Supporting Information

Lukacik et al. 10.1073/pnas.1203472109

SI Text

SI Materials and Methods. ***Strains, plasmids, and cloning.*** *E. coli* BL21 (DE3) and T7 express cells were obtained from EMD Biosciences and New England Biolabs, respectively. *Yersinia pestis* KIM6+ (1) and KIM10+ were provided by B.J. Hinnebusch (NIAID, NIH). *Y. pseudotuberculosis* 2I (type 1) was provided by S. Adhya (NCI, NIH). *Y. pseudotuberculosis* contains a 4.74 Mbp circular chromosome, a 27 Kbp plasmid (pYptb32953), and a 68 Kbp plasmid (pYV; homologous to pCD1 in *Y. pestis*). The pYV plasmid is associated with virulence, enabling Ca^{2+} dependent growth at 37 °C, expression of V and W antigens and expression of certain outer membrane proteins including those involved in type III secretion. All pathogenic species of *Yersinia* contain the pCD1virulence plasmid. *Y. pestis* also contains two unique plasmids, pPCP1 and pMT1, that encode a variety of virulence determinants. The pPCP1 plasmid encodes the plasminogen activator Pla essential for virulence through the subcutaneous route. Pesticin (*pst*) and pesticin immunity protein (*pim*) are also encoded on pPCP1. The pMT1 plasmid encodes murine toxin Ymt and the F1 capsular protein that have been shown to play a role in the transmission of plague. *Y. pestis* KIM6+ and KIM10+ contain the pathogenicity island on which *fyuA* is located (thus the + designation); but, they do not contain the 75 kb low calcium response virulence plasmid, pCD1. In addition, KIM10+ has been cured of pPCP1 while KIM6+ has not.

A tandem operon including the open reading frames for *pst* and *pim* was amplified from *Y. pestis* genomic DNA (strain 195/P) by PCR. The amplified product was cloned into a pET16b vector (Novagen) using the XhoI and BamHI restriction sites such that the plasmid contained an N-terminal 7-His tag and Factor Xa protease site followed by the *pst* gene that was followed by the *pim* gene. The *pim* gene has the reverse orientation relative to *pst* so a putative promoter region upstream of the *pim* start codon was also included at the 3′ end.

The nucleotides encoding the FyuA TonB type transporter were amplified from *Y. pestis* (strain 195/P) genomic DNA and cloned into pET20b (Novagen) using the NcoI and HindIII restriction sites such that the expressed protein contained only residues of the mature receptor (23–673) plus two extra N-terminal residues (Met and Gly) as a cloning artifact. The pET20b plasmid contains a PelB signal sequence that allows targeting of FyuA to the *E. coli* outer membrane. This construct will be referred to subsequently as pET20b-*fyuA*$^+$. To create pTrc99A-*fyuA*, a gene coding for the OmpA signal sequence fused to mature wild-type FyuA was synthesized (Biobasic Inc.) and cloned into the NcoI and HindIII sites of pTrc99A (2).

All remaining genes used in this study were created by gene synthesis (BioBasic Inc.). Pesticin mutants and the pesticin-T4 lysozyme hybrid were cloned into pET16b. To coexpress FyuA and Pim, a DNA fragment was synthesized that contained the T7 promoter followed by a ribosome binding site and the *pim* gene. This fragment was cloned into the HindIII and XhoI sites of pET20b-*fyuA*$^+$ in the same direction and downstream of *fyuA*. To express and purify T4 lysozyme, a gene fragment containing a 10X histidine tag followed by a TEV site and T4 lysozyme was synthesized and cloned into the NcoI and HindIII sites of pBAD/HisA (Invitrogen). The wild type sequence of T4 lysozyme (3) was modified to substitute cysteines at position 54 and 97 with Thr and Ala, respectively (4) in order to prevent possible intermolecular disulfide formation. We also cloned pesticin and the hybrid lysin into pET28a. For these constructs a 10-His tag followed by a TEV protease site is directly upstream of the pesticin or the hybrid gene. These constructs were used to express and purify pesticin and hybrid for the bactericidal assays in Fig. 2 *A–C*. For all other experiments, pesticin, the pesticin mutants, and the hybrid lysin were expressed and purified using the pET16b constructs.

Purification of pesticin and FyuA for X-ray crystallography. Cells were grown in SelenoMet Medium (Molecular Dimensions Limited) supplemented with 100 μg/mL carbenicillin and 40 μg/mL L-selenomethionine to an $OD_{600} = 0.6$ at 37 °C with a subsequent induction using 1 mM IPTG and continued growth at 25 °C for 12 h for pesticin. Growth for FyuA was the same except that no IPTG was added and the cells were grown at 37 °C for approximately 60 h to an OD_{600} of about 2.

For pesticin purification, 20 g of cells were resuspended in 100 mL of 50 mM K_2HPO_4 at pH 7.5, 200 mM NaCl, 10 mM $MgCl_2$, 10 μg/mL DNase I, and 100 μM AEBSF. The cells were then disrupted in an Emulsiflex-C5 (Avestin) homogenizer (four passes, 18,000 psi, 4 °C). Following centrifugation (160,000 × *g*, 1 hour, 4 °C) the clarified supernatant was applied to a 15 ml Ni-NTA column (Qiagen) equilibrated with 50 mM K_2HPO_4 at pH 7.5, 200 mM NaCl and 10% glycerol (vol/vol). Pesticin was eluted using an imidazole step gradient. Protein containing fractions were then pooled and dialyzed at 4 °C for 12 hours against 50 mM Tris-HCl at pH 8.0, 100 mM NaCl, 1 mM EDTA. The sample was then filtered using a 0.22 μm filter and further purified by anion exchange using a 10 ml Q-sepharose high performance column (GE Healthcare) equilibrated in 50 mM Tris-HCl at pH 8.0, and 1 mM EDTA. A linear NaCl gradient was used to elute the protein. Peak fractions were pooled and applied to a Sephacryl 300 16/60 column (GE Healthcare) equilibrated with 20 mM Tris HCl at pH 8.0, 200 mM NaCl, 1 mM EDTA, and 0.02 % NaN_3 (wt/vol). The essentially pure pesticin peak was concentrated (Centriprep YM-10) to a final concentration of 12 mg/mL for use in crystallization.

