Coevolution of the ATPase ClpV, the Sheath Proteins TssB and TssC, and the Accessory Protein TagJ/HsiE1 Distinguishes Type VI Secretion Classes

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Background: The bacterial type VI secretion apparatus is a phage tail-like structure that secretes toxins/effectors.

Results: Four components of the type VI secretion system have coevolved and interact.

Conclusion: Distinct classes of type VI secretion systems emerge from the phylogenetic data.

Significance: Molecular understanding of type VI secretion might make it amenable to targeting by antimicrobials.

The type VI secretion system (T6SS) is a bacterial nanomachine for the transport of effector molecules into prokaryotic and eukaryotic cells. It involves the assembly of a tubular structure composed of TssB and TssC that is similar to the tail sheath of bacteriophages. The sheath contracts to provide the energy needed for effector delivery. The AAA+ ATPase ClpV disassembles the contracted sheath, which resets the systems for reassembly of an extended sheath that is ready to fire again. This mechanism is crucial for T6SS function. In Vibrio cholerae, ClpV binds the N terminus of TssC within a hydrophobic groove. In this study, we resolved the crystal structure of the N-terminal domain of Pseudomonas aeruginosa ClpV1 and observed structural alterations in the hydrophobic groove. The modification in the ClpV1 groove is matched by a change in the N terminus of TssC, suggesting the existence of distinct T6SS classes. An accessory T6SS component, TagJ/HsiE1, exists predominantly in one of the classes. Using bacterial two-hybrid approaches, we showed that the P. aeruginosa homolog HsiE1 interacts strongly with ClpV1. We then resolved the crystal structure of HsiE1 in complex with the N terminus of HsiB1, a TssB homolog and component of the contractile sheath. Phylogenetic analysis confirmed that these differences distinguish T6SS classes that resulted from a functional co-evolution between TssB, TssC, TagJ/HsiE1, and ClpV. The interaction of TagJ/HsiE with the sheath as well as with ClpV suggests an alternative mode of disassembly in which HsiE recruits the ATPase to the sheath.

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Gram-negative bacteria have evolved various strategies to compete in hostile environments. Among them, secretion systems have attracted a lot of attention because of their clinical importance but also because of their complex architecture and regulation. The conserved bacterial type VI secretion system (T6SS) is associated with bacteria-host interaction (3-6) but subsequently found to inject toxins into bacterial targets (7-12). These toxins have various activities, the best characterized of which are amidases (13), phospholipases (12), and nucleases (14). However, the list of T6SS toxins and their cognate immunity is expanding (15, 16), whereas their role in bacterial competition during host colonization is beginning to be established (14, 17).

A striking feature of the T6SS is its homology to the tail of the T4 phage. The gp19 tail tube is the conduit through which the phage DNA is injected, whereas the gp5-gp27 heterotrimeric complex is the puncturing device that penetrates the host cell membrane (18). The force needed to push the tube and puncturing device is provided by the contraction of the gp18 sheath (19). In the T6SS, TssB and TssC oligomerize into a sheath-like structure, similar to gp18 (20-24), whereas VgrG and Hcp share features with the puncturing device, gp5-gp27, and the tail tube, gp19, respectively (25).

A fundamental distinction between phage and T6SS is the dynamic nature of injection. The bacteriophage sheath contraction is a singular event, which empties the phage head of its DNA. In this case, return to the original state is unnecessary, and no energy is required to reset the system. In contrast, in T6SS, a series of assembly and contraction events of the TssB/TssC sheath are observed (20, 26, 27), which result in several bursts of toxin injection. ClpV, an AAA+ (ATPase associated with various cellular activities) protein, provides energy for sheath disassembly (21, 27, 28).

AAAn proteins are hexameric ring-shaped complexes involved in a variety of functions, including protein quality control (29). In the T6SS, ClpV is proposed to disassemble the contracted sheath. In support of this model, it was shown that a...
**TABLE 1**

