Supporting Information

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SI Materials and Methods.

Media, Culture Growth, and General DNA Manipulations. Cultures were grown in standard Luria–Bertani (LB) media supplemented with ampicillin (100 μg/mL), chloramphenicol (10 μg/mL), and kanamycin (40 μg/mL) as indicated. IPTG and arabinose were used for inductions of plasmid-borne promoters at final concentrations of 1 mM and 0.2%, respectively. Lysis profiles were obtained by monitoring A_{550} after thermal or IPTG/arabinose inductions, as described previously (1, 2). Site-directed mutagenesis, cloning steps and DNA sequencing have been described (1).

Bacterial Strains, Bacteriophages, and Plasmids. The key features of the bacterial strains, bacteriophages, and plasmids used in this work are listed in Table S1.

For cysteine modification and cross-linking experiments, derivatives of the plasmid pS^{21}68 were used (Fig. S8*A*). The plasmid, pS^{21}68 was derived from pBP121 (3), which has the entire phage 21 lysis gene cassette, genes (*SRRzRz1*)^{21} (4), cloned in pBR322 under its native promoter, the late gene promoter pR′^{21}. Also, the *S*^{21} gene is modified by deletion of its first three codons, creating the allele *S*^{21}*68*, the Shine–Dalgarno sequence serving the *S*^{21}*68* gene is changed from 5′-CGGAGGC-3′ to 5′-CGGAGGT-3′, and the other three lysis genes were inactivated by introducing amber nonsense codons into *R*^{21} (positions Tyr39 and Tyr42), *Rz*^{21} (Gln100), and *Rz1*^{21} (Trp39). All pS^{21}68 based plasmids were transformed into the strain MDS12Δ*tonA* (RY 17341) (5), carrying the prophage λ*Q*^{21}Δ(*SRRzRz1*)^{21} (Fig. S8*C*), which, by thermal-induction, provides the phage 21 late gene activator, Q^{21}, to trans activate the pS^{21}68. The bacteriophage λ*Q*^{21}Δ(*SRRzRz1*)^{21} was generated by recombination between λhy21*Kan* (3) and pBR-Cam^{R} (Fig. S8*B*). The plasmid, pBR-Cam^{R}, was derived from pBR121 (3) by replacing the DNA between the EcoRI site upstream of the *S*^{21} gene and the ClaI site within the *kan*^{R} gene with a *cam*^{R} cassette (6). MDS12Δ*tonA*(λhy21*Kan*) was transformed with pBR-Cam^{R} and a lysate was obtained by thermal induction. The lysate was used to infect MDS12Δ*tonA* at low multiplicity and the lysogen MDS12Δ*tonA*(λ*Q*^{21}Δ(*SRRzRz1*)^{21}) was recovered by selecting on LB-Cam (10 μg/mL chloramphenicol) and screening for sensitivity to kanamycin.

The *S*^{21}*68*$_{\Delta TMD1}$ and *irsS*^{21}*68* alleles are expressed from plasmids pS^{21}68$_{\Delta TMD1}$ and pirsS^{21}68, respectively. Plasmid pS^{21}68$_{\Delta TMD1}$ is a derivative of pTP2 (7), with the codons encoding transmembrane domain (TMD)1 of *S*^{21}*68* deleted (Fig. 1*A*), and with the introduction of nonsense codons into the (*RRzRz1*)^{21} lysis genes, as described above. The same nonsense codons were inserted into pTP7 (7), generating the plasmid pirsS^{21}68, encoding S^{21}68 with the *irs* epitope inserted after the Met4 start codon (Fig. 1*A*). Each plasmid was transformed into the strain MG1655 *lacI*^{q1} *tonA*::Tn*10* (7, 8) carrying pQ, a low copy plasmid carrying the λ gene *Q*, encoding the late gene activator, under an IPTG and arabinose inducible promoter (9).

For experiments requiring coexpression of the wt *S*^{21}*68* with certain derivative alleles, strain MDS12Δ*tonA* carrying the inducible prophage λ*S*^{21}*68* (Fig. S8*C*) was used as the host. The lysogen MDS12Δ*tonA*(λ*S*^{21}*68*) was obtained by infecting MDS12Δ*tonA* with the lysate obtained by thermal induction of MDS12Δ*tonA*(λ*Q*^{21}Δ(*SRRzRz1*)^{21}) carrying pS^{21}68, selecting for lysogens on LB-Kan (40 μg/mL kanamycin), and screening for sensitivity to chloramphenicol. Induction of this prophage results in the production of the Q^{21} late gene activator and S^{21}68 *in trans* to coresident plasmids.

The plasmid pS^{21}68$_a$ was used to provide the other *S*^{21}*68* allele *in trans* to λ*S*^{21}*68*. It was constructed by inserting the DNA between the middle of the *Q*^{21} gene and the PstI site downstream of the 21 lysis cassette of the plasmid pS^{21}68 into the NcoI and PstI sites of plasmid pRE (Fig. S8*A*) (7). Thus, the plasmid pS^{21}68$_a$ has the pR′^{21} promoter and the entire lysis gene cassette, identical to pS^{21}68, except that it carries *Amp*^{R}, instead of *Kan*^{R}. The plasmid pirsS^{21}68* was used to provide a copy of *irsS*^{21}*68* allele *in trans* to λ*S*^{21}*68*. It was derived from plasmid pS^{21}68$_a$, with the codons encoding the *irs* epitope inserted after the Met4 codon of the *S*^{21}*68* gene.