The FyuA protein was detergent solubilized and purified by three chromatography steps (5). In brief, FyuA was extracted from the cell membrane with the use of 5% vol/vol ELUGENT (Calbiochem). Then, the protein was applied to a nickel column in the presence of 0.01% dodecyl maltoside (DDM), the His_{10}-tag was cleaved with TEV protease , and the cleavage step was followed by ion exchange in the presence of 0.01% DDM. The final gel filtration step was carried out on a column equilibrated with 20 mM Tris·HCl at pH 7.5, 200 mM NaCl, 0.02% NaN3, and 0.05% dodecyl dimethylamine oxide. 0.45% wt/vol octyl tetraethylene glycol monoether was added to the purified peak fraction, and the protein was concentrated to 12 mg/mL. Final protein yields ranged from 5 to 10 mg of purified FyuA per 30 g of frozen cells.

Purification of pesticin, pesticin point mutants, and the hybrid protein for bactericidal assays. The above protocol was slightly modified to prepare pesticin and its point mutants for in vivo bacteriocidal assays. Cells were grown in Novagen Overnight Express TB Autoinduction Media at 37 °C to stationary phase densities. After Ni-NTA purification, the N-terminal His tag was cleaved to avoid the possibility that the tag would interfere with the function of the TonB box that is found close to the N-terminus. To cleave the tag, 5 mM $CaCl_2$ and Factor Xa protease (10 U per 1 mg of pesticin) were added to the dialyzed protein and incubated at room temperature for 12 h. The anion exchange step was omitted in the purification protocol, and the cleaved protein was applied directly to a Sephacryl 300 16/60 column equilibrated with

20 mM Tris HCl at pH 8.0, 200 mM NaCl. The protein peak was concentrated to between 2–10 mg/mL.

Further alterations to the protocol were made to reduce precipitation of the hybrid protein to negligible amounts: Cells were grown to stationary phase densities at 20 °C instead of 37 °C. All purification steps were performed strictly at 4 °C including the Factor Xa cleavage step. Consequently, the incubation period where the hybrid protein was incubated with Factor Xa was extended to 48 h. The salt content of all buffers was raised to 500 mM NaCl, and the pH of all buffers was maintained at pH 7.5. The dialysis buffer also contained 10% glycerol (vol/vol). The final hybrid protein peak was concentrated to 2 mg/mL. Pesticin and the hybrid lysin were expressed and purified using the pET28a constructs as described above except that TEV protease was used to cleave off the N-terminal tag instead of factor Xa.

Purification of T4 lysozyme for bactericidal assays. Cells were grown in 1 liter of 2x YT broth (MP Biomedicals) supplemented with 100 μg/mL carbenicillin to OD_{600} of 2.0 at 37 °C. Protein expression was induced by the addition of 0.04% arabinose (wt/vol), and the cells were cultured for a further 2 h at 37 °C. The cultures were then centrifuged at 4,800 rpm for 20 min at 4 °C. During centrifugation, the cells underwent a partial autolysis. Two micrograms per milliliter of DNAse I was then added to the centrifugation supernatant, and the supernatant was filtered through a 0.2 μm filter. Fifteen milliliters of Ni-NTA agarose (Qiagen) resin was then added to the supernatant and briefly agitated. The resin was then collected in a column that was subsequently equilibrated with 50 mM K_2HPO_4 at pH 7.5, 200 mM NaCl and 10% glycerol (vol/vol). T4 lysozyme was eluted using an imidazole step gradient. The collected peak fractions were then dialyzed against 20 mM Tris HCl at pH 8.0, 200 mM NaCl. Finally, the dialyzed protein was concentrated to 5 mg/mL.

Crystallization and structure determination. For the pesticin structure, crystals were grown by the hanging drop vapor diffusion method in 24-well Linbro Plates (Hampton Research) at 21 °C. The concentrated pesticin protein (1 μL) was mixed with 1 μL of 20% wt/vol PEG3350 and 0.25 M $CaCl_2$. Prior to freezing, the crystals were cryoprotected by the addition of 20% glycerol (vol/vol) to the mother liquor. The crystals were flash frozen in liquid propane, and X-ray diffraction data were collected at 100 K, at beamline SER-CAT 22-ID, Advanced Photon Source (Argonne, IL). A MAD data set was collected at the peak, edge, and remote wavelengths of 0.97925, 0.97939, and 0.97172 Å, respectively. Data were integrated and scaled with HKL2000 (6). Programs implemented by PHENIX (7) were used to locate the selenium sites for density modification and initial autobuilding. The initial model was improved by iterative cycles of manual rebuilding in Coot (8) and refinement in Refmac (9). Six hundred and forty eight residues in the asymmetric unit were evaluated in total by Molprobity Ramachandran analysis (10); 97.8% of all residues were in favored regions. One hundred percent of all residues were in allowed regions. There were no Ramachandran outliers.

Crystals for the hybrid lysin and thermostable (TS) hybrid lysin were grown using conditions established for pesticin and diffracted to 2.6 Å and 1.8 Å resolution, respectively. We collected diffraction data at SER-CAT and GM/CA-CAT beamlines and solved both structures by molecular replacement using the pesticin and T4 lysozyme coordinates (PDB code 2LYZ).

FyuA was crystallized by mixing 0.5 μL protein with 0.5 μL of well solution containing 2.4 M Ammonium Formate and 0.1 M Hepes at pH 6.8. Diffraction data were collected at the SER-CAT 22-ID beamline at the Advanced Photon Source (Argonne, IL). We solved the structure of FyuA in its unliganded form by the SAD method to a resolution of 3.2 Å.