| Strains and plasmids used in this study | Relevant characteristics/Description Source/Reference |
|----------------------------------------|------------------------------------------------------|
| **Strain** (E. coli)                   |                                                      |
| One-shotTOP10                          |                                                      |
| DHM1                                   |                                                      |
| B834(DE3)                              |                                                      |
|                                      | F – mcrA Δ(mrr-issRMS-issC)Δ80lacZAM15 16lacX74 recA1 araD139 Δ(araleu) Invitrogen |
|                                      | cyt-554 recA1 gyrA436 (Nal) his1 hsdR17 (mp8 T1 rfd1) glvV44(AS) Karimova (41) |
|                                      | F – ompT hsdSB (8 – m6b – ) gal dcm met (DE3) Laboratory collection |
| **Plasmid**                            |                                                      |
| pET28                                  | Expression vector used for expression of N-terminal 6-histidine tagged proteins Novagen |
| pET28-hsiE1                             | pET28 expressing HsiE1 with an N-terminal histidine tag This study |
| pET28-hsiE1B1                          | pET28 expressing HsiE1 with an N-terminal histidine tag in tandem with untagged HsiB1 This study |
| pET28-ClpV1-N                          | pET28 expressing ClpV1-N with an N-terminal histidine tag This study |
| pKT25                                  | pET15B vector for fusion of target proteins to B. pertussis cya gene T25 fragment; Pcya=cyt-33 p15ori, KmR Karimova et al. (41) |
| pUT18C                                  | pET15B vector for fusion of target proteins to B. pertussis cya gene T18 fragment; Pcya=cyt-33 pUCori, ApR Karimova et al. (41) |
| pKT25-zip                              | Fusion of zip encoding leucine zipper from GCN4 to cya gene T18 fragment in pUT18C, ApR Karimova et al. (41) |
| pKT25-hsiE1                            | Fusion of hsiE1 to cya gene T25 fragment in pKT25, KmR Lossi et al. (33) |
| pKT25-hsiB1                            | Fusion of hsiB1 to cya gene T25 fragment in pKT25, KmR Lossi et al. (33) |
| pKT25-hsiC1                            | Fusion of hsiC1 to cya gene T18 fragment in pUT18C, ApR Lossi et al. (33) |
| pKT25-hsiC1B1                          | Fusion of hsiC1B1 to cya gene T18 fragment in pUT18C, ApR Lossi et al. (33) |
| pKT25-ClpV1-N                          | Fusion of ClpV1 to cya gene T25 fragment in pKT25, KmR This study |
| pKT25-NClpV1                           | Fusion of NClpV1 to cya gene T25 fragment in pUT18C, ApR This study |
| pKT25-NClpV1                            | Fusion of NClpV1 to cya gene T25 fragment in pKT25, KmR This study |
| pKT25-NClpV1                            | Fusion of NClpV1 to cya gene T18 fragment in pUT18C, ApR This study |
| pKT25-NClpV1                            | Fusion of NClpV1 to cya gene T25 fragment in pKT25, KmR This study |
| pKT25-NClpV1                            | Fusion of NClpV1 to cya gene T18 fragment in pUT18C, ApR This study |
| pKT25-NClpV1                            | Fusion of NClpV1 to cya gene T18 fragment in pKT25, KmR This study |
| pKT25-NClpV1                            | Fusion of NClpV1 to cya gene T18 fragment in pKT25, KmR This study |
| pKT25-hsiC1Δ1-10                        | Fusion of hsiC1Δ1-10 to cya gene T25 fragment in pKT25, KmR This study |
| pKT25-hsiC1Δ1-10                        | Fusion of hsiC1Δ1-10 to cya gene T18 fragment in pUT18C, ApR This study |
| pKT25-hsiC1Δ1-33                        | Fusion of hsiC1Δ1-33 to cya gene T25 fragment in pKT25, KmR This study |
| pKT25-hsiC1Δ1-33                        | Fusion of hsiC1Δ1-33 to cya gene T18 fragment in pUT18C, ApR This study |
| pKT25-hsiC1Δ2-30                        | Fusion of hsiC1Δ2-30 to cya gene T18 fragment in pKT25, KmR This study |
| pKT25-hsiC1Δ2-30                        | Fusion of hsiC1Δ2-30 to cya gene T18 fragment in pUT18C, ApR This study |