Plasmids pETS^{21}68^{his}, pETS^{21}68$_{\Delta TMD1}$^{his}, and pETirsS^{21}68^{his} were used for overexpression of *S*^{21}*68*^{his}, *S*^{21}*68*$_{\Delta TMD1}$^{his}, and *irsS*^{21}*68*^{his}, respectively. The pETS^{21}68^{his} carries *S*^{21}*68* inserted into the hyperproduction plasmid pET11a, with codons encoding the purification tag GGH$_6$GG inserted between codons 66 and 67 (7). The plasmids pETS^{21}68$_{\Delta TMD1}$^{his} and pETirsS^{21}68^{his} are isogenic to pETS^{21}68^{his}, but encode the ΔTMD1 and *irs*-tagged derivatives, respectively. Each plasmid was transformed into *Escherichia coli* strain C43(DE3) (10).

For in vivo characterization of *S*^{21}*68*^{his} and *irsS*^{21}*68*^{his}, plasmids pS^{21}68^{his} and pirsS^{21}68^{his} were used, respectively. The pS^{21}68^{his} is identical to pS^{21}68$_a$, except that the DNA sequence encoding the his-tag was inserted between codons 66 and 67, as in the plasmid pETS^{21}68^{his}. Similarly, the plasmid pirsS^{21}68^{his} was derived from pirsS^{21}68*. Each plasmid was transformed into lysogen MDS12Δ*tonA*(λ*Q*^{21}Δ(*SRRzRz1*)^{21}), for thermal induction.

Purification of S^{21}68^{his}. Induction, detergent extraction and purification of the his-tagged proteins were performed according to Savva et al. (11). Briefly, cells overexpressing protein S^{21}68^{his} or its variants were pelleted and resuspended in lysis buffer (20 mM Tris, pH 7.9/150 mM NaCl/1 mM DTT/1 mM EDTA/1 mM PMSF) and lysed by passing through an French pressure cell (Spectronic Instruments) at 16,000 psi. Whole cells and cell debris were removed by centrifugation at 10,000 × *g* in a Sorvall SS-34 rotor for 15 min at 4 °C. Membranes were harvested by ultracentrifugation at 130,000 × *g* for 90 min at 4 °C in a Beckman Ti50.2 rotor, and extracted overnight at 4 °C in buffer containing 20 mM Tris pH 7.9/150 mM NaCl/10 mM $MgCl_2$/1 mM PMSF/1% (wt/vol) n-dodecyl-β-D-maltopyranoside (DDM; Anatrace). Soluble material was separated from insoluble material by ultracentrifugation in 4 °C at 130,000 × *g* for 90 min, and was applied to Talon Metal Affinity Resin (Clontech) for Immobilized Metal Affinity Chromatography (IMAC). S^{21}68^{his} or its variants was eluted in buffer containing 20 mM Tris, pH 7.9/150 mM NaCl/500 mM imidazole/0.1% (wt/vol) DDM.

Gel Filtration Chromatography. Gel filtration analysis was performed on an AKTA FPLC workstation (Amersham Pharmacia). Briefly, purified protein samples in elution buffer [20 mM Tris, pH 7.9/150 mM NaCl/500 mM imidazole/0.1% (wt/vol) DDM] were centrifuged in 4 °C at 130,000 × *g* for 10 min to remove any aggregates. A 300-μL volume of each supernatant was loaded on a Superdex 200 10/300 GL column [Amersham Pharmacia; bed dimensions 10 × 300 mm, bed volume 24 mL, preequilibrated with buffer containing 20 mM Tris, pH 7.9/150 mM NaCl/0.1% (wt/vol) DDM] with flow rate 0.4 mL/min at

room temperature, eluted with the same preequilibration buffer, and collected in 1 mL fractions.

SDS/PAGE and Western Blotting. SDS/PAGE and Western blotting was performed as described (7). Briefly, 10% Tris-Tricine gels were used to separate protein samples. Proteins were then transferred to either 0.2 μm PVDF membranes (Pall), for $S^{21}68$ and $irsS^{21}68$ protein variants; or 0.1 μm nitrocellulose membranes (Whatman) for $S^{21}68_{\Delta TMD1}$ protein variants. An antibody raised in rabbit against the S^{21} C-terminal peptide KIREDRR-KAARGE was used as primary antibody to detect S^{21} protein variants (3), at a dilution of 1:1,000, and preincubated with *E. coli* strain MG1655 *lacI*^{q1} *tonA*::Tn*10* or RY17341 cell lysates to reduce background. Horseradish peroxidase-conjugated goat-anti-rabbit secondary antibody (Pierce) was used at a dilution of 1:4,000. Except Fig. S7, blots were developed by using the Supersignal West Femto maximum sensitivity substrate kit (Thermo Scientific) according to the manufacturer's instructions. Images were obtained by using the Molecular Imager Gel Doc XR system (Bio-Rad) and analyzed by software Quantity One (Bio-Rad). The blot in Fig. S7 was developed by the chromogenic substrate 4-chloro-1-naphthol (Sigma).