Bactericidal and growth inhibition assays. T7 express cells containing empty pET20b, pET20b-*fyuA*$^+$, or pET20b-*fyuA*$^+$*pim*$^+$ were grown to an OD600 of ∼0.3 in LB medium supplemented with 100 μg/mL carbenicillin. The cells were then washed and adjusted to an OD600 of 0.1 with PBS containing 100 μg/mL carbenicillin. Pesticin or hybrid protein (diluted in PBS) was added to the treated samples to a final concentration of 100 μg/mL. The cells were then shaken at 37 °C. At each time point, the cells were washed, diluted with cold PBS, and then pipetted onto LB plates containing 100 μg/mL carbenicillin. After overnight incubation at 37 °C, colonies were counted to determine cell survival. To test the relative bactericidal activities the pesticin active site mutants and T4 lysozyme, pET20b-*fyuA*$^+$ cells were cultured to an OD_{600} of 0.3 in LB medium supplemented with 100 μg/mL carbenicillin. Pesticin, hybrid, and pesticin mutants E178A, E178A + T201A, T201A, P178D were added to individual culture tubes to a concentration of 100 μg/mL and T4 lysozyme to a final concentration of 53 μg/mL. As a positive control, 53 μg/mL of T4 lysozyme and 800 μg/mL EDTA were added to the same culture tube (Fig. S3). All tubes were incubated for 20 min before dilution and plating.

Bactericidal activity of pesticin and hybrid lysin was also tested against *E. coli* clinical isolates. We examined whether the bactericidal activity of pesticin and hybrid lysin is species specific or whether their bactericidal functions are *fyuA* specific and, therefore, generalizable to other *fyuA*$^+$ strains. We tested the bactericidal effects of both proteins against three uropathogenic *E. coli* (UPEC) laboratory strains and 18 clinical isolates whose *fyuA* genetype has been previously determined (11). For these assays, the clinical isolates were first grown in LB medium and then subcultured into M63 minimal medium containing 0.2% glycerol. The cells were then shaken O \ N at 37 °C and adjusted to an OD_{600} of 0.1 with fresh M63 minimal medium. Pesticin or the hybrid lysin was added to a final concentration of 100 μg/mL, and the cells were incubated at 37 °C with shaking for 100 min. The cells were then diluted in PBS, dispensed onto Luria broth (LB) agar plates, and the surviving colonies counted the next day.

For the pesticin growth inhibition assay, MG1655 (F- lambda-*ilvG*- *rfb*-50 *rph*-1) cells harboring pTrc99A-*fyuA* were grown in LB medium containing 100 μg/mL carbenicillin at 37 °C with shaking. When the culture reached an OD_{600} of approximately 0.1, it was split into 2 mL aliquots, and purified pesticin was added to varying concentrations. The OD_{600} of the cultures was measured approximately every 20 min to monitor cell growth.

Top agar plate assays. LB top agar was prepared (12). The following strains were tested: *E. coli* BL21(DE3) transformed with pET20b-*fyuA*$^+$ or pET20b only. The cells were grown in LB media supplemented with 100 μg/mL carbenicillin. Molten LB top agar (3 mL) was added to $8{\cdot}10^8$ cells and poured onto LB agar plates supplemented with 100 μg/mL carbenicillin. Serial dilutions of pesticin or the hybrid protein were applied to the plates and the plates were incubated at 37 °C for 16 h. Appearance of clear zones was taken as evidence of pesticin or hybrid sensitivity.

In the case of *Yersinae*—*Y. pestis* KIM6+, *Y. pestis* KIM10+, and *Y. pseudotuberculosis*—the cells were grown in iron-deficient (ID) media prepared as described by Perry and Brubaker (13) with the exception that the final iron extraction was carried out with Chelex-100 (Sigma) instead of 8-hydroxyquinoline. Forty grams of Chelex-100 was washed with 1.5 L of water and then added to 0.5 L of ID media. The mixture was then stirred for 1.5 h, and the Chelex-100 was removed by filtration. The pH was adjusted to 7.5 with NaOH. To prepare ID plates, the media was solidified by the addition of 1.5% agar (wt/vol) for the bottom layer and 0.7% for the top agar layer (wt/vol).

Cultures of *Yersiniae* were grown 24 h in LB media and subcultured into ID media. After 24 h growth in ID media, the cells

were subcultured into fresh ID media, grown for 24 h and used in top agar plate assays. Molten ID top agar (3 mL) was added to $8{\cdot}10^8$ cells and poured over ID agar plates. Dilutions of pesticin or the hybrid protein were spotted onto the top agar and the plates were incubated for 16 h at 37 °C

Using the plate assay described above, we also tested three Gram-positive strains provided by D. Nelson (U. Maryland Biotechnology Institute) for sensitivity to pesticin and hybrid. These were *Streptococcus pyogenes*, strain D471 (group A *Streptococcus*), *Staphylococcus aureus*, strain RN4220, and *Enterococcus faecalis*, strain EF24. Strains were grown in brain-heart infusion broth. None of the strains exhibited sensitivity to either toxin perhaps because pesticin and the hybrid lack the carbohydrate binding domains found in lysins that target Gram-positive organisms and/or because peptidoglycans differ chemically (*S. aureus* contains 6-*O*-acetylated peptidoglycans that are not cleaved by T4-type lysins).

