# SUPPORTING INFORMATION

## Functional characterization of a subtilisin-like serine protease from *Vibrio cholerae*

Matthew Howell[1,2,†], Daniel G. Dumitrescu[1-3,†], Lauren R. Blankenship[1,2], Darby Herkert[1,2], and Stavroula K. Hatzios[1-3,*]

[1]Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT 06511, USA
[2]Microbial Sciences Institute, Yale University, West Haven, CT 06516, USA
[3]Department of Chemistry, Yale University, New Haven, CT 06511, USA
[†]These authors contributed equally to this work.
*Corresponding author: stavroula.hatzios@yale.edu

## Table of Contents

**Supplementary Table S1.** Strain list.

| Strain | Genotype/Description[a] | Source/Reference |
|---|---|---|
| WT | WT El Tor O1 clinical isolate of *V. cholerae* C6706 ($Sm^R$) | 1 |
| Δ | C6706 Δ*ivaP* | 2 |
| S361A | C6706 Δ*ivaP::*C6706 *ivaP*$^{S361A}$ | 2 |
| pBAD | C6706 Δ*ivaP* pBAD33 | This study |
| WT* | C6706 Δ*ivaP* pBAD33*SP-His$_6$-ivaP(nt 70-1605)-FLAG* | This study |
| S361A* | C6706 Δ*ivaP* pBAD33*SP-His$_6$-ivaP$^{S361A}$(nt 70-1605)-FLAG* | This study |
| WT-FLAG | C6706 Δ*ivaP* pBAD33*ivaP-FLAG* | This study |
| C9Y-FLAG | C6706 Δ*ivaP* pBAD33*ivaP$^{C9Y}$-FLAG* | This study |
| S361A-$His_6$ | C6706 Δ*ivaP* pBAD33*ivaP$^{S361A}$-His$_6$* | This study |
| *lacZ::S361A* | C6706 *lacZ::*$P_{TAC}$*:ivaP$^{S361A}$-FLAG* | This study |
| Δ *lacZ::S361A* | C6706 Δ*ivaP lacZ::*$P_{TAC}$*:ivaP$^{S361A}$-FLAG* | This study |
| Haiti | WT El Tor O1 clinical isolate of *V. cholerae*, strain H1, isolated during the 2010 Haitian cholera outbreak ($Sm^R$) | 3 |
| Haiti Δ | Haiti Δ*ivaP* | This study |
| Y9C | Haiti Δ*ivaP::ivaP*$^{Y9C}$ | This study |
| E7946 | WT El Tor O1 clinical isolate ($Sm^R$) | 4 |
| E7946 Δ | E7946 Δ*ivaP* | This study |
| N16961 | WT El Tor O1 clinical isolate ($Sm^R$) | 5 |
| DH5α*λpir* | *Escherichia coli* cloning strain | |
| SM10*λpir* | *E. coli* conjugation donor ($Kan^R$) | 6 |
| OneShot™BL21(DE3)pLysS | *E. coli* OneShot™BL21(DE3)pLysS chemically competent protein expression strain ($Cm^R$) | Thermo Fisher |
| *E. coli* pET28b*His$_6$-I9* | *E. coli* OneShot™BL21(DE3)pLysS pET28b*His$_6$-ivaP(nt 70-402)* (note: *ivaP* gene encoding aa 24-134 of IvaP) | This study |

[a]*ivaP* refers to the *vc0157* gene of *V. cholerae* (NCBI Gene ID 2614886). The FLAG tag used in this study refers to the amino acid sequence DDDDKDDDDK. SP refers to the IvaP signal peptide, aa 1-23, encoded by nt 1-69 of the *ivaP* gene.