**Vibrio cholerae clpV** mutant has a reduced T6SS-dependent killing activity toward *Escherichia coli*, suggesting that in this mutant sheath, contraction happens only once, and no subsequent toxin burst occurs (30). Disassembly of the *V. cholerae* VipA/VipB sheath (TssB/TssC homologs) (20, 21, 27) is dependent on a direct interaction between ClpV and the N-terminal helix of VipB (TssC homolog), which docks into a hydrophobic groove in the N-terminal domain of the ATPase (28). *Pseudomonas aeruginosa* is an opportunistic pathogen, which has three T6SSs designated H1- to H3-T6SS (31, 32). Besides 13 conserved core genes, the *P. aeruginosa* H1-T6SS contains accessory genes, among them *tagl/hsiE1*, which is not found in the H2- and H3-T6SSs. Although HsiE1 (also called Tagl) was found to interact with the essential sheath component HsiB1 (TssB/VipA homolog) (33), its exact role in T6SS remains unclear. Here we used a combination of structural and *in vivo* protein-protein interaction approaches to characterize molecular aspects of the H1-T6SS of *P. aeruginosa*. Structural characterization of the N domain of ClpV1 and analysis of its interaction with HsiC1 (TssC/VipB homolog) suggest that the H1-T6SS functions differently from the *V. cholerae* system. We solved the crystal structure of HsiE1 in complex with an N-terminal fragment of HsiB1 and observed that in addition to binding to HsiB1, HsiE1 is capable of interacting with ClpV1. We thus found evidence for distinct T6SS classes, which is confirmed through phylogenetic analysis of the four T6SS components, ClpV, HsiE/Tagl, TssB, and TssC.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Culture Conditions**—Strains were cultivated in Luria-Bertani (LB) or Terrific broth at 37 °C. The antibiotics were added at the following concentrations: ampicillin, 100 µg/ml; kanamycin, 50 µg/ml. All bacterial strains and plasmids used are listed in Table 1.

**Expression and Protein Purification**—The hsiE1 (PA0086) and hsiB1 (PA0083) genes and the sequence corresponding to the first 159 residues of ClpV1 (PA0090) were amplified from *P. aeruginosa* PA01 genomic DNA and cloned into pET28. pET28-E1 encodes HsiE1 with an N-terminal His tag cleavable with thrombin. pET28-E1B1 contained hsiE1 preceded by sequence coding for a cleavable N-terminal His tag in frame with hsiB1. pET28-ClpV1-N encodes N-terminally His-tagged ClpV1-N. In all cases, transformed *E. coli* B834(DE3) cells were grown at 37 °C to an A600 of about 0.6 in Terrific broth. Expression of proteins was induced with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside, and cells were grown overnight at 18 °C before centrifugation (4,000 × g, 15 min at 4 °C). Cell pellets were resuspended in buffer A (50 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole (pH 8.0)) and lysed by French press after the addition of an anti-protease mixture (Sigma). Cell debris was eliminated by centrifugation (40,000 × g, 40 min).

Proteins were purified by IMAC chromatography using nickel-Sepharose resin (GE Healthcare) equilibrated in buffer A. Proteins were eluted with buffer A containing 500 mM instead of 20 mM imidazole and were further purified by size exclusion chromatography using a HiLoad Superdex 75 column equilibrated in 50 mM Tris-HCl and 250 mM NaCl (pH 8). All chromatographic steps were performed on an AKTA Prime Plus system (GE Healthcare) at 4 °C. Protein purity was checked by SDS-PAGE. Proteins were concentrated to at least 10 mg/ml using centrifugal concentrators (Millipore) and stored at −80 °C.
**Crystallization and Structure Determination**—HsiE1 (at 15 mg/ml) crystallized in 30% 2-methyl 2,4-pentanediol, 0.1 M sodium acetate (pH 4.6), and 20 mM CaCl₂. For co-crystallization, HsiB1 peptide (MGSTTSSQKFIARNRAPRVQ; Eurogentec) was added in a 5-fold molar excess to HsiE1 concentrated to 22 mg/ml. Crystals grew in 50 mM MES (pH 6.5), 100 mM NaCl, 0.8 M PEG 3350. Crystals that were not already cryoprotected by the crystallization solution were transferred into buffer containing 25% glycerol.

Data were collected on a MicroMax-007 HF rotating anode (Rigaku) and at Diamond Light Source beamlines I03, I04, and I04-1 and reduced in XDS (35) and iMosflm (35). Structures were solved in Phaser (36). VPA1052 (37) was used as a search model to solve HsiE1, which was in turn used to solve the structure of the HsiE1-HsiB1 complexes. ClpV-N (28) was used as a search model for the ClpV1-N domain. Structures were refined in Refmac5 (38) and phenix.refine (39) and rebuilt in Coot (40) until convergence. Crystallographic statistics are summarized in Table 2. All models and structure factors were deposited to the Protein Data Bank with codes 4UQX (ClpV N domain), 4UQY (HsiE1), 4UQZ (HsiE1 + HsiB1 peptide), and 4UQW (HsiE1 + HsiB1 fragment).