Electron microscopy. *E. coli* T7 express cells transformed with pET20b-*fyuA*$^+$ were cultured at 37 °C, 220 rpm to an OD_{600} of 0.6. The hybrid, T4 lysozyme, or pesticin protein was added to the cells to a final concentration of 100 μg/mL (hybrid, pesticin) or 53 μg/mL (T4 lysozyme) and the cells were incubated at 37 °C for 10 min. Control cells were incubated in media at 37 °C for ten minutes without protein treatment. Solutions of *E. coli* samples were applied to Quantifoil holey carbon grids (Quantifoil Micro Tools GmbH, Germany), blotted and plunge-frozen in liquid ethane with a Vitrobot (FEI, Hillsboro, OR, USA). Grids were imaged using a Technai T12 (FEI, Hillsboro, OR) electron microscope operating at 120 kV equipped with an energy filter operated in zero-loss mode. Images were captured on a 2kx2k CCD camera (Gatan, Pleasanton, CA) at magnifications ranging from 21,000× to 38,500× (14.3–7.8-Å pixel size). Electron dose per image ranged from 5–10 $e^-/Å^2$ and images were recorded at −4 to −6 μm defocus.

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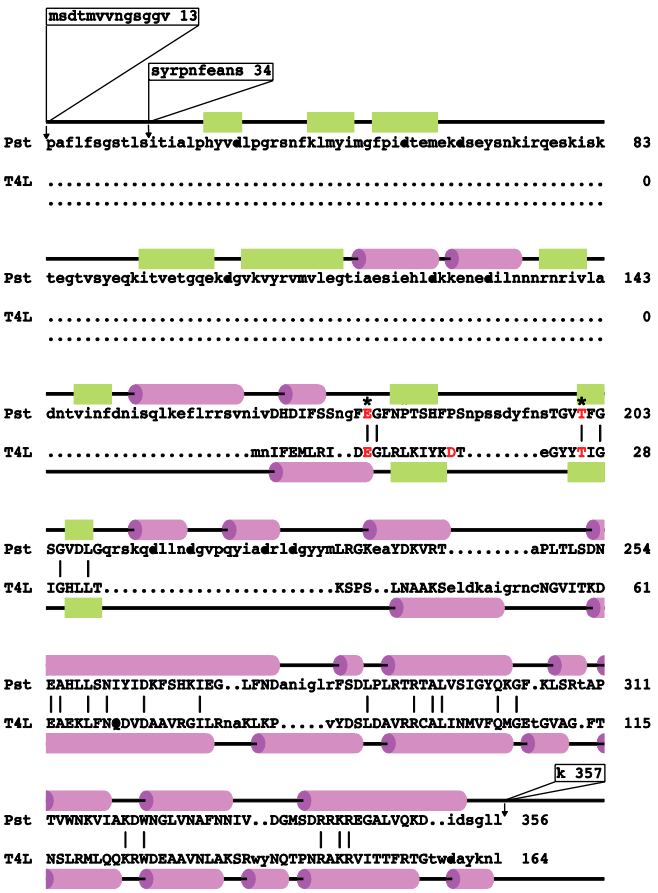

Fig. S1. The C-terminal domain of pesticin resembles T4 lysozyme structurally. Pesticin does not share sequence homology with any other bacterial colicin (except for the TonB box motif) or any other protein currently in the Protein Databank (PDB). Therefore, the pesticin fold was analyzed using the DALI server in order to identify structurally similar proteins. For the N-terminal domain, DALI analysis did not yield an unambiguous structural alignment. Only 62 residues out of the 164 residues of the N-terminal domain could be structurally aligned with a significant Z score of 3.1 to the C2 binding domain of the intracellular CD2

binding protein CD2BP2 (1). A much more definitive alignment was obtained for the C-terminal domain of pesticin. Here, a high Z score match of 8.9 was obtained to the lysozyme domain of the gp5 component of the cell-puncturing device of bacteriophage T4 (2). Therefore, despite not sharing any sequence similarity to T4 or other phage lysozymes, based on its structure the C-terminal domain of pesticin can be assigned to the phage lysozyme family (within the lysozyme-like superfamily) according to SCOP classification (3).

DaliLite structure-based sequence alignment of the pesticin C-domain with T4 lysozyme (PDB = 2LZM). The alignment identified 23 identical residues that are shared between T4 lysozyme and the C-terminal pesticin domain comprising 14% of the residues in T4 lysozyme. Active site residues are highlighted in red, and the residues mutated in this study are indicated with an asterisk. A comparison of the secondary structure elements between the two structures is also shown. α-helices are represented in pink and β-strands in light green. For completeness, the N-terminal domain of pesticin is also shown. Residues in boxes represent parts of the structure having poor electron density (i.e., not aligned in Dalilite).

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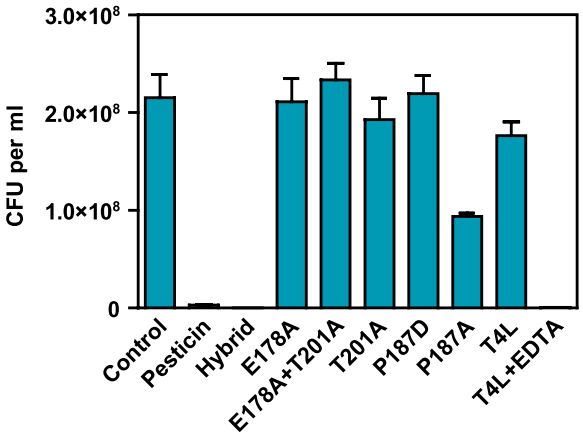

Fig. S2. Bactericidal assays for pesticin active site mutants. Pesticin, mutant pesticin, hybrid lysin, or T4 lysozyme was added to *E. coli* transformed with pET20b-*fyuA*$^{+}$. Samples were taken after a 20 min incubation, plated, and surviving bacterial colonies were counted. Mutating pesticin residues E178 or T201 to Ala completely abolishes activity; whereas, mutating P187 to Ala results in partial inactivation. Mutating P178 to Asp (mimicking T4 lysozyme) was expected to increase pesticin's activity but also results in inactivation. These experiments suggest that E178 and T201 are of primary importance in the reaction with P187 having a secondary role. To demonstrate that T4 lysozyme does not kill cells unless it gains access to the periplasm, cells were treated with 53 μg/mL T4 lysozyme or 53 μg/mL T4 lysozyme plus 800 μg/mL EDTA. EDTA permeabilizes the outer membrane allowing T4 lysozyme to degrade the peptidoglycan layer. This results in cell death. Compared to other bacteriocins, relatively high levels of pesticin and the hybrid were required to kill the *E. coli* in this assay; however, concentrations used here are similar to HEWL lysozyme concentrations routinely used to lyse Gram-negative cells in combination with EDTA (1).