1. Smith DL, Young R (1998) Oligohistidine tag mutagenesis of the lambda holin gene. *J Bacteriol* 180:4199–4211.
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9. Gründling A, Manson MD, Young R (2001) Holins kill without warning. *Proc Natl Acad Sci USA* 98:9348–9352.
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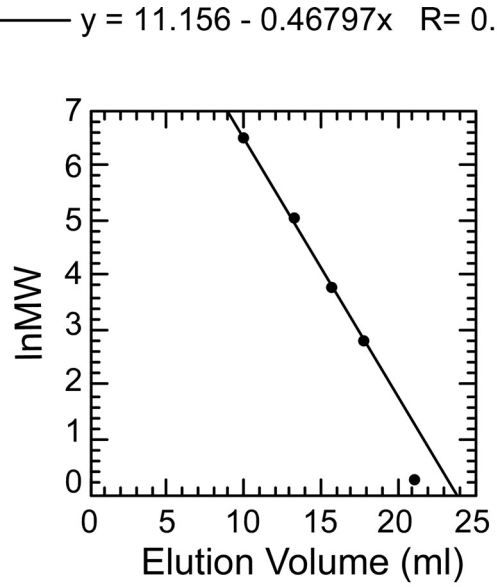
12. Gründling A, Manson MD, Young R (2001) Holins kill without warning. *Proc Natl Acad Sci USA* 98:9348–9352.



	Elution Vol (ml)	MW (kDa)	lnMW
Thyroglobulin	9.829	670	6.51
Bovine γ-globulin	13.241	158	5.06
Chicken ovalbumin	15.669	44	3.78
Equine myoglobin	17.75	17	2.83
Vitamin B_{12}	20.988	1.35	0.30
$S^{21}68^{his}$	13.907	104	4.65

Fig. S1. Calculation of the size of the $S^{21}68$ in gel-filtration chromatography. The natural logarithm of the molecular mass of each protein standard was plotted against elution volume on a Superdex 200 gel-filtration column. The elution volume of vitamin B_{12} was close to the bed volume, and ignored in the plot. The apparent molecular mass of $S^{21}68$ was calculated by fitting the equation generated from the protein standard.

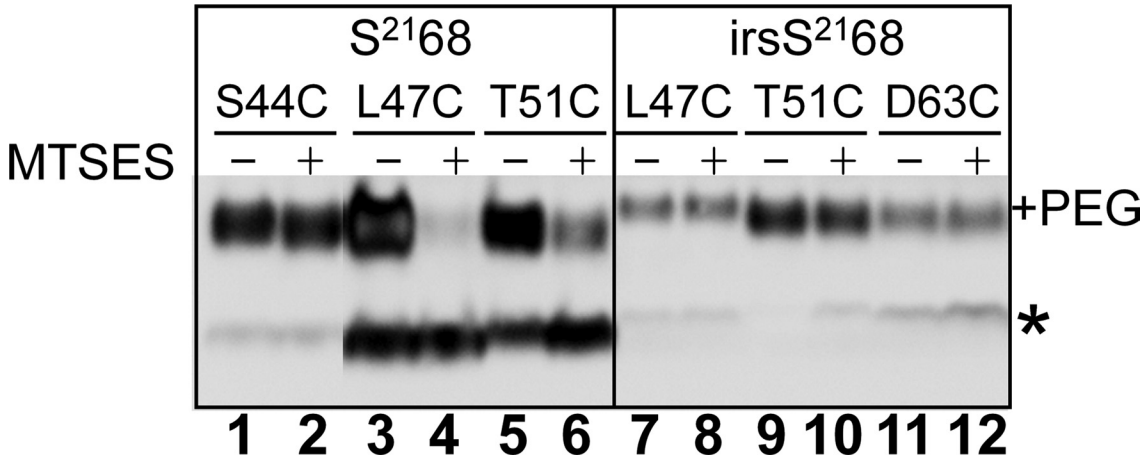


Fig. S2. Membrane depolarization by 2,4-dinitrophenol (DNP) treatment does not affect the methanethiosulfonate (MTSES) protection. Experiment was as described in Fig. 3, except that 1 mM DNP was added into each culture just before harvesting and into the phosphate buffer used in each step until the L-cysteine quenching reaction. (*Left*) $S^{21}68$ carrying the lysis-defective mutation S44C (lanes 1 and 2), and cysteine substitutions at the lumen-facing residues L47 (lanes 3 and 4) and T51 (lanes 5 and 6); (*Right*) $irsS^{21}68$ carrying the cysteine substitutions at L47 (lanes 7 and 8), T51 (lanes 9 and 10), and also at D63, which is located in the cytoplasmic tail (lanes 11 and 12).