**Bacterial Two-hybrid Assay**—The genes of interest were amplified from *P. aeruginosa* PAO1 genomic DNA, adding appropriate restriction sites. The resulting PCR products were ligated into either bacterial two-hybrid (BTH) plasmid pKT25 or pUT18C, leading to in-frame fusions of the protein of interest with the T25 or T18 subunit of the *Bordetella pertussis* adenylate cyclase, respectively (41). Recombinant pKT25 and pUT18C plasmids were co-transformed into the reporter *E. coli* DHM1 strain. Four independent colonies for each co-transformation were inoculated into LB medium supplemented with ampicillin and kanamycin. After overnight growth at 37 °C, 10 μl of each culture were spotted onto MacConkey agar plates with 1% maltose and LB agar plates supplemented with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal), both in the presence of ampicillin, kanamycin, and 1 mM isopropyl 1-thio-β-D-galactopyranoside, and incubated for at least 48 h at 30 °C. The pKT25 and pUT18C derivatives encoding the leucine zipper from GCN4, which readily dimerizes, were used as a positive control in all experiments. The experiments were done at least in duplicate, and a representative result is shown.

For quantification of BTH interactions, β-galactosidase activity from co-transformants picked from X-gal LB agar plates was measured as described previously (42). The β-galactosidase activity is calculated in Miller units.

**Bioinformatics Analysis**—For analysis of the groove residues, protein sequences were retrieved by BlastP searches using each of ClpV, ClpV1, ClpV2, and ClpV3 as queries. After pruning of duplicates, a total of 1,593 sequences were aligned with MAFFT (43). For the phylogenetic analysis, sequences from 68 T6SSs were retrieved from the Kyoto Encyclopedia of Genes and Genomes. Strains and accession codes are shown in Table 3. Sequences were aligned with MAFFT. For ClpV and TssC, the e-ins-i option (multiple conserved domains and long gaps) was used. TssB and TagJ/HsiE were aligned with default parameters. In all four cases, the Blosum62 scoring matrix was used. Maximum likelihood phylogenies were calculated with phyML (44) with the LG substitution model, no invariable sites, nearest neighbor interchange tree improvement, topology and branch optimization, and aBayes branch support calculation. Trees were visualized with TreeDyn (45). The weblogs were created at WebLogo 3 Web site.
RESULTS

Distinct Structural Features of the N-terminal Domain of P. aeruginosa ClpV1—The structure of the N-terminal domain of V. cholerae ClpV (ClpV-N) has previously been solved in complex with a peptide corresponding to the N-terminal helix of the TssC homolog VipB (Table 4) (28). A hydrophobic groove in ClpV-N provides the binding site for VipB. To investigate whether the N-terminal domain of P. aeruginosa ClpV1 (ClpV1-N) can interact with the TssC homolog HsiC1 in a similar manner, we solved the structure of ClpV1-N from crystals diffracting to 1.5 Å, using the Vibrio cholerae structure (Protein Data Bank code 3ZRJ) as a model for molecular replacement (Table 2). The asymmetric unit contains two copies of ClpV1-N in nearly identical conformations (r.m.s. deviation for all Cαs).
atoms of 0.5 Å). The overall structure of ClpV1-N is similar to that of the *Vibrio* ClpV-N, with matching secondary structure elements and an r.m.s. deviation of 1.6 Å for 121 equivalent Ca atoms (Fig. 1A). Both structures possess an N-terminal helix α0 that distinguishes ClpV-type ATPases from other Hsp100 ATPases (e.g. ClpA) (46). A hydrophobic cleft between this helix and helix α1 forms the binding site for VipB in *V. cholerae* (Fig. 1A).

The residues lining the VipB binding groove in ClpV-N are partially conserved in ClpV1-N. For example, Ile10 and Phe87 lie at the bottom of the groove. The corresponding residues in ClpV1-N are Phe10 and Leu88 (Fig. 1B). However, other residues are not conserved. Tyr84, which packs against Glu22 of VipB in *V. cholerae* (Fig. 1B), is Ile85 in *P. aeruginosa*, a residue that could not participate in the same interaction. The two aliphatic residues Leu86 and Ala86, which sit at the top of the groove in *Vibrio* ClpV-N, are replaced by large residues of opposite charge in ClpV1-N (i.e. Arg86 and Asp87) (Fig. 1B). In one of the two ClpV1-N molecules in the asymmetric unit, these residues form a salt bridge across the groove, whereas in the other, they point into the groove. Both conformations not only diminish the hydrophobic character of the groove but also are incompatible with binding of an α-helix, as observed in *V. cholerae* (Fig. 1, B and C). Consistent with the altered conformation of the binding groove, we were unable to obtain a complex of ClpV1-N bound to a peptide corresponding to the N terminus of HsiC1.

