Supporting Information

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SI Text

Circular Dichroism Spectroscopy. Circular dichroism (CD) spectra were recorded using a J-715 Jasco CD spectropolarimeter. Wavelength scans were performed between 205 and 240 nm in PBS in 1-mm quartz cuvettetes using 5.22 μM protein. Five traces were collected for each protein. Data were analyzed using the CONTINLL, SELCON3, and CDSSTR algorithms from the CDPro software package and the SP43 reference protein database (1, 2).

Tryptophan Emission. Intrinsic fluorescence of the tryptophan residues in PFO and its derivatives was measured to assess the relative conformation of the D4 loops of PFO loop mutants compared to wild-type toxin. Protein samples (88.23 pM) were incubated at room temperature in HBS for 5 min before measuring their tryptophan emission. Emission spectra were scanned between 310 and 400 nm with an excitation wavelength of 295 nm. The emission scans from a total of four separate experiments were averaged.

1. Kabsch W, Sander C (1983) Dictionary of protein secondary structure: Pattern recognition of hydrogen-bonded and geometrical features. *Biopolymers* 22:2577–2637.
2. Sreerama N, Woody RW (1994) Poly(pro)II helices in globular proteins: Identification and circular dichroic analysis. *Biochemistry* 33:10022–10025.

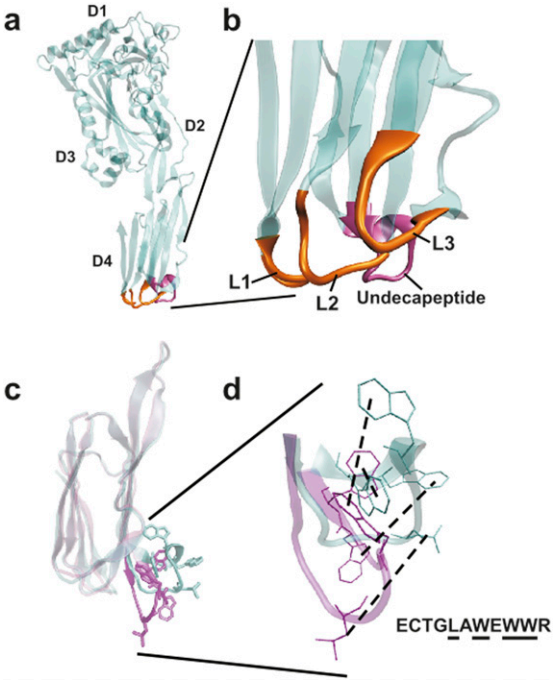

Fig. S1. The molecular structures of domain 4 and the undecapeptide from PFO and ALO. Shown is a ribbon representation of the crystal structure of PFO (1) (*A*), PFO domain 4 showing the locations of loops L1–L3 (orange), and the undecapeptide (purple) (*B*), and overlays of the domain 4 (*C*) and undecapeptide (*D*) structures of PFO (cyan) and ALO (2) (purple). Shown in *D* are the relative positions of the leucine and three tryptophans of the conserved undecapeptide (ECTGLAWEWWR) from PFO and ALO. The dashed lines connect the equivalent undecapeptide leucine and tryptophan residues of PFO and ALO. D1–D4, domains 1–4 of PFO.

1. Rossjohn J, Feil SC, McKinstry WJ, Tweten RK, Parker MW (1997) Structure of a cholesterol-binding thiol-activated cytolysin and a model of its membrane form. *Cell* 89:685–692.
2. Bourdeau RW, et al. (2009) Cellular functions and X-ray structure of anthrolysin O, a cholesterol-dependent cytolysin secreted by *Bacillus anthracis*. *J Biol Chem* 284:14645–14656.

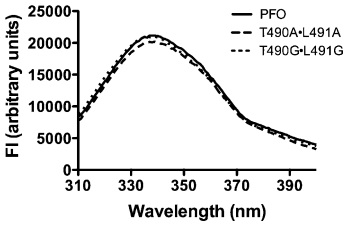

Fig. S2. Tryptophan emission of PFO, PFO$^{T490A•L490A}$, and PFO$^{T490G•L490G}$. The tryptophan emission of PFO, PFO$^{T490A•L491A}$, and PFO$^{T490G•L491G}$ were determined. Protein samples (88.23 pM) were incubated at room temperature in HBS for 5 min. Tryptophan emission measurements were taken between 310 and 400 nm with an excitation wavelength of 295 nm. Four separate experiments were performed and the data averaged.

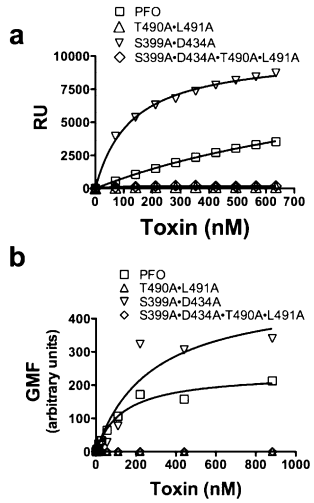

Fig. S3. Contribution of residues of loops 2 and 3 to cholesterol recognition. Ser-399 and Asp-434 of loops 2 and 3 increased binding of PFO to cholesterol-rich liposomes when substituted with alanine (Fig. 1). We determined whether these second site mutations could partially or fully restore binding of PFO$^{T490A•L491A}$ to cholesterol-rich membranes. Binding of PFO and PFO$^{T490A•L491A}$ without and with the alanine substitutions at Ser-399 and Asp-434 was measured on POPC-cholesterol liposomes (*A*) and to human erythrocytes (*B*). In both cases binding of PFO was increased whereas PFO$^{T490A•L491A}$ with or without the Ser-399 and Asp-434 mutations was undetectable (note that the symbols for each overlap along the *x* axis in *A* and *B*).