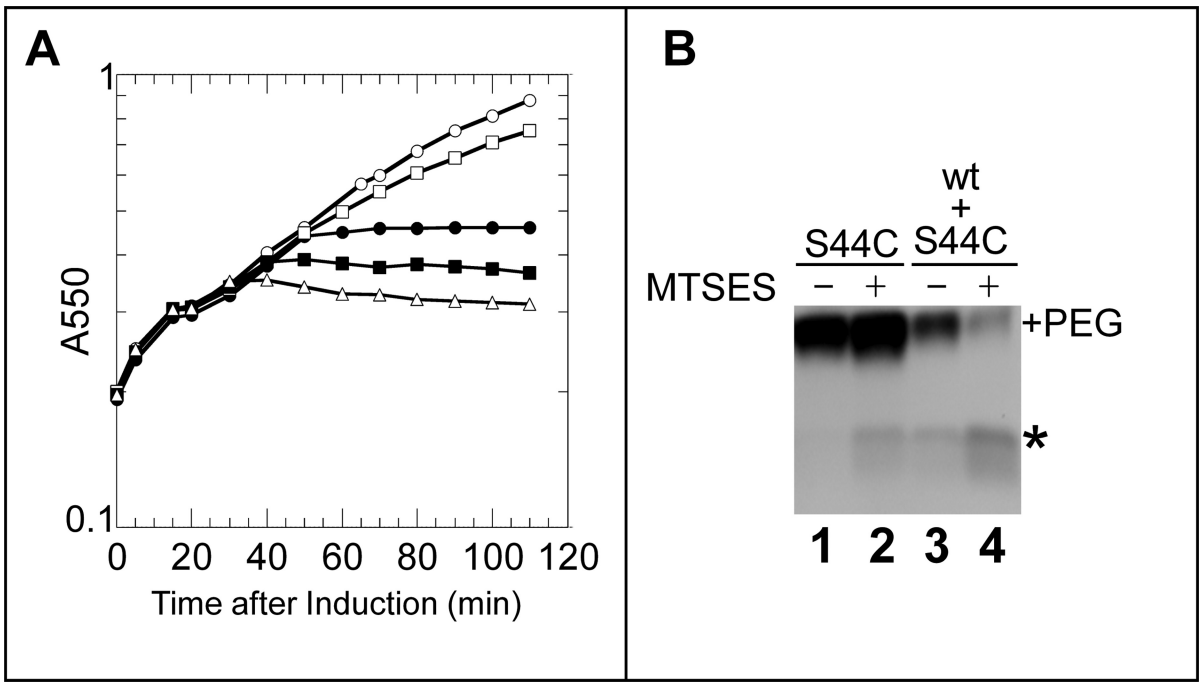
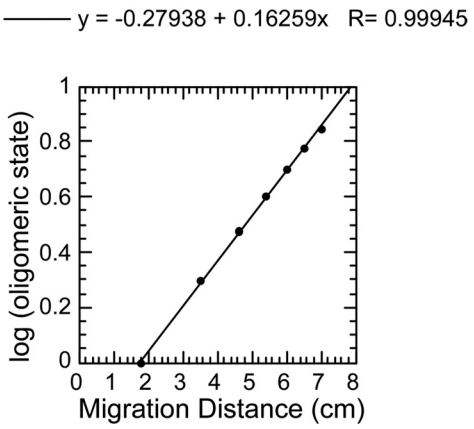


Fig. S3. $S^{21}68_{S44C}$ is lysis-defective but can participate in pinhole formation with the WT protein. (*A*) Triggering of *$S^{21}68$* alleles. Cultures carrying indicated plasmids and prophages were induced at t = 0 and monitored for culture growth as A_{550}. Open circles: prophage $\lambda Q^{21}\Delta(SRRzRz1)^{21}$, no plasmid. Closed circles: prophage $\lambda S^{21}68$, no plasmid. Open squares: prophage $\lambda Q^{21}\Delta(SRRzRz1)^{21}$, plasmid $pS^{21}68_{aS44C}$. Closed squares: prophage $\lambda S^{21}68$, plasmid $pS^{21}68_{a}$. Open triangles: prophage $\lambda S^{21}68$, plasmid $pS^{21}68_{aS44C}$. (*B*) MTSES-protection of position 44. Experiment as described in Fig. 3, except that the cells carried $pS^{21}68_{aS44C}$ with either (lanes 1 and 2) prophage $\lambda Q^{21}\Delta(SRRzRz1)^{21}$ or (lanes 3 and 4) prophage $\lambda S^{21}68$.



Migration distance (cm)	Oligomeric state	Calc. Oligomeric state
1.8	1	1.03
3.5	2	1.95
4.6	3	2.94
5.4	4	3.97
6	5	4.97
6.5	6	5.99
7	7	7.22

Fig. S4. The DSP cross-linked $S^{21}68$ bands correspond to their oligomeric states. The decadic logarithm of the apparent oligomeric state of each cross-linked $S^{21}68$ band was plotted against the migration distance of each band on the gel. A linear equation was applied. In the table, the calculated oligomeric state was obtained by putting each migration distance into the equation.