**Phylogenetic Analysis Identifies Distinct T6SS Classes Based on the ClpV Structure**—Alignment of ClpV sequences shows a division based on sequence conservation within the binding groove in the N-terminal domain (Fig. 2, weblogos). There are homologs with an arginine and an aspartate (rarely glutamate) as in *P. aeruginosa* ClpV1 (Arg6 and Asp87, respectively) and homologs with two aliphatic residues as in *V. cholerae* ClpV (Leu6 and Ala86, respectively). Phylogenetic analysis of ClpV sequences from 68 T6SSs covering a wide range of bacterial species shows evolutionary separation based on the groove residues. ClpV homologs with charged residues group with *P. aeruginosa* ClpV1 on the left side of the phylogenetic tree.
FIGURE 2. Division of T6SSs into distinct phylogenetic classes. Maximum likelihood phylogenetic tree generated from 68 aligned ClpV sequences belonging to the indicated bacterial species. ClpV sequences separate into two classes according to two residues in the hydrophobic groove between helices α0 and α1. Homologs where these residues are uncharged like in V. cholerae are shown in black, whereas homologs with charged residues are shown in pink. Blue boxes indicate the presence of an HsiE1 homolog on the same secretion cluster. Boldface type denotes the presence of the LLDEII motif in the TssC homolog on the same cluster. The branch that is highly enriched in T6SSs containing ClpV homologs with charged residues and HsiE1 homologs is shown against a pink background. The branch support value is shown. The three ClpV homologs from P. aeruginosa and V. cholerae ClpV are indicated in red. The weblogo at the bottom show the sequence conservation of residues in the hydrophobic groove of ClpV, depending on the presence (left) or absence (right) of the pair of charged residues. On the left, the two charged residues are numbered according to the P. aeruginosa ClpV1 sequence as in our structure. On the right, the uncharged residues in the same positions are numbered according to the V. cholerae ClpV sequence, as in the published structure (28). The scale bar shows amino acid changes per site. At the bottom is a brief key to the labeling used.
Distinct Classes of Type VI Secretion Systems

(Fig. 2, shown in pink). ClpV homologs with aliphatic residues group with \textit{V. cholerae} ClpV on the right side of the tree (Fig. 2, shown in black). It is noticeable that the two other \textit{P. aeruginosa} ClpVs, ClpV2 and ClpV3, which are associated with the H2- and H3-T6SSs, respectively, lack the charged residues found in the hydrophobic groove of ClpV1 and group with \textit{V. cholerae} ClpV (Fig. 2, shown in black).

\textbf{TssC Has Coevolved with ClpV—}In \textit{V. cholerae}, the peptide corresponding to the N-terminal \(\alpha\)-helix of VipB that binds to ClpV contains a short conserved motif (\textit{\(1^\text{st} \text{LLDEIM}^{24}\)}) (28). By analyzing the N termini of the \textit{P. aeruginosa} VipB homologs, HsiC1, HsiC2, and HsiC3, we found this motif to be conserved in C2 (\textit{\(3^\text{rd} \text{ILDSII}^{33}\)}) and C3 (\textit{\(3^\text{rd} \text{LLDEIF}^{35}\)}) but not C1 (\textit{\(2^\text{nd} \text{EFASLL}^{36}\)}) (Fig. 3A). Mapping the presence of the LLDEII motif in TssC proteins onto the phylogenetic tree of ClpV sequences suggests that TssC homologs have coevolved with ClpV ATPases (Fig. 2, boldface type). TssC homologs with the LLDEII motif are associated only with ClpV homologs having aliphatic residues in their hydrophobic groove.

To investigate these differences and the interaction between the ClpV and TssC partners, we used the BTH assay. Although full-length ClpV1 interacts with HsiC1, the isolated N-terminal domain does not (Fig. 3B), in contrast to what was observed in \textit{V. cholerae} (28). The two other \textit{P. aeruginosa} ClpV proteins display binding grooves that are closely related to the one observed in \textit{Vibrio} ClpV-N (Phyre homology models; data not shown). In contrast to ClpV1 and HsiC1, the N-terminal domain of ClpV2 interacts with HsiC2 to the same degree as full-length ClpV2 (Fig. 3B), suggesting that the interaction mode between ClpV2 and HsiC2 is similar to the \textit{Vibrio} ClpV-VipB interaction.

