Structure of the Type VI Effector-Immunity Complex (Tae4-Tai4) Provides Novel Insights into the Inhibition Mechanism of the Effector by Its Immunity Protein*

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The type VI secretion system (T6SS), a multisubunit needle-like apparatus, has recently been found to play a role in interspecies interactions. The Gram-negative bacteria harboring T6SS (donor) deliver the effectors into their neighboring cells (recipient) to kill them. Meanwhile, the cognate immunity proteins were employed to protect the donor cells against the toxic effectors. Tae4 (type VI amidase effector 4) immunity proteins were employed to protect the donor cells of Tae4, whereas a protruding loop (L4) in the other subunit is mainly responsible for inhibiting Tae4 activity. The Tai4 dimer is responsible for inhibiting Tae4 activity. The inactivation of Tae4 is required by collaboration of both subunits of Tai4 dimer. Our results add new insights into the effector-immunity interaction module.

**Background:** The bacteria effector Tae4 is injected into the recipient cells to kill them and the immunity protein Tai4 is produced to inactivate Tae4.

**Results:** Tae4 displays a papain-like fold, and Tai4 dimer is responsible for inhibiting Tae4 activity.

**Conclusion:** The inactivation of Tae4 is required by collaboration of both subunits of Tai4 dimer.

**Significance:** Our results add new insights into the effector-immunity interaction module.

The atomic coordinates and structure factors (codes 4HFL, 4HFK, and 4HFF) have been deposited in the Protein Data Bank (http://wwpdb.org/).

*This article contains supplemental Fig. 1.

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**The abbreviations used are:** T6SS, type VI secretion system; CHAP, cysteine, histidine-dependent amidohydrolases/peptidase; mDAP, meso-diaminopimelic acid; PDB, Protein Data Bank; PG, peptidoglycan; r.m.s.d., root mean square deviation; SAXS, small angle x-ray scattering; SPR, surface plasmon resonance; Tae4, type VI amidase effector 4; Tai4, type VI amidase immunity 4; Tse1, type VI secretion exported 1; Tsi1, type VI secretion immunity 1; Tse2, type VI secretion exported 2; Tsi2, type VI secretion immunity 2; Tse3, type VI secretion exported 3; Tsi3, type VI secretion immunity 3.
Crystal and Solution Structures of Tae4-Tai4 Complex

Research and Qiagen kits using sitting-drop vapor-diffusion method at 293 K. EcTae4 crystals suitable for x-ray diffraction grew from 0.5 M ammonium sulfate, 0.1 M sodium citrate tribasic dehydrate, pH 5.6, 1.0 M lithium sulfate monohydrate. Tae4-Tai4 from E. cloacae were crystallized using 0.2 M sodium chloride, 0.1 M sodium/potassium phosphate, pH 6.5, 25% (w/v) PEG1000. The SeMet S. typhimurium Tae4-Tai4 crystal was obtained in the mixture solution of 0.2 M ammonium acetate, 0.1 M sodium citrate tribasic dehydrate, pH 5.6, and 30% v/v (+/−)-2-methyl-2,4-pentanediol in 2–3 days.

Data Collection, Structure Determination, and Refinement—All of the data were collected on the beamline 3W1A at BSRF (Beijing Synchrotron Radiation Facility) with a mounted MAR-165 CCD detector. Before data collection, the crystals were soaked in the reservoir solution supplemented with 20% (v/v) glycerol for a few seconds and then flash-frozen in liquid nitrogen.

All the data were processed by HKL2000 (12). The SeMet crystal structure of the Tae4-Tai4 complex from S. typhimurium was determined by the single wavelength anomalous dispersion method. The selenium atoms were located by the program Shelxd (13) and then used to calculate the initial phases in Shelxe. The phases from Shelxe were improved in Resolve (14) and then used in Buccaneer (15) for model building. Coot (16) and Phenix.refine (17) were used for manually building and refinement, respectively. The Tae4 and Tae4-Tai4 complex structures from E. cloacae were determined by molecular replacement using the program Phaser (18) with the SeMet one as the searching model. All of the structures were validated by Molprobity (19).

Small Angle X-ray Scattering (SAXS) and Low Resolution Model Building—SAXS data were collected on the beamline station 1W2A in BSRF using a MARCCD165 detector. The scattering was recorded in the range of the momentum transfer 0.023 < s < 0.22 Å−1, in which s = (4πsinθ)/λ, 2θ represents the scattering angle, and the x-ray wavelength λ is 1.54 Å. The measurements were performed in a cuvette (100 μl) with exposure time of 100 s to diminish the parasitic scattering.