**Supplementary Table S2.** Plasmid list.

| Plasmid | Description[a] | Source/Reference |
|---|---|---|
| pCVD442 | Allele exchange vector | 7 |
| pCVD442Δ*ivaP* | Allele exchange vector for deletion of *V. cholerae ivaP* | 2 |
| pCVD442*ivaP*$^{Y9C}$ | Allele exchange vector for chromosomal replacement of *ivaP* with the C6706 *ivaP* gene, which encodes a Cys at position 9 of the IvaP protein | This study |
| pBAD33 | Arabinose-inducible expression vector | 8 |
| pBAD33*ivaP-FLAG* | Expression vector for *ivaP-FLAG* | This study |
| pBAD33*ivaP*$^{S361A}$*-FLAG* | Expression vector for *ivaP*$^{S361A}$*-FLAG* (note: used exclusively as a cloning template in this study) | This study |
| pBAD33*ivaP*$^{C9Y}$*-FLAG* | Expression vector for *ivaP*$^{C9Y}$*-FLAG* | This study |
| pBAD33*ivaP*$^{S361A}$*-His*$_6$ | Expression vector for *ivaP*$^{S361A}$*-His*$_6$ | This study |
| pBAD33*SP-His*$_6$*-ivaP-FLAG* | Expression vector for *SP-His*$_6$*-ivaP(nt 70-1605)-FLAG* | This study |
| pBAD33*SP-His*$_6$*-ivaP*$^{S361A}$*-FLAG* | Expression vector for *SP-His*$_6$*-ivaP*$^{S361A}$*(nt 70-1605)-FLAG* | This study |
| pTD101 | *lacZ* integration plasmid with *lacI*$_q$, $P_{TAC}$, and a multiple cloning site | Tobias Dörr, Cornell University (9) |
| pTD101*ivaP*$^{S361A}$*-FLAG* | *lacZ* integration plasmid for chromosomal replacement of *lacZ* with *ivaP*$^{S361A}$*-FLAG* | This study |
| pET28b | IPTG-inducible expression vector | EMD Millipore |
| pET28b*ivaP-His*$_6$ | Expression vector for *ivaP-His*$_6$ (note: used exclusively as a cloning template in this study) | This study |
| pET28b*ivaP*$^{S361A}$*-His*$_6$ | Expression vector for *ivaP*$^{S361A}$*-His*$_6$ (note: used exclusively as a cloning template in this study) | This study |
| pET28b*His*$_6$*-ivaP(nt 70-1605)-His*$_6$ | Expression vector for *His*$_6$*-ivaP(nt 70-1605)-His*$_6$ (note: *ivaP* gene encoding aa 24-535 of IvaP; used exclusively as a cloning template in this study) | This study |
| pET28b*His*$_6$*-I9* | Expression vector for *His*$_6$*-ivaP(nt 70-402)* (note: *ivaP* gene encoding predicted I9 domain of IvaP, aa 24-134 of IvaP) | This study |

[a]The FLAG tag used in this study refers to the amino acid sequence DDDDKDDDDK. SP refers to the IvaP signal peptide, aa 1-23, encoded by nt 1-69 of the *ivaP* gene.