1 Dean CR Ward OP (1992) The use of EDTA or polymyxin with lysozyme for the recovery of intracellular products from *Escherichia coli*. *Biotechnol Tech* 6:133–138.

PESTICIN

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        -15         -5▼          6          16          26          36
mgplyhhhhh hhssghiegr hmlemsdtmv vngsggvpaf lfsgstlssy rpnfeansit

         46          56          66          76          86          96
ialphyvdlp grsnfklmyi mgfpidteme kdseysnkir qeskiskteg tvsyeqkitv

        106         116         126         136         146         156
etgqekdgvk vyrvmvlegt iaesiehldk kenedilnnn rnrivladnt vinfdnisql

        166         176         186         196         206         216
keflrrsvni vdhdifssng fegfnptshf psnpssdyfn stgvtfgsgv dlgqrskqdl
                             *           *           *
        226         236         246         256         266         276
lndgvpqyia drldgyymlr gkeaydkvrt apltlsdnea hllsniyidk fshkieglfn

        286         296         306         316         326         336
daniglrfsd lplrtrtalv sigyqkgfkl srtaptvwnk viakdwnglv nafnnivdgm

        346         356
sdrrkregal vqkdidsgll k
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T4 LYSOZYME

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        -10           1          11          21          31          41
MGHHHHHHHH HHENLYFQGM NIFEMLRIDE RLRLKIYKDT EGYYTIGIGH LLTKSPSLNA

         51          61          71          81          91         101
AKSELDKAIG RNTNGVITKD EAEKLFNQDV DAAVRGILRN AKLKPVYDSL DAVRRAALIN

        130         140         150         160         170         180
MVFQMGETGV AGFTNSLRML QQKRWDEAAV NLAKSIWYNQ TPNRAKRVIT TFRTGTWDAY KNL
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HYBRID

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        -15         -5▼          6          16          26          36
mgplyhhhhh hhssghiegr hmlemsdtmv vngsggvpaf lfsgstlssy rpnfeansit

         46          56          66          76          86          96
ialphyvdlp grsnfklmyi mgfpidteme kdseysnkir qeskiskteg tvsyeqkitv

        106         116         126         136         146         156
etgqekdgvk vyrvmvlegt iaesiehldk kenedilnnn rnrivladnt vinfdnisql

        166         176         186         196         206         216
keflrrsvni vIFEMLRIDE RLRLKIYKDT EGYYTIGIGH LLTKSPSLNA AKSELDKAIG

        226         236         246         256         266         276
RNTNGVITKD EAEKLFNQDV DAAVRGILRN AKLKPVYDSL DAVRRAALIN MVFQMGETGV

        286         296         306         316         326
AGFTNSLRML QQKRWDEAAV NLAKSIWYNQ TPNRAKRVIT TFRTGTWDAY KNL
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Fig. S3. Sequence details of the proteins purified in this study. His tag and cloning artifacts for the pET16b constructs are highlighted in cyan, the TonB box motif in magenta, and active site residues in orange. The residues mutated in this study are marked with an asterisk. The position of the Factor Xa cleavage site is indicated with a triangle symbol. For the hybrid protein, the portion derived from pesticin is shown in lowercase and the portion derived from T4 lysozyme in uppercase. For the pET28a constructs expressing pesticin or the hybrid lysin, the sequence highlighted in cyan was replaced with a 10-His tag followed by a TEV protease cleavage site (mhhhhhhhhhhenlyfqs).