The PRIMUS program was used to process the scattering curves (20). The sample was measured at different protein concentrations (1, 3, and 5 mg/ml for S. typhimurium Tae4-Tai4 proteins; 2, 4, and 6 mg/ml for E. cloacae Tae4-Tai4 proteins) to exclude concentration dependence. The distance distribution functions p (r) was computed with experimental data by the program GNOM (21). The theoretical curves were calculated by the program CRYSOL (22).

The program GASBOR was used to build the ab initio low resolution shapes of the complex in solution (23). The protein structure is represented by an ensemble of dummy residues.

Analytical Ultracentrifugation—The sedimentation velocity measurements were carried out using a Beckman Optima XL-I analytical ultracentrifugation (Beckman-Coulter Instruments) with a Ti rotor at 293 K. Both the Tae4-Tai4 proteins were diluted to an A280 of 0.8. The SEDFIT program was used to analyze the sedimentation coefficient (24).

Surface Plasmon Resonance (SPR) Experiments—The interactions between EcTae4 and EcTai4 were explored using a BIACore 3000 instrument at 298 K. EcTae4 (~2 μg/ml) was

mDAP bond. However, it targets the acceptor stem rather than the donor stem.

Our group and others have solved the crystal structures Tae1 and its complex with Tai1 from P. aeruginosa and revealed the structural mechanisms for the inhibition of Tae1 by Tai1 (7–10). But so far the structure of Tae4 has not been reported. Both Tae4 and Tai4 bear very low sequence homology with Tae1 and Tai1, and the effectors have different substrate specificities. Therefore, the substrate recognition and catalysis by Tae4 may be different from that of Tae1. The mechanism of how Tai4 specifically inactivates Tae4 remains unknown. Structural exploration of Tae4 and the Tae4-Tai4 complex will aid the development of new antibacterial agents and our understanding of the role of T6SS in interspecies competition.

In this paper, we have determined the crystal structure of Tae4 from Enterobacter cloacae. The Tae4-Tai4 structures from E. cloacae and Salmonella typhimurium were also solved by x-ray crystallography. Tae4 displays several distinct features different from its structural homologues. A flexible loop may contribute to substrate specificity based on structural and mutagenesis analysis. The Tae4-Tai4 complex forms a heterotetramer that is closely linked to the biological function. A biochemical approach was used to reveal the species specificity of the Tae4 effector inhibited by Tai4, and the common nature of the effector inhibited by an immunity protein through binding to a lid loop represents a characteristic toxin-antitoxin interaction model.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification—The genes encoding full-length EcTae4 and truncated EcTai4 (residues 19–117 without the N-terminal 18-residue signal peptide) were amplified from the E. cloacae genomic DNA. The PCR products were digested and then cloned into the pET28at-plus vector (11), introducing an N-terminal His tag followed by a tobacco etch virus cleavage site. These expression plasmids were transformed into Escherichia coli strain BL21 (DE3). EcTae4 was also ligated into the pET21b vector (Novagen) between the Nhel and Xhol sites. The two recombinant plasmids (pET21b-EcTae4 and pET28at-plus-EcTai4) were co-transformed into BL21 (DE3) cells for co-expression. The genes encoding full-length StTae4 and truncated StTai4 (without the N-terminal 24-residue signal peptide) were PCR-amplified from S. typhimurium LT2 genomic DNA and cloned using the same procedure as above.

Recombinant proteins were purified as described previously (10). Briefly, the protein expression was induced by adding 0.5 mM isopropyl β-D-thiogalactopyranoside at 289 K. The supernatant was loaded onto a 2-ml Ni-nitrilotriacetic acid resin column (GE Healthcare) and eluted with buffer B (25 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5% v/v glycerol) containing 250 mM imidazole. The proteins were further purified by ion-exchange chromatography and subsequent gel-filtration chromatography. All the mutants were generated by the overlap PCR method.

Crystallization—EcTae4 and the Tae4-Tai4 complex were concentrated to ~20 mg/ml using Millipore Amicon Ultra 10 KD. Crystallization screens were performed with Hampton Research and Qiagen kits using sitting-drop vapor-diffusion method at 293 K. EcTae4 crystals suitable for x-ray diffraction grew from 0.5 M ammonium sulfate, 0.1 M sodium citrate tribasic dehydrate, pH 5.6, 1.0 M lithium sulfate monohydrate. Tae4-Tai4 from E. cloacae were crystallized using 0.2 M sodium chloride, 0.1 M sodium/potassium phosphate, pH 6.5, 25% (w/v) PEG1000. The SeMet S. typhimurium Tae4-Tai4 crystal was obtained in the mixture solution of 0.2 M ammonium acetate, 0.1 M sodium citrate tribasic dehydrate, pH 5.6, and 30% v/v (+/−)-2-methyl-2,4-pentanediol in 2–3 days.