**Supplementary Table S3.** Primer list.

| Primer | Sequence (5' to 3')[a] | Description |
|---|---|---|
| SKH-1 | ATCCTACCTGACGCTTTTTATCG | Forward primer for sequencing pBAD33 constructs |
| SKH-35 | GGCAAATTCTGTTTTATCAGACCGC | Reverse primer for sequencing pBAD33 constructs |
| SKH-55 | CCAGCCCTCCTGTTTGAAGATG | Forward primer for sequencing pCVD442 constructs |
| SKH-56 | ACTGAGAAGCCCTTAGAGCC | Reverse primer for sequencing pCVD442 constructs |
| SKH-147 | ATAG**TCTAGA**GCCTTGCTAGCAATCAGGGAGGC | Forward primer for 5' flanking region of *ivaP*; XbaI site |
| SKH-150 | TGTA**TCTAGA**AGTGGCAACAATGGCTGTTGGAGG | Reverse primer for 3' flanking region of *ivaP*; XbaI site |
| SKH-151 | GCTGCTACATTAGGCAATCCACCCC | Forward primer for sequencing pCVD442 constructs containing 5' flanking region of *ivaP* |
| SKH-152 | CATTACAGCGTCGTGCTGAGCATA | Reverse primer for sequencing pCVD442 constructs containing 3' flanking region of *ivaP* |
| SKH-163 | CCGCTTATGTGATCGATACCGGTATTG | Forward primer for sequencing constructs containing *ivaP* |
| SKH-170 | GAGTGGTACGGCGATGGCCTCACCCCATGTAGC | Forward primer for mutating Ser361 codon of *ivaP* (TCT) to Ala (GCG) |
| SKH-171 | GGTGAGGCCATCGCCGTACCACTCATAGTCGTCG | Reverse primer for mutating Ser361 codon of *ivaP* (TCT) to Ala (GCG) |
| SKH-176 | TGACGTCAGATCCTGGTGCGAAGA | Reverse primer for sequencing constructs containing *ivaP* |
| SKH-196 | TACT**CCATGG**GCATGTTTAAAAAGTTTTTAAGCTTATGCATTGTTTCG | Forward primer for cloning *ivaP* into pET28b; NcoI site |
| SKH-197 | TACT**CTCGAG**GTAGCGAGCCACCAAACTGACATTGT | Reverse primer for cloning *ivaP* into pET28b with a C-terminal $His_6$ tag; XhoI site |
| SKH-198 | TGCT**CATATG**CAACCCAATCAGCTTGTTGGCAAATCAT | Forward primer for cloning *ivaP(nt 70-1605)* into pET28b with an N-terminal $His_6$ tag; NdeI site |
| SKH-199 | TACT**CTCGAG**GTAGCGAGCCACCAAACTGACATTGT | Reverse primer for cloning *ivaP(nt 70-1605)* into pET28b with a C-terminal $His_6$ tag; XhoI site |
| SKH-231 | CTCCATACCCGTTTTTTTGGGCTAGCGAATTCGAGATGTTTAAAAAGTTTTTAAGCTTATGCATTGTTTCG | Forward primer for cloning *ivaP* into pBAD33 via Gibson Assembly |
| SKH-232 | TGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGTCAGTGGTGGTGGTGGTGGTGCTC | Reverse primer for cloning *ivaP* with a C-terminal $His_6$ tag into pBAD33 via Gibson Assembly |
| SKH-233 | TGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAGTCATTTGTCATCATCGTCCTTATCATCATCATCGTAGCGAGCCACCAAACTGACATTGT | Reverse primer for cloning *ivaP* into pBAD33 with a C-terminal FLAG tag via Gibson Assembly |
| SKH-236 | CACCACCACCACCACCACCAACCCAATCAGCTTGTTGGCAAATCAT | Forward primer for adding an N-terminal $His_6$ tag to *ivaP(nt 70-1605)* |
| SKH-239 | GTTTTTTTGGGCTAGCGAATTCGAGATGTTTAAAAAGTTTTTAAGCTTATGCATTGTTTCGACGTTTTCCGTCGCAGCAACCTCAGCACTTGCCCACCACCACCACCACCACCAACCCA | Forward primer for cloning *ivaP(nt 1-69)-$His_6$-ivaP(nt 70-1605)-FLAG* into pBAD33 via Gibson Assembly |

| | | |
|---|---|---|
| SKH-240 | TGCAGGTCGACTCTAGAGGATCCC | Reverse primer for cloning *ivaP(nt 1-69)-His$_6$-ivaP(nt 70-1605)-FLAG* into pBAD33 via Gibson Assembly |
| SKH-246 | CAGGAAACAGACCATGGAATTCGAGCTCGGTACCCATGTTTAAAAAGTTTTTAAGCTTATGCATTGTTTCG | Forward primer for cloning *ivaP* into pTD101 via Gibson Assembly |
| SKH-247 | CTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCTCATTTGTCATCATCGTCCTTATCATCATCAT | Reverse primer for cloning *ivaP* with a C-terminal FLAG (i.e., 2XDDDDK) tag into pTD101 via Gibson Assembly |
| SKH-248 | CTCGTATAATGTGTGGAATTGT | Forward primer for sequencing pTD101 constructs |
| SKH-249 | TCAGGCTGAAAATCTTCTCT | Reverse primer for sequencing pTD101 constructs |
| SKH-255 | CCCTTTACACATTTTAGGTCTTGCCTGC | Reverse primer for confirming integration of *ivaP$^{S361A}$-FLAG* at *lacZ* locus |
| DD-3 | AATTTTGTTTAACTTTAAGAAGGAGATATACATGCACCACCACCACCACCACCAACCCAATCAGCTTGTTGGC | Forward primer for cloning *ivaP(nt 70-402)* into pET28b with an N-terminal His$_6$ tag via Gibson Assembly |
| DD-4 | TGATGATGATGATGATGGCTGCTGCCCATGTCAGATGATCGGATTAACCGTAATAATTT | Reverse primer for cloning *ivaP(nt 70-402)* into pET28b via Gibson Assembly |
| DMH-1 | AGTTTTTAAGCTTATACATTGTTTCGACGTTTTCCG | Forward primer for mutating Cys9 codon of *ivaP* (TGC) to Tyr (TAC) |
| DMH-2 | GTCGAAACAATGTATAAGCTTAAAAACTTTTTAAAC | Reverse primer for mutating Cys9 codon of *ivaP* (TGC) to Tyr (TAC) |
| LRB-12 | GCTAGTTATTGCTCAGCGG | Reverse primer for sequencing pET28b constructs |
| LRB-13 | TAATACGACTCACTATAGGG | Forward primer for sequencing pET28b constructs |

[a]Enzyme restriction sites appear in boldface text.