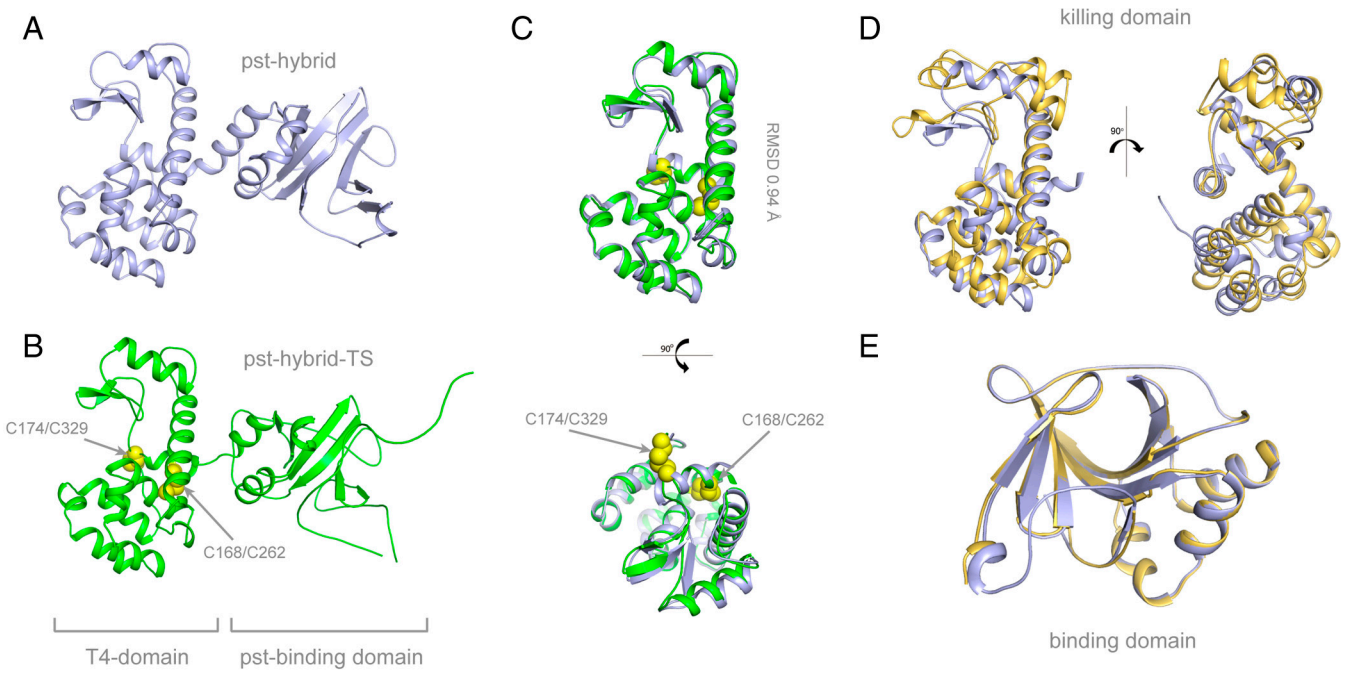


Fig. S4. Structure determination of the hybrid and thermostable hybrid. (*A*) The structure of the pesticin-hybrid (pst-hybrid) is shown in purple. (*B*) The structure of the thermostable pesticin-hybrid (pst-hybrid-TS) is shown in green. The locations of the disulfide bonds are indicated as yellow spheres. Numbering refers to the hybrid sequence. For comparison, the disulfides are located at residues 3–97 and 9–164 in T4 lysozyme. (*C*). A structural superposition of the T4-lysozyme domains from the pst-hybrid and pst-hybrid-TS crystal structures (rmsd 0.94 Å) with the view at a 90° rotation along the *x*-axis shown in the lower panel. (*D*) Alignment of the killing domains for pesticin (pst) and the hybrid-pst T4. Pesticin is shown in yellow and the hybrid-pst T4 structure is shown in purple. (*E*) Alignment of the pesticin and hybrid binding domains, with colors as in (*D*).

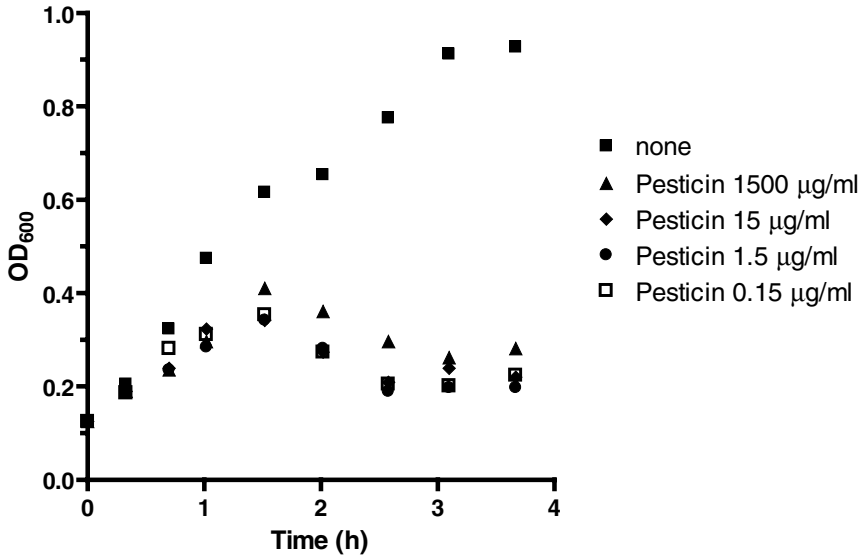


Fig. S5. Pesticin was added at varying concentrations to early log phase cultures of MG1655 cells expressing *fyuA*. The OD_{600} of these cultures was then measured approximately every 20 min. Pesticin inhibited growth similarly when it was added to a high (1500 μg/mL) or low (0.15 μg/mL) final concentration. In contrast, a final concentration of 0.15 μg/mL of colicin Ia completely inhibited cell growth; whereas, a higher final concentration (15 μg/mL) allowed cell growth that was similar to the untreated control (1). In the same study, Jakes et al. showed that each molecule of colicin Ia requires two Cir receptors: one for binding and one for cell entry and subsequent killing. Thus, at high concentrations, colicin Ia inhibits itself by binding to all of the available Cir receptors leaving none for outer membrane translocation. Because pesticin inhibits growth equally at high and low concentrations, our data suggest that pesticin does not require two copies of its receptor (FyuA) for cell binding and entry.

1 Jakes KS Finkelstein A (2010) The colicin Ia receptor, Cir, is also the translocator for colicin Ia. *Mol Microbiol* 75:567–578.