We then engineered a N-terminal truncation of HsiC1 to remove the first 33 residues (HsiC1\(\Delta_{1-33}\)). HsiC1\(\Delta_{1-33}\) interacts with ClpV1 (Fig. 3C), which suggests that the N terminus of HsiC1 is not essential for a stable interaction, further highlighting the difference with \textit{V. cholerae}. In contrast, the truncation of the corresponding N-terminal residues of HsiC2 (HsiC2\(\Delta_{1-33}\)) has a detrimental effect (10-fold reduction) on binding to ClpV2 (Fig. 3C). These data provide evidence for at least two distinct classes of TssC homologs that specifically interact with cognate ClpVs, which is consistent with the phylogenetic analysis. One mode of interaction, exemplified by \textit{V. cholerae}, involves a primary interaction between the ClpV N domain and the N-terminal helix of TssC (VipB/HsiC) within a hydrophobic groove. The other mode involves additional features beyond the N domain of ClpV or additional protein partners, which may modulate the TssC-ClpV interaction.

\textbf{HsiE1/TagJ Is a Direct T6SS Partner for ClpV1—}T6SS clusters vary in genetic organization and are composed of core and accessory genes. The \textit{hsei1} \textit{tagJ} gene is found in the \textit{P. aeruginosa} H1-T6SS cluster but is lacking in the H2- and H3-T6SSs or in the \textit{V. cholerae} T6SSs (Table 4). We performed BTH experiments showing that HseI1 interacts with full-length ClpV1 (Fig. 4). The interaction between ClpV1 and HsiE1 distinguishes \textit{P. aeruginosa} from \textit{V. cholerae} where no HseI/TagJ homolog exists. Analysis of the ClpV phylogenetic tree further showed that when the T6SS involves a TssC protein containing the LLDEII motif, such as in \textit{V. cholerae}, there is no associated

HsiE1 homolog (Fig. 2, boldface type and not boxed). There is only one exception in \textit{Solibacter usitatus}. This suggests that these two features are mutually exclusive and that HsiE1 plays a role in the interaction between ClpV and the TssC/TssB sheath.
Crystal Structure of HsiE1 in Complex with the N Terminus of HsiB1—We previously showed that P. aeruginosa HsiE1 interacts with the TssB sheath component HsiB1 (Fig. 4) (33). Here, we solved the HsiE1 structure from crystals diffracting to 1.2 Å (Table 2). Vibrio parahaemolyticus VPA1032, a protein of unknown function (37) with 26% sequence identity to HsiE1 (Protein Data Bank code 1ZBP), served as a model for molecular replacement. Both VPA1032 and HsiE1 belong to the “ImpE family” (47). Their structures are similar, as indicated by the r.m.s. deviation of 1.5 Å over 199 equivalent Ca atoms and the conserved N-terminal tetratricopeptide repeat domain and C-terminal “ImpE fold” (Fig. 5A).

We crystallized HsiE1 in complex with a peptide corresponding to the first 20 residues of HsiB1 (\(1\text{MGSTTSSQFIARN-RAPRVQ}^{20}\)) or an HsiB1 degradation product, which appeared during purification of the E1-B1 complex. Both the E1-B1 peptide and E1-B1 fragment structures were solved by molecular replacement (Table 2 and Fig. 5, B and C). In the E1-B1 complex structures, there is clear electron density for residues 8–20 of the extraneously added B1 peptide (Fig. 5B) and for residues 8–30 of the co-purified B1 fragment (Fig. 5C), respectively. Both structures are nearly identical to the structure of HsiE1 alone, with an r.m.s. deviation of 0.4 Å in either case, and to each other with an r.m.s. deviation of 0.3 Å. The B1 fragment (residues 8–30) wraps around E1 in an extended conformation (Fig. 6A, main panel), following a prominent surface groove that originates in a deep hydrophobic pocket between the 12-stranded β-barrel of the “ImpE fold” and the two α-helices just preceding it (Fig. 6A, left inset). The B1 residue Phe\(^{19}\) nestles in that pocket, which is lined with residues Phe\(^{154}\), Trp\(^{171}\), and Pro\(^{181}\).