MFKKFLSLCIVSTFSVAATSALAQPNQLVGKSSPQQLAPLMKAASGKGIKNQYIVVLKQP
TTIMSNDLQAFQQFTQRSVNALANKHALEIKNVFDSALSGFSAELTAEQLQALRADPNVD
YIEQNQIITVNPIISASAN**AAQDNV**TWGIDRIDQRDLPLNRSYNYNYDGSGVTAYVIDTG
IAFNHPEFGGRAKSGYDFIDNDNDASDCQGHGTHVAGTIGGAQYGVAKNVNLVGVRVLGC
DGSGSTEAIARGIDWVAQNASGPSVANLSLGGGISQAMDQAVARLVQRGVTAVIAAGNDN
KDACQVSPAREPSGITVGSTTNNDGRSNFSNWGNCVQIFAPGSDVTSASHKGGTTTMSGT
SMASPHVAGVAALYLQENKNLSPNQIKTLLSDRSTKGKVSDTQGTPNKLLYSLTDNNTTP
NPEPNPQPEPQPQPDSQLTNGKVVTGISGKQGELKKFYIDVPAGRRLSIETNGGTGNLDL
YVRLGIEPEPFAWDCASYRNGNNEVCTFPNTREGRHFITLYGTTEFNNVSLVARY

**Figure S1. IvaP primary sequence.** The 23-amino-acid IvaP signal peptide predicted by the SignalP server (version 4.1) appears in red. The I9 domain of IvaP predicted by the Simple Modular Architecture Research Tool (version 8.0) is underlined (10). Tryptic peptides identified by activity-based proteomic analyses of *V. cholerae* C6706 biofilm culture supernatants are highlighted in yellow (2). The N-terminal peptide sequence of mature IvaP determined by Edman degradation analysis appears in boldface type.