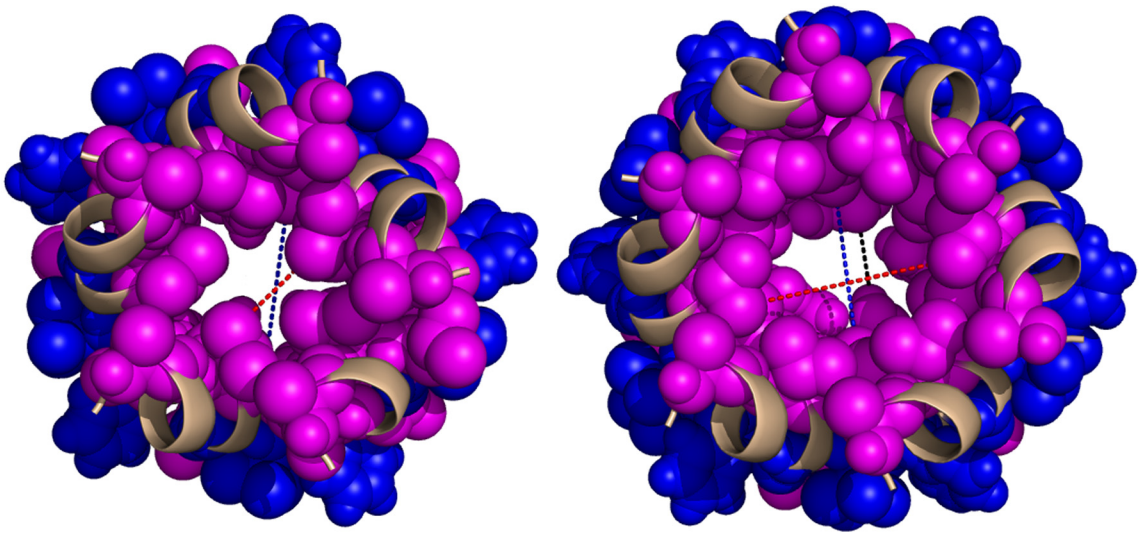


Fig. S5. Computational models for the pentameric (*Left*) and hexameric (*Right*) pinholes. MTSES sensitive and insensitive positions are shown in magenta and blue, respectively. Colored dashed lines show pore distances at various depths. In the pentamer: red, 6.4 Å at Val41; blue, 11.6 Å at Leu45. In the hexamer: red, 12.6 Å at Val41; blue, 10.5 Å at Leu45; black, 6.7 Å at Tyr52.

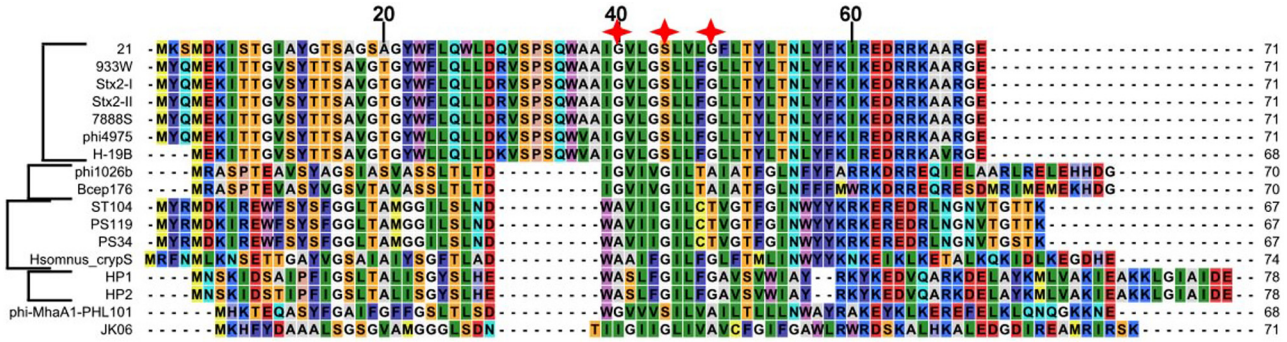
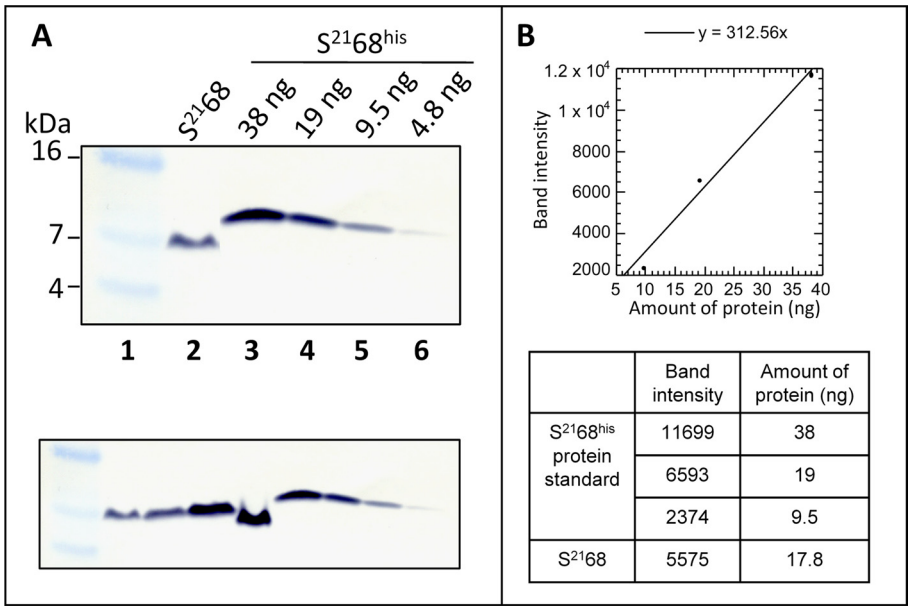