The amino group of Lys\(^{9}\) of HsiB1 interacts with the negative dipole of helix α7 and forms hydrogen bonds with two carbonyl oxygens at the end of that helix (residues 155 and 157). Another key residue for the interaction is Arg\(^{18}\) of B1, which forms two salt bridges with the carboxyl group of Glu\(^{273}\) in HsiE1 (Fig. 6A, top right inset). In addition, most of the E1-B1 binding interface is hydrophobic in nature, involving Ala\(^{12}\), Gln\(^{20}\), Tyr\(^{23}\), Val\(^{25}\), and Leu\(^{27}\) from B1.

The residues of B1 between 8 and 29 that are involved in specific E1 interactions are highly conserved (Fig. 6B, top). HsiE1 homologs also show conservation of residues that line the hydrophobic pocket with residues 171 and 181 always tryptophan and proline, respectively, and residue 154 always a large hydrophobic residue (e.g. Phe, Leu, or Trp). Residue 273, which forms two salt bridges with the conserved Arg\(^{18}\) in HsiB1, is always glutamic or aspartic acid.

The N Terminus of TssB Homologs Coevolved with HsiE1 Homologs—Because we showed that P. aeruginosa HsiE1 interacts both with ClpV1 and HsiB1, it is tempting to generalize that once HsiE1 binds onto the sheath via an interaction with TssB, it is an ideal docking point for ClpV1 to be recruited to the sheath. There are features in the N termini of TssB proteins that support this idea. The conservation of the N termini of TssB homologs associated with HsiE1 has been described above (Fig. 6B, top). The N termini of TssB homologs not associated with HsiE1 do not show this striking conservation (Fig. 6B, bottom), and there is very little similarity between the two sets. Features
in the N termini of TssB homologs, therefore, also appear to differentiate between the two T6SS classes.

**DISCUSSION**

Repeated injection of T6SS-dependent toxins is essential for effective killing of target cells, and rebuilding of the secretion sheath is necessary in this process. Here, we present molecular details of T6SS components involved in the sheath dynamics and propose the existence of distinct T6SS classes based on their interaction and co-evolution. It has been previously shown that the formation of the contractile sheath of the T6SS requires the co-polymerization of TssB/HsiB1/VipA and TssC/HsiC1/VipB components (Table 4) (20, 21, 23, 24). For sheath disassembly, the AAA+ ATPase ClpV is required (21, 30). In *V. cholerae*, the ClpV N-domain directly interacts with the VipB component (TssC/HsiC1 homolog) of the sheath via a conserved hydrophobic groove and the N-terminal helix of VipB (28). We identified two key residues that have diverged in the N termini of TssB homologs, therefore, also appear to differentiate between the two T6SS classes.
from their equivalents in *V. cholerae* in a subset of T6SSs. These residues are of complementary charge and alter the shape and accessibility of the ClpV1-N groove, as shown in the structure of the N-terminal domain of ClpV1 from *P. aeruginosa* that we solved. The presence of these residues is incompatible with the mode of binding observed for ClpV-VipB. Consequently, we found that the N domain of the *Pseudomonas* ATPase ClpV1 is not sufficient for interaction with HsiC1, nor is the presence of the N-terminal helix of HsiC1 required for it. This is markedly different from what was observed in *V. cholerae*, where VipB binds to ClpV via its N-terminal helix containing a LLDEII motif (28). The differences in the interaction between ClpV and the sheath component TssC in the H1-T6SS of *P. aeruginosa* versus *V. cholerae* lead us to hypothesize that sheath disassembly differs mechanistically in these systems.

To provide further evidence for this hypothesis, we carried out phylogenetic analysis of T6SSs and mapped our structural and interaction data on the resulting tree. Our phylogenetic tree of ClpV divides into two main branches based on the absence of residues of opposite charge on top of the hydrophobic binding groove of the N domain. We found that TssC sequences divide similarly according to the presence of the N-terminal LLDEII motif, which is not observed in secretion systems where the ClpV homolog contains charged residues in the hydrophobic groove. On these features we could superimpose the observation that the accessory protein TagJ/HsiE co-occurs only with the *Pseudomonas* ClpV1 type and thus co-evolved with TssC homologs that lack the LLDEII motif.