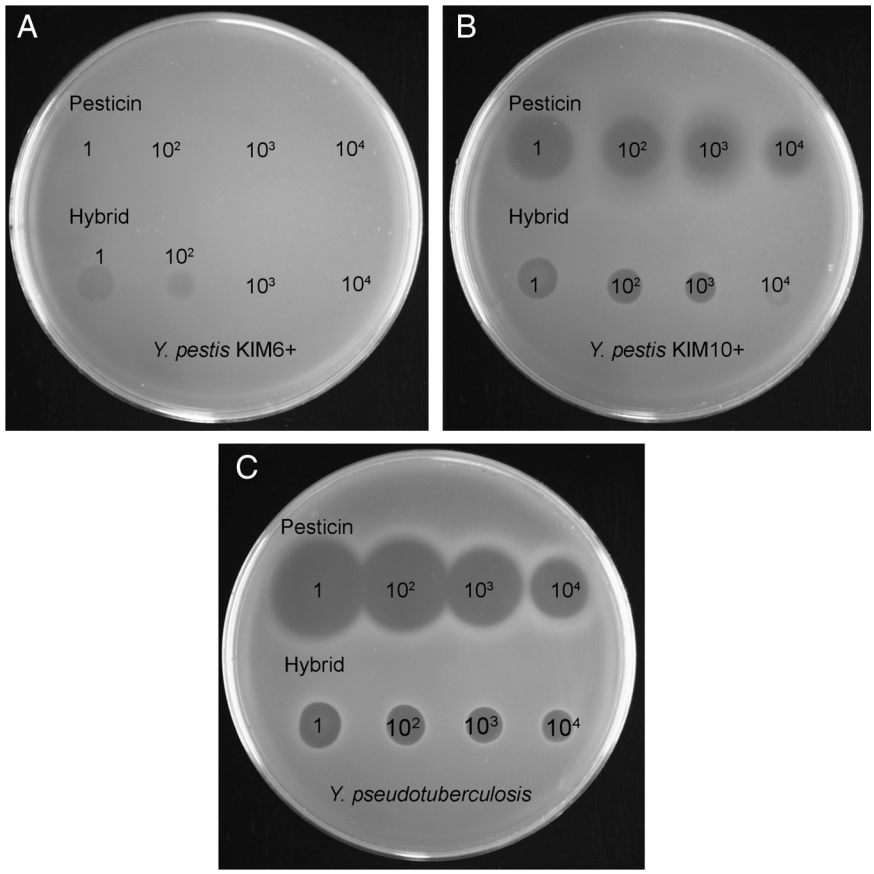
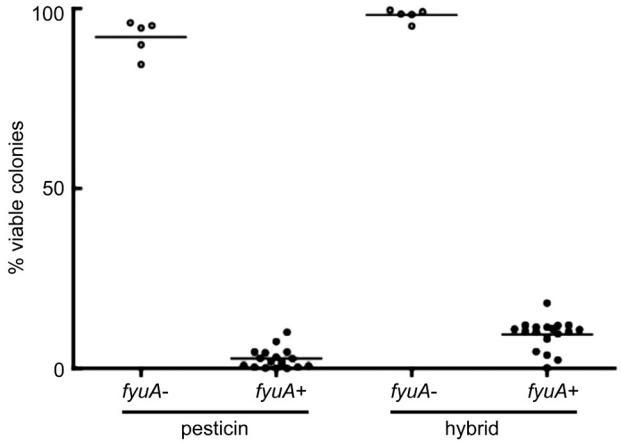


Fig. S6. *Yersiniae* plated on ID top agar plates. Serial dilutions of pesticin were performed from a starting concentration of 10 mg/mL, and 4 μL droplets of each dilution were applied to the top row of the plates (from *left* to *right*: 1×, 10×, 100×, and 1000× dilutions of pesticin). The hybrid protein was applied to the *bottom* row using the same procedure except that the starting concentration of the hybrid was 2 mg/mL. (*A*) *Y. pestis* KIM6+, (*B*) *Y. pestis* KIM10+, and (*C*) *Y. pseudotuberculosis* 2I (type 1).



Strain	*fyuA*	sensitivity to pesticin / hybrid	
E. coli			
MG1655	-	-	-
UT189	+	+	+
CFT073	+	+	+
ASB1	+	+	+
ASB2	+	+	+
ASB3	+	+	+
ASB4	-	-	-
ASB5	+	+	+
acute1	+	+	+
acute2	-	-	-
acute3	+	+	+
acute4	+	+	+
rUTI1	-	-	-
rUTI2	+	+	+
rUTI3	+	+	+
rUTI4	+	+	+
rUTI5	+	+	+
pyelo1	+	+	+
pyelo2	-	-	-
pyelo3	+	+	+
pyelo4	+	+	+

Fig. S7. Bactericidal culture assays for uropathogenic *E. coli* (UPEC) strains. To provide additional direct evidence that the FyuA receptor is essential for killing, we assessed three fully sequenced *E. coli* strains (MG1655, UTI189, and CFT073) and eighteen previously characterized UPEC strains (clinical isolates) (11) for susceptibility to pesticin and hybrid. The chart demonstrates that sensitivity to pesticin and hybrid correlates with expression of *fyuA*.