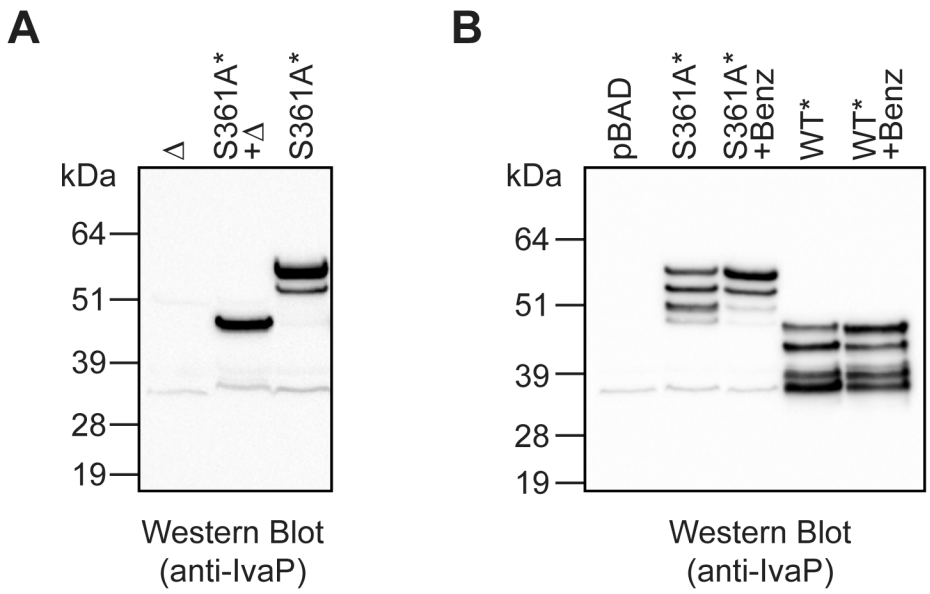


**Figure S2. IvaP$^{S361A*}$ can be partially processed by other *V. cholerae* proteases.** (**A**) Western blot analysis of *V. cholerae* C6706 culture supernatants from Δ*ivaP* (Δ) biofilms or stationary-phase cultures of S361A*. Cultures of S361A* were supplemented with ethanol to decrease proteolysis of IvaP$^{S361A*}$ as described under "Experimental procedures". Equal protein amounts of Δ and S361A* supernatants were co-incubated for 1 h at 37 ˚C prior to analysis. (**B**) Western blot analysis of stationary-phase culture supernatants from Δ*ivaP V. cholerae* C6706 expressing empty vector (pBAD), IvaP$^{S361A*}$ (S361A*), or IvaP* (WT*). Cultures were supplemented with either benzamidine (Benz) or vehicle control (water) as described under "Experimental procedures". These analyses were repeated three times with consistent results.

```
IvaP_C6706      ATGTTTAAAAAGTTTTTAAGCTTATGCATTGTTTCGACGTTTTCCGTCGCAGCAACCTCA    60
IvaP_Haiti      ATGTTTAAAAAGTTTTTAAGCTTATACATTGTTTCGACGTTTTCCGTCGCAGCAACCTCA    60
                ************************ **********************************

IvaP_C6706      GCACTTGCC       69
IvaP_Haiti      GCACTTGCC       69
                *********
```

**Figure S3. *ivaP* sequence alignment showing G-to-A mutation in *V. cholerae* Haiti.** Nucleotide sequences encoding the 23-amino-acid IvaP signal peptide from *V. cholerae* C6706 and *V. cholerae* Haiti (nt 1-69 of *ivaP*) were aligned using Clustal Omega (version 1.2.4). The G-to-A mutation is highlighted in red.

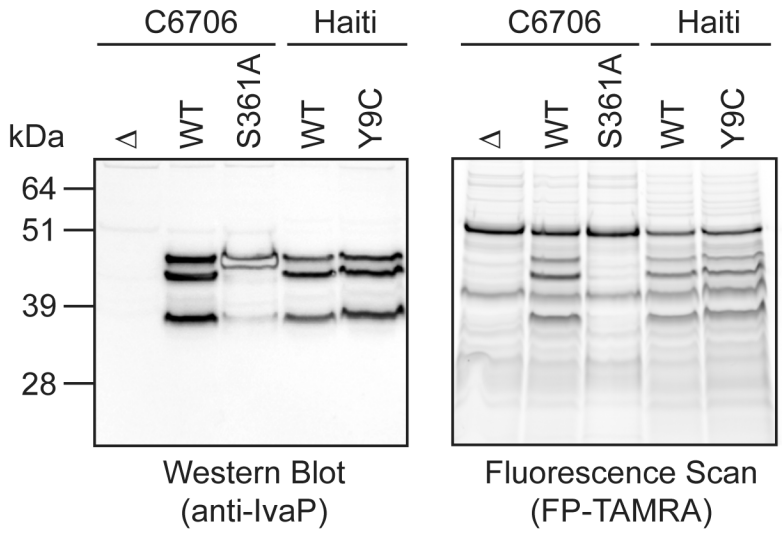


**Figure S4. IvaP is similarly processed by stationary-phase cultures of *V. cholerae* C6706 and Haiti.** Western blot (left) and in-gel fluorescence (right) analysis of FP-TAMRA-labeled supernatants from stationary-phase cultures of Δ, WT, and S361A *V. cholerae* C6706 and WT and Y9C *V. cholerae* Haiti. These analyses were repeated three times with consistent results.