The importance of the co-evolution between TagJ/HsiE and the two classes of ClpV and TssC is reinforced by two observations. First, we were able to show that HsiE1 directly interacts with ClpV1, which suggests that the function of these two proteins is coordinated. Second, we have shown previously that HsiE1 directly interact with the other sheath component HsiB1/TssB, suggesting that ClpV1 and HsiE1 function is associated with sheath disassembly. Here, we have solved the structure of HsiE1 in complex with residues 8–30 of the *P. aeruginosa* HsiB1. The N terminus of HsiB1 wraps around HsiE1 in an extended conformation, and all HsiB1 residues visible in the crystal structure are in close proximity to HsiE1. Residues 8–29 are highly conserved in HsiB1 homologs (TssB) from secretion systems containing HsiE1. It is thus clear from our HsiB1-HsiE1 crystal structure that in the context of the intact sheath, the N terminus of HsiB1 would need to be accessible for interaction with HsiE1. In contrast, the interaction between HsiB1/TssB and the other sheath component HsiC1/TssC is mostly mediated by a conserved hydrophobic motif in a helix near the C terminus of TssB (24, 48).

Our phylogenetic analysis does not show a strict division into just two classes of T6SSs. Instead, there are mixed clades and isolated branches. For example, we identified four T6SS clusters containing HsiE1 and a ClpV homolog without charged residues in the groove (Fig. 2, black and boxed), among them *V. parahemolyticus*. They are notable for two reasons. First, the N termini of their TssB homologs do not display the strict amino acid conservation (Fig. 6B, middle) seen in T6SSs with a *P. aeruginosa*-type ATPase and a HsiE1 homolog (Fig. 6B, top). For example, neither Lys9 nor Phe10, two key residues for the interaction with HsiE1 that we identified in the crystal structure of the HsiB1-HsiE1 complex, are present in those TssB homologs. Second, the cognate HsiE1 homologs lack key residues involved in the TssB-HsiE1 interaction (Fig. 6B, middle). From the sequence, we predict the absence of a hydrophobic pocket to accommodate a TssB peptide. The structure of VPA1032, the HsiE1 homolog from *V. parahemolyticus*, bears this out (Fig. 6A, bottom right inset). This observation suggests that VPA1032 may not interact with its cognate TssB homolog at all, and this HsiE1 homolog may be an evolutionary vestige whose function has been lost.

All four T6SS components studied here are involved in the dynamics of sheath assembly and disassembly. The close evolutionary link among these four proteins suggests a functional connection. At the same time, the evolutionary data suggest that interactions with sheath components and sheath disassembly differ in different T6SS classes. It is possible that the selective pressure imposed by the environment, target cells, and/or competitors has led to the evolution of subtle mechanical differences that dramatically altered the efficacy and speed of the T6SS machine.
Distinct Classes of Type VI Secretion Systems

In V. cholerae, a simple system exists. ClpV directly recognizes the sheath component VipB and drives ATP-dependent disassembly after sheath contraction and toxin ejection (Fig. 7A). It is likely that the conformational change associated with contraction (e.g. exposing the N terminus of VipB) is the signal for ClpV binding (28). In P. aeruginosa, the system is more complex. Free ClpV1 can still interact with HsIC1, but HsIE1 is more likely to recruit ClpV1 to the sheath by recognizing the N terminus of HsIB1 (Fig. 7B). It is not clear whether HsIE1 chaperones ClpV1 to the sheath or whether it binds first and provides increased avidity for ClpV1 binding. In either case, proximity of ClpV1 to the sheath would allow the ATPase to recognize and bind HsIC1 and engage in its ATP-dependent disassembly activity.

Because ClpV hexamers bind all along the sheath but in substoichiometric numbers in V. cholerae (21), it is interesting to speculate that disassembly of the sheath leads to the accumulation of oligomeric fragments that would either need to be broken down further for reassembly or cleared from the cell. In P. aeruginosa, HsIE1-mediated disassembly depends on accessible N termini of HsIB1. It has been suggested in previous studies that the N termini of V. cholerae VipA (HsIB) are only accessible at tubule ends (22). The overall similarity of the sheath in P. aeruginosa tempts us to speculate on a two-step mechanism, where the initial explosive disassembly mediated by direct interaction between ClpV1 and HsIC1 leads to the production of sheath fragments. These fragments may offer an increased accessibility of HsIB N termini and could then be broken down into their components by sequential extraction of HsIC1 upon targeting of ClpV1 to HsIB1 mediated by HsIE1. This model provides ideas to further elucidate the mechanism of sheath disassembly and how it is regulated within the two different classes of T6SSs identified in this study. The T6SS is used by bacteria to fight against each other in a strategic and efficient manner. We can learn from this molecular process and attempt to reroute it to our advantage by designing T6SS-based antimicrobial strategies against bacterial pathogens.

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