Fig. S6. Conservation of ''glycine-zipper'' motif in class II pinholins. The genomes of phages and prophages of Gram-negative bacteria were scanned for annotated holin genes. Among the class II holins (two predicted TMDs), those with putative signal-anchor release (SAR) domains were selected by the presence of a dual start motif, MxxM, or by homology to sequences with dual start motifs. The brackets indicate families of related sequences. The red stars indicate the positions of the glycine-zipper motif in S^{21}. Numbering at the top refers to S^{21}, and the length in amino acids, of each sequence is to the right. The GenBank identification numbers of these sequences are: 21: 215467; 933W: 9632510; Stx2-1: 20065950; Stx2-II: 32171159; 7888S: 10799915; phi4975: 30910957; H-19B: 2668771; phi1026b: 38707913; Bcep176: 77864684; ST104: 46358688; PS119: 30910957; PS34: 3676080; Hsomnus_crypS: 915370; HP1: 9628629; HP2: 17981839; phi-MhaA1-PHL101: 90110550; JK06: unannotated holin gene found in phage JK06 genome, GI: 71149512.



	Band intensity	Amount of protein (ng)
$S^{21}68^{his}$ protein standard	11699	38
	6593	19
	2374	9.5
$S^{21}68$	5575	17.8

Fig. S7. Measurement of $S^{21}68$ expression level at the time of triggering. (*A*) MDS12Δ*tonA* (λ*S*^{21}*68*) was thermally induced and an aliquot of 2.1×10^8 cells was precipitated by trichloroacetic acid immediately after $S^{21}68$ triggering. The sample was subjected to SDS/PAGE and western blotting in parallel with samples containing a known amount of purified $S^{21}68^{his}$. Lane 1, molecular weight standard; lane 2, $S^{21}68$ from MDS12Δ*tonA*(λ*S*^{21}*68*); lanes 3–6, purified $S^{21}68^{his}$, amount indicated at the top of each lane. Band intensities were measured using the ImageJ program (http://rsb.info.nih.gov/ij/). (*B*) The amount of $S^{21}68$ present in the induced cultures was calculated using the standard curve generated from samples containing the purified $S^{21}68^{his}$ protein. Based on 17.8 ng $S^{21}68$ accumulated in 2.1×10^8 cells, approximately 6,400 $S^{21}68$ molecules are present at the normal triggering time.