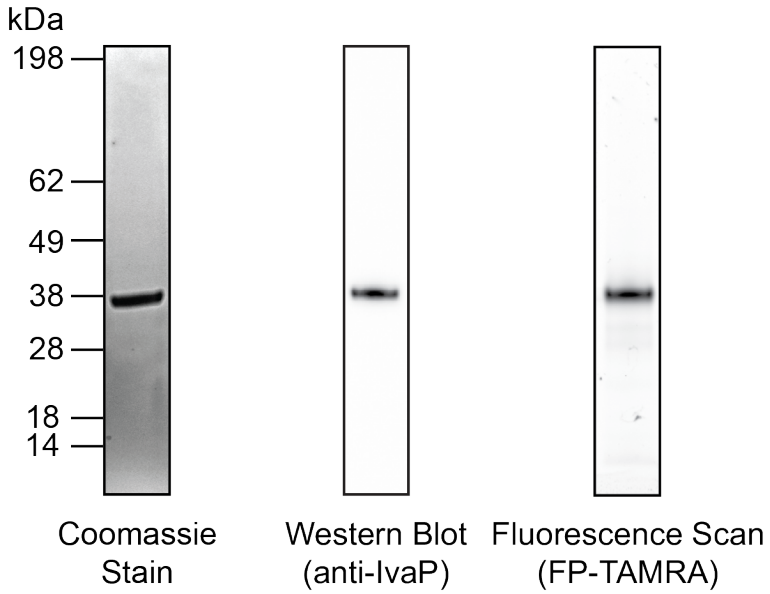


**Figure S5. Purification of mature IvaP.** Mature IvaP was purified from the culture supernatants of *V. cholerae* C6706 Δ*ivaP* expressing pBAD33*ivaP-FLAG* by anion-exchange chromatography. The purified protein was resolved by SDS-PAGE and visualized by Coomassie staining or labeled with 2 μM FP-TAMRA for 1 h at room temperature prior to Western blot and in-gel fluorescence analysis.

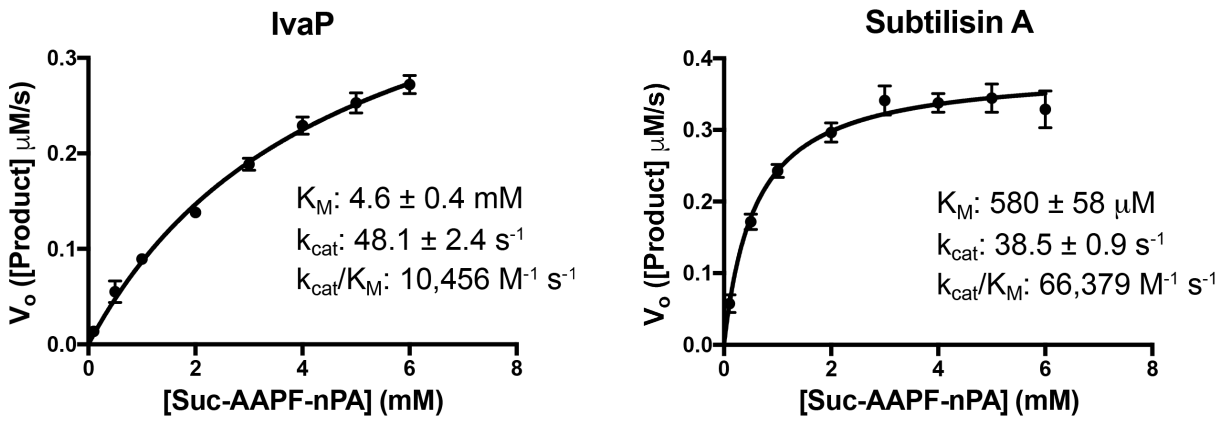


**Figure S6. Hydrolysis of *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide by IvaP and Subtilisin A.** IvaP (10 nM) or Subtilisin A (10 nM) from *Bacillus licheniformis* was incubated with the colorimetric substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Suc-AAPF-nPA; 0.1 to 6 mM) at room temperature in 100 mM Tris-HCl, pH 8. Initial velocity ($V_o$) values were calculated by measuring product absorbance at 410 nm. Michaelis-Menten parameters were calculated using GraphPad Prism (version 7.0). Data represent the mean ± S.D. (error bars) of three independent experiments.