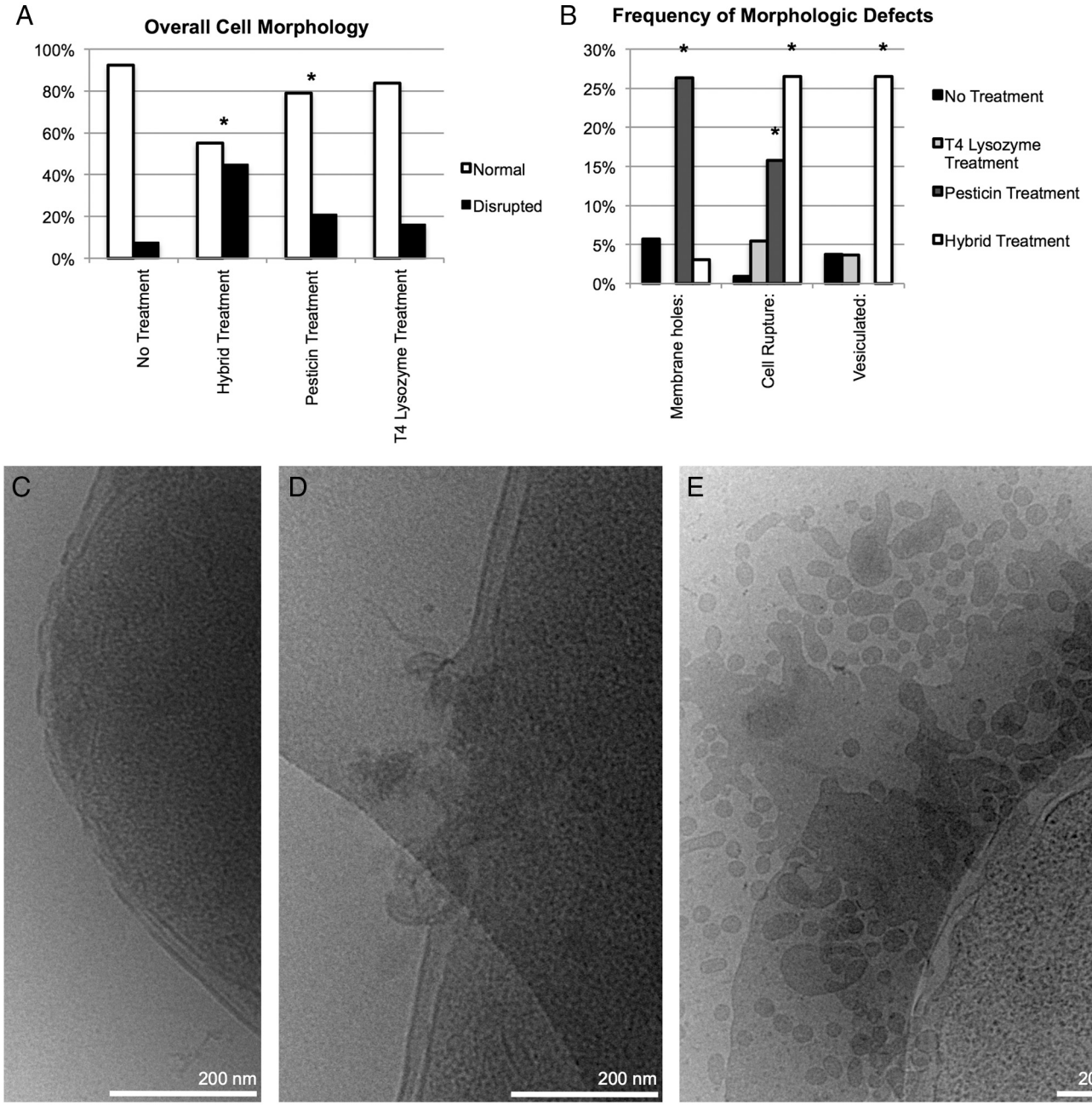


Fig. S8. Morphological effects of protein treatment observed by electron microscopy. T7 Express cells expressing FyuA were treated with protein and observed by cryo-EM as described. (*A*) Individual cell morphology was classified as "normal" or "disrupted" based on overall cell condition. Untreated control cells were >90% "normal" ($n = 106$). A significant percentage of hybrid and pesticin treated cells were classified as "disrupted" (45%, $n = 98$; 21%, $n = 76$, respectively). Over 80% of T4 lysozyme treated cells appeared to be normal, a difference that was not significant from control cells ($n = 55$). (*B*). Individual morphological defects were also tracked; several were found to correlate with protein treatment. Treatment with pesticin caused a significant rise in the appearance of holes in the outer membrane and ruptured cells. Treatment with hybrid protein caused a significant rise in ruptured cells and cells with vesiculated membranes. Statistically significant result (*) ($p < 0.01$, chi square test). (*C*) Holes in outer membrane seen in pesticin treated cells. (*D*). Ruptured cell. Inner and outer membranes are disrupted exposing cell contents seen in cells treated with pesticin or hybrid. (*E*) Vesiculation of inner and outer cell membranes seen in hybrid treated cells.

Table S1. Summary of Data Collection, Phasing, and Refinement Statistics

	pesticin (Se-MAD)			FyuA (Se-SAD)	pst-hybrid	pst-TS-hybrid
	peak	*edge*	*remote*	*peak*		
Data Collection						
Wavelength (Å)	0.97925	0.97939	0.97172	0.97912	1.0	1.0
Space group		P21		I4122	P32	P21212
a (Å)		36.50		181.18	108.43	66.40
b (Å)		86.90		181.18	108.43	122.88
c (Å)		122.23		319.86	109.75	52.30
α (°)		90		90	90	90
β (°)		96.2		90	90	90
γ (°)		90		90	120	90
Resolution (Å)*	50–2.11 (2.19–2.11)	37–2.09 (2.15–2.09)	50–2.10 (2.18–2.10)	50–3.2 (3.28–3.20)	50–2.60 (2.69–2.60)	50–1.74 (1.78–1.74)
R_{sym}*	0.052 (0.254)	0.052 (0.257)	0.054 (0.364)	0.25 (0.65)	0.073 (0.773)	0.085 (0.577)
$I/\sigma I$*	20.4 (4.12)	20.5 (4.1)	19.4 (2.7)	12.4 (1.5)	19.5 (1.9)	21.6 (3)
Completeness*	97.1 (87.4)	97.6 (91.5)	97.1 (90.7)	97.9 (83.0)	99.9 (99.7)	95.9 (94.7)
Redundancy*	3.6 (3.0)	3.6 (3.1)	3.5 (3.0)	12.7 (5.9)	4.9 (4.5)	8.3 (7.8)
Refinement						
Resolution (Å)		2.09		3.20	2.60	1.74
R_{work}/R_{free}		0.21/0.25		0.23/0.24	0.20/0.25	0.14/0.20
Number of atoms						
Protein		5291		4863	10300	2676
Ligand/ion		-		21	-	9
Water		211		-	69	561
Average B-factors						
Protein		50.7		43.3	75.1	17.7
Ligand/ion		-		55.9	-	37.6
Water		46.6		-	61.9	48.7
R.m.s deviations						
Bond lengths (Å)		0.011		0.013	0.007	0.014
Bond angles (°)		1.227		1.440	1.110	1.338
PDB code		4EPF		4EPA	4EXM	4EPI

*Statistics for the highest resolution shell are given in parentheses