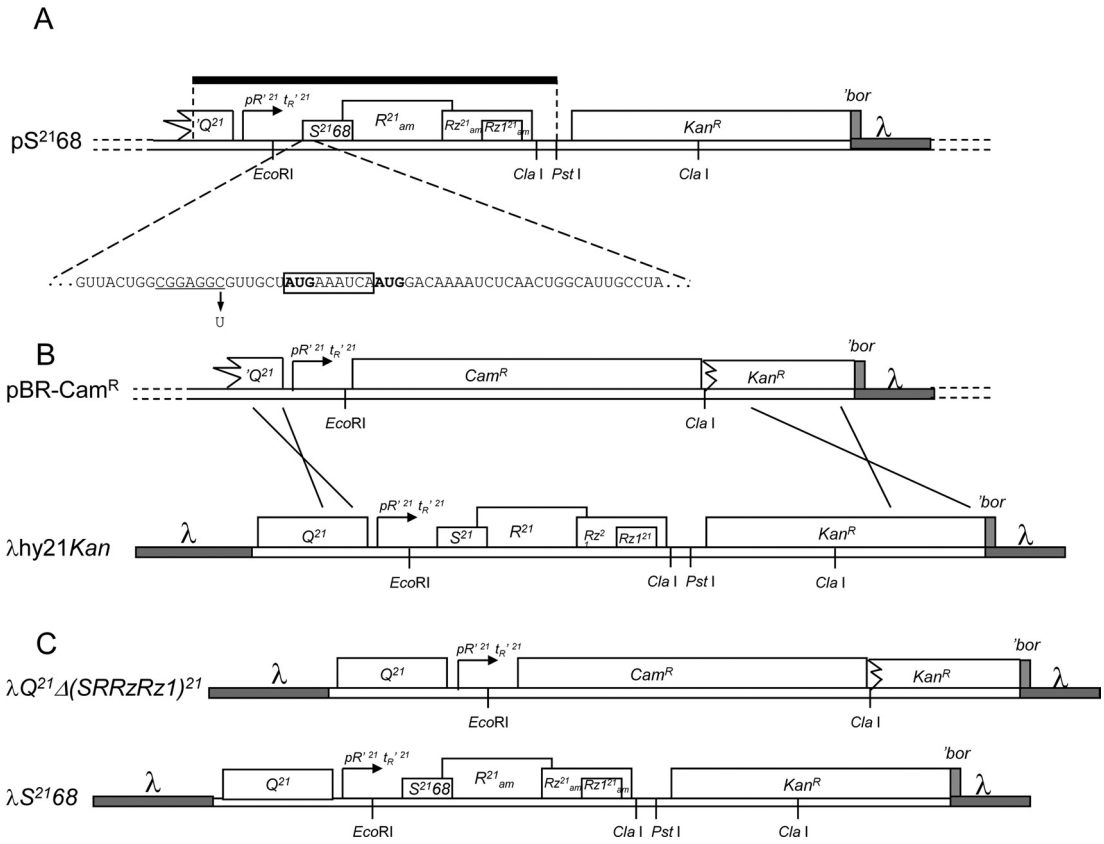


Fig. S8. Structure of plasmids and phages. (*A*) Structure of the plasmid pS^{21}68. The black bar above the 21 lysis gene cassette represents the DNA fragment inserted between the NcoI and PstI sites of plasmid pRE to construct plasmid pS^{21}68$_a$. In this construct, the first three codons (boxed) of *S^{21}* are deleted, so that the holin S^{21}68, but not the antiholin S^{21}71, is encoded. The Shine-Dalgarno sequence serving the *S^{21}68* gene is changed from 5′-CGGAGGC-3′ to 5′-CGGAGGT-3′, and amber nonsense codons were introduced into *R^{21}* (positions Tyr39 and Tyr42), *Rz^{21}* (Gln100), and *Rz1^{21}* (Trp39). See *SI Materials and Methods* for details of construction. (*B*) Structures of the plasmid pBR-Cam^R and phage λhy21*Kan*. Crosses between the two constructs indicate the regions of homology. (*C*) Structures of the phage *λQ^{21}Δ(SRRzRz1)^{21}* and the phage *λS^{21}68*. Phage *λQ^{21}Δ(SRRzRz1)^{21}* was obtained by homologous recombination between the plasmid pBR-Cam^R and the phage λhy21*Kan*. Phage *λS^{21}68* was obtained by homologous recombination between the plasmid pS^{21}68 and the phage *λQ^{21}Δ(SRRzRz1)^{21}*. For details, see *SI Materials and Methods*.

