colonies expressing LukF and LukS (four of each subunit constitute the Luk pore) with a scalpel and placed them on a separate agar plate. Typically, we collected about 50 μl of material for each monomer, although the exact volume of material was not critical. The two blobs were mixed thoroughly using a scalpel but were not lysed. A glass tip was inserted into the mixture, and the probe was immediately placed into the *cis* chamber of the bilayer recording cell. The formation of single Luk pores usually required longer probe engagements than those used for αHL colonies, which might be expected as Luk has a much lower activity than αHL in hemolytic assays[23]. Typically, a tip from freshly mixed colonies might be engaged for several seconds before a single pore was inserted. However, mixed colonies often became more potent with time; some yielded as many pores per second as αHL colonies. Mixtures of Luk colonies could be stored for at least 1 month at 4 °C on agar plates and still produce protein pores by the probe method.

Kinetic data analysis. We made current amplitude and dwell time histograms using ClampFit 9.0. We determined the average dwell times τ_{off} from dwell-time histograms by fitting distributions to an exponential probability function with one term.

Note: Supplementary information is available on the Nature Chemical Biology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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