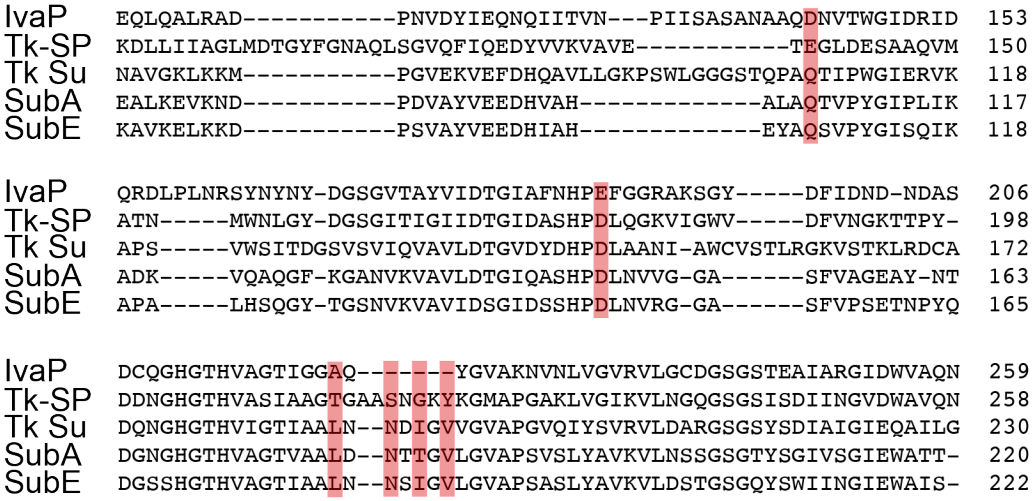


**Figure S7. Multiple sequence alignment of bacterial subtilases.** Amino acid sequences of VC0157 from *V. cholerae* (IvaP; UniProt accession number Q9KVI8), subtilisin-like serine protease from *Thermococcus kodakarensis* (Tk-SP; UniProt accession number Q5JIZ5), subtilisin from *Thermococcus kodakarensis* (Tk Su; UniProt accession number P58502), Subtilisin Carlsberg from *Bacillus licheniformis* (SubA; UniProt accession number P00780), and Subtilisin E from *Bacillus subtilis* (SubE; UniProt accession number P04189) were aligned using Clustal Omega (version 1.2.4). Residues highlighted in red participate in calcium binding in SubE (11).

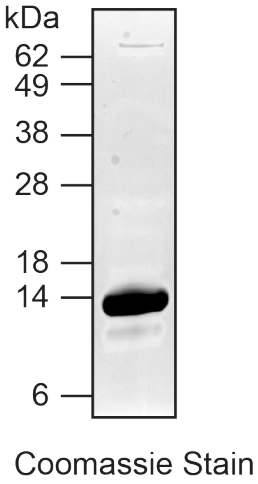


**Figure S8. Purification of the IvaP I9 domain.** A 111-amino-acid region of IvaP (aa 24-134) containing the predicted I9 domain was expressed with an N-terminal $His_6$ tag in *Escherichia coli*, purified by Ni-NTA affinity chromatography, and visualized following SDS-PAGE by Coomassie staining.

## Supplementary References

**1)** Mandlik, A., *et al.* (2011) RNA-Seq-based monitoring of infection-linked changes in *Vibrio cholerae* gene expression. Cell Host Microbe *10*, 165-174.

**2)** Hatzios, S. K., *et al*. (2016) Chemoproteomic profiling of host and pathogen enzymes active in cholera. Nat Chem Biol *12*, 268-274.

**3)** Chin, C. S., *et al*. (2011) The origin of the Haitian cholera outbreak strain. N Engl J Med *364,* 33-42.

**4)** Miller, V.L., *et al.* (1989) Identification of *toxS*, a regulatory gene whose product enhances *toxR*-mediated activation of the cholera toxin promoter. J Bacteriol *171*, 1288-1293.

**5)** Dörr, T. D., *et al.* (2016) A cell wall damage response mediated by a sensor kinase/response regulator pair enables beta-lactam tolerance. Proc Natl Acad Sci USA *113*, 404-409.

**6)** Miller, V. L. and Mekalanos, J. J. (1988) A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J Bacteriol *170*, 2575-2583.

**7)** Donnenberg, M. S. and Kaper, J. B. (1991) Construction of an *eae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. Infect Immun *59*, 4310-4317.

**8)** Guzman, L. M., *et al*. (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. J Bacteriol *177*, 4121-4130.

**9)** Weaver, A. I., *et al*. (2018) Genetic determinants of penicillin tolerance in *Vibrio cholerae*. Antimicrob Agents Chemother *62*, e01326-18.

**10)** Letunic, I. and Bork, P. (2018) 20 years of the SMART protein domain annotation resource. Nucleic Acids Res *46*, D493-D496.

**11)** Jain, S. C., *et al*. (1998) The crystal structure of an autoprocessed Ser221Cys-subtilisin E-propeptide complex at 2.0 Å resolution. J Mol Biol *284*, 137-144.