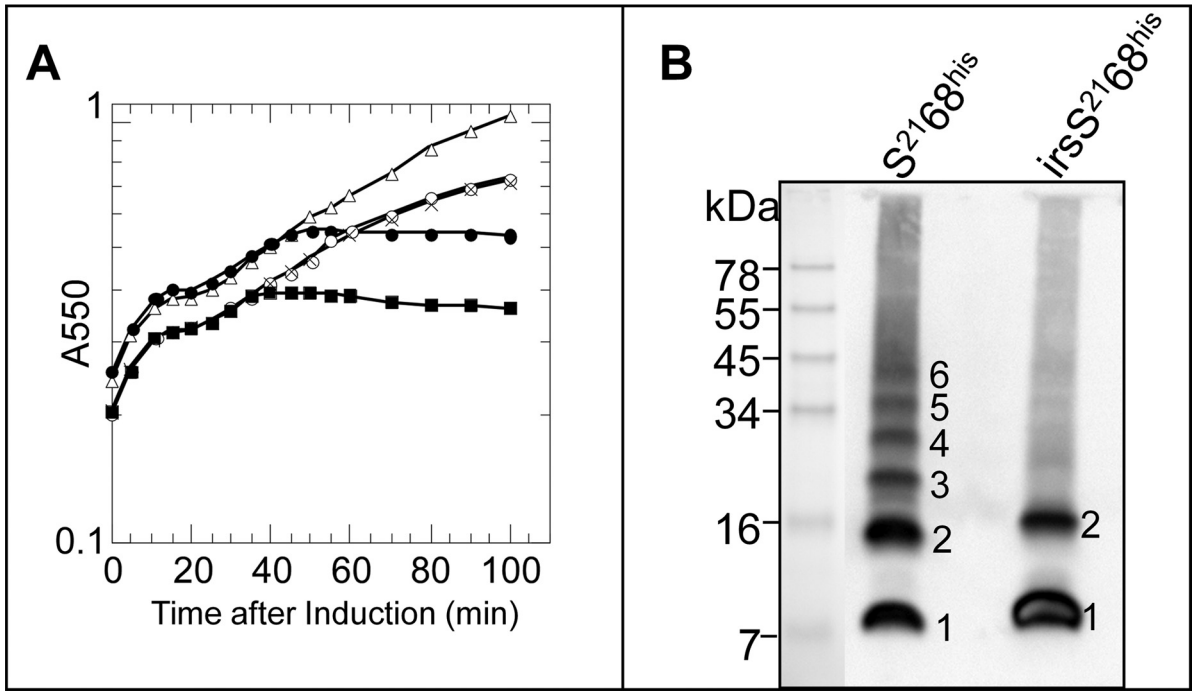


Fig. S9. The addition of his-tag does not affect the function of $S^{21}68$. (*A*) Triggering of $S^{21}68^{his}$ alleles. Cultures carrying prophage $\lambda Q^{21}\Delta(SRRzRz1)^{21}$ and indicated plasmids were induced at t = 0 and monitored for culture growth as A_{550}. Open triangles: no plasmid. Closed circles: plasmid $pS^{21}68$. Open circles: plasmid $pirsS^{21}68$*. Closed squares: plasmid $pS^{21}68^{his}$; crosses: $pirsS^{21}68^{his}$. (*B*) DSP cross-linking of $S^{21}68^{his}$ and $S^{21}68^{his}$ in vivo. Experiment was as described in Fig. 4 *Left*, except that whole cells carrying prophage $\lambda Q^{21}\Delta(SRRzRz1)^{21}$ with either plasmid $pS^{21}68^{his}$ or $pirsS^{21}68^{his}$ were used.

Table S1. Bacterial strains, bacteriophages, and plasmids

	Genotype and relevant features	Source/reference
Phages		
λhy21*Kan*	λ *cI857* hy(*QSRRzRz1*)^{21} *bor*::*kan*	3
λ$Q^{21}\Delta$*(SRRzRz1)*^{21}	λ *cI857* hy(*Q*$^{21\Delta}$*(SRRzRz1)* ^{21}::*Cam*^{R}*) bor*::*kan′*	This study
λS^{21}*68*	λhy21*Kan* S^{21}*68*(R_{am}/Rz_{am}/$Rz1_{am}$)^{21}	This study
E. coli strains		
MG1655	F^{-} *ilvG rfb50 rph1*	8
MG1655*lacI*^{q1}*tonA*::Tn*10*	—	7
MDS12	MG1655 with 12 deletions, totalling 376,180 nt, including cryptic prophages	5
RY 17341	MDS12Δ*tonA*	Lab stock
C43(DE3)	Derivative of BL21(DE3) [*E.coli* F^{-} *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* λ(DE3)], used for overexpression of membrane proteins	10
MDS12Δ*tonA*(λhy21*Kan*)	Lysogen carrying prophage λhy21*Kan*	This study
MDS12Δ*tonA*(λ$Q^{21\Delta}$*(SRRzRz1)*^{21})	Lysogen carrying prophage λ$Q^{21}\Delta$*(SRRzRz1)*^{21}	This study
MDS12Δ*tonA*(λS^{21}*68*)	Lysogen carrying prophage λS^{21}*68*	This study
Plasmids		
pBR121	pBR322::(*Q′SRRzRz1*)^{21}	3
pBR-Cam^{R}	pBR121 with the DNA between the *Eco*RI site upstream of the S^{21} gene and the *Cla*I site within the *kan*^{R} gene replaced with a *cam*^{R} cassette	This study
pBP68	pBR121 but S^{21}*68* instead of S^{21}	3
pS^{21}68	pBP68 (R_{am}/Rz_{am}/$Rz1_{am}$)^{21}, Shine-Dalgarno sequence of S^{21}*68* changed.	This study
pTP2	pBR322 origin, pR′ promoter, (*S68*/*R*/*Rz*/*Rz1*)^{21}	7
p$S^{21}68_{\Delta TMD1}$	pTP2 with the codons encoding TMD1 of S^{21}*68* deleted, (R_{am}/Rz_{am}/$Rz1_{am}$)^{21}	This study
pTP7	pTP2 encoding RYIRS fusion to N terminus of S^{21}68	7
pirsS^{21}68	pTP7 (R_{am}/Rz_{am}/$Rz1_{am}$)^{21}	This study
pQ	pSC101 origin with modification, $P_{lac/ara-1}$ promoter, *Q* from λ	12
pRE	pJF118EH with *lacI*^{Q} and P_{tac} replaced by pR′ promoter	7
p$S^{21}68_a$	pRE with pR′^{21}, (*S68*R_{am}/Rz_{am}/$Rz1_{am}$)^{21} *Amp*^{R}, instead of *Kan*^{R}	This study
pirsS^{21}68*	p$S^{21}68_a$ with codons encoding RYIRS fused to N terminus of S^{21}*68*	This study
pET$S^{21}68^{his}$	pET11a carrying S^{21}*68* gene, with introduction of codons of GGH_6GG between codons 66 and 67	7
pET$S^{21}68_{\Delta TMD1}{}^{his}$	pET$S^{21}68^{his}$ but $S^{21}68_{\Delta TMD1}$ instead of the S^{21}*68*	This study
pETirs$S^{21}68^{his}$	pET$S^{21}68^{his}$ with codons encoding RYIRS fused to N terminus of S^{21}*68*	This study
p$S^{21}68^{his}$	p$S^{21}68_a$ with codons encoding GGH_6GG inserted between codons 66 and 67	This study
pirs$S^{21}68^{his}$	p$S^{21}68^{his}$ with codons encoding RYIRS fused to N terminus of S^{21}*68*	This study