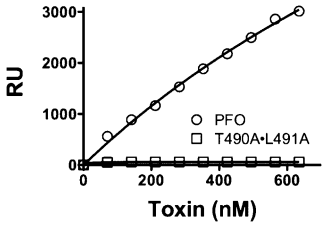

Fig. S4. Binding of PFO and PFO$^{T490A•L491A}$ to membranes with increased levels of available cholesterol. The cholesterol-dependent binding of PFO is sensitive to the structure of the membrane lipid. The introduction of lipids with unsaturated fatty acyl chains has been shown to dramatically reduce the level of membrane cholesterol required for PFO binding (1, 2). Substitution of DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) for POPC reduces the concentration of cholesterol required for maximal PFO binding from ≈55 mol % to 25–30 mol %. We examined binding of PFO and PFO$^{T490A•L491A}$ to liposomes where POPC was replace by DOPC. The DOPC-cholesterol liposomes contained 55 mol % cholesterol and 45 mol % DOPC, a concentration of cholesterol 25–30 mol % greater than required for maximal binding of native PFO to these liposomes. As is evident, no detectable binding of PFO$^{T490A•L491A}$ was observed, even though these liposomes maximize cholesterol availability.

1. Flanagan JJ, Tweten RK, Johnson AE, Heuck AP (2009) Cholesterol exposure at the membrane surface is necessary and sufficient to trigger perfringolysin O binding. *Biochemistry* 48: 3977–3987.
2. Nelson LD, Johnson AE, London E (2008) How interaction of perfringolysin O with membranes is controlled by sterol structure, lipid structure, and physiological low pH: Insights into the origin of perfringolysin O-lipid raft interaction. *J Biol Chem* 283:4632–4642.

Table S1. CDC loop L1–L3 residues

CDC	Loop 1	Loop 2	Loop 3
Anthrolysin	GTTLYP	HYGAYVA	DKTAH
Cereolysin	GTTLYP	HYGAYVA	DKTAH
Thuringiolysin	GTTLYP	HYGAYVA	DKTAH
Sphaericolysin	GTTLYP	HYGAYVA	DKTAH
Alveolysin	GTTLYP	HSGAYVA	DRSAH
Perfringolysin	GTTLYP	HSGAYVA	DKTAH
Caniolysin	GSTLSP	HQGAYVA	SKTSP
Equisimilysin	GSTLSP	HQGAYVA	SKTSP
Streptolysin	GSTLSP	HQGAYVA	SKTSP
Botulinolysin	GTTLYP	HSGAYVA	DKTAH
Tetanolysin	GTTLYP	HSGAYVA	DRTAH
Novyiolysin	GTTLYP	HRGAYVA	GRTAH
Ivanolysin	GTTLYP	HSGAYVA	DKLAH
Listeriolysin	GTTLYP	HSGGYVA	SKLAH
Seeligeriolysin	GTTLYP	HSGGYVA	SKLAH
Vaginolysin	GTTLWP	HRGAYVA	YRTAH
Lectinolysin	GTTLNP	HKGAYIA	NRTSG
Intermedilysin	GTTLHP	HDGAFVA	NRGAH
Suilysin	GTTLYP	HSGAYVA	NLTSH
Pneumolysin	GTTLYP	HSGAYVA	DLTAH
Pyolysin	GTTLNP	HGGGYVA	ARTLG

Shown are the residues that comprise loops L1–L3 in the CDCs and those conserved in all CDCs (gray background). Alignments of the loops were derived from the alignment of the full-length CDC primary structures using CLC Sequence Viewer.

Table S2. Circular dichroism analysis of the secondary structure of PFO and its derivatives

	Circular dichroism (SE)			
Toxin	α-Helix	β-Sheet	Turn	Unordered
PFO	21 ± 2.3	34 ± 1.3	21 ± 0.1	25 ± 1.1
T490A•L491A	20 ± 2.3 (P = 0.7650)	33 ± 2.4 (P = 0.7342)	21 ± 0.2 (P = 0.2720)	26 ± 1.4 (P = 0.7508)
T490G•L491G	20 ± 3.3 (P = 0.6320)	35 ± 1.3 (P = 0.4599)	21 ± 0.3 (P = 0.0789)	26 ± 1.3 (P = 0.7052)
T490L•L491T	22 ± 4.2 (P = 0.6950)	29 ± 4.9 (P = 0.3767)	21 ± 0.4 (P = 0.1710)	27 ± 1.7 (P = 0.4751)

Shown are the results of CD analysis of the secondary structure of PFO and the mutants of the cholesterol binding motif. In parenthesis are the P values for a paired t test comparing PFO with each mutant (n = 5). No significant differences in the secondary structure were observed.