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Surface Plasmon Resonance (SPR) Experiments—The interactions between EcTae4 and EcTai4 were explored using a BIACore 3000 instrument at 298 K. EcTae4 (~2 μg/ml) was
coupled to a CM5 sensor chip in 10 mM sodium acetate, pH 5.5, using the amine coupling kit. EcTai4, and its mutants were injected at a flow rate of 40 μl/min for 90 s, followed by washing with running buffer (50 mM HEPES, pH 8.0, 150 mM NaCl, and 0.005% (v/v) Tween 20) for 10 min. The CM5 surface was regenerated with 100 mM phosphoric acid at a flow rate of 40 μl/min for 50 s. The kinetic parameters were modeled with the BIAevaluation 4.1 software.

**Ligand-Protein Docking**—t-Ala-d-Glu-mDAP was docked into the EcTae4 structure by the AutoDock 4.2 program (25). Docking studies were carried out using a Lamarckian genetic algorithm with 25,000 energy evaluations. The grid maps contained 50 × 50 × 50 points with a grid points spacing of 0.375 Å in each dimension. Cys-46 and His-128 were treated as flexible residues in the process of modeling. The model with the lowest binding energy was selected for analysis.

**Cell Toxicity Assay**—Tae4/Tai4 mutants from *E. cloacae* were subcloned into the pET22b/pET26b vectors containing signal peptide sequences. A single colony harboring the expressing plasmid was grown in LB medium at 310 K. After signal peptide sequences. A single colony harboring the expressing plasmid was grown in LB medium at 310 K. After

**RESULTS**

**Overall Structure of Tae4**—The crystal structure of Tae4 from *E. cloacae* (EcTae4) has been determined at a resolution of 2.0 Å. Residues 145–148 could not be seen because of the poor electron density (Fig. 1A). Tae4 crystallizes in the space group *P*3_121 with one molecule in the asymmetric unit (Table 1). The overall structure of Tae4 demonstrates a common architecture of the NlpC/P60 domain (26–28), arranged in an antiparallel β-sheet flanked by a short α-helix. The N-terminal subdomain is composed of an antiparallel β-sheet flanked by a short α-helix. The C-terminal subdomain is formed by five α-helices and a β-hairpin (Fig. 1, A and B). According to previous studies, the substrate binding sites are located in a concave between these two subdomains (6, 27) (see Fig. 3A). The Dali server was used to find the structural neighbors to Tae4 (29). There are two CHAP (cysteine, histidine-dependent amido
drases/peptidase) family functional proteins with Z-scores greater than 5, including endopeptidase YkIC from *Bacillus cereus* (PDB ID code 3H41) (30) and bacteriolytic effector Tae1 (Tse1) from *P. aeruginosa* (PDB ID code 4FOV) (10). Despite the low sequence similarity, superfamilies of Tae4 with the two proteins give r.m.s.d. values of 2.720 and 3.068 Å, respectively, suggesting that these structures share the same fold (31) (Fig. 2, A and B).

The Crystal and Solution Structures of Tae4-Tai4 Complex

**Crystal and Solution Structures of Tae4-Tai4 Complex**—The crystal structure of Tae4 in complex with Tai4 from *E. cloacae* (EcTae4-Tai4) was solved at 2.1 Å resolution and belonged to the C121 space group. There are four molecules in an asymmetric unit. However, the Tae4-Tai4 complex from *S. typhimurium* (StTae4-Tai4) was crystallized in the *P*6_22 space group and
diffracted to 2.4 Å resolution (Table 1). The asymmetric unit is composed of two molecules.

Both Tae4-Tai4 proteins migrated on size-exclusion chromatography with a molecular mass of ~49 kDa compared with its calculated heterodimer molecular mass of ~31 kDa (Fig. 4A, StTae4-Tai4 data not shown). Analytical ultracentrifugation was performed to evaluate the oligomeric state of the complex. The Ec- and St-complex proteins showed a sedimenting boundary at 60.3 and 61.7 kDa, which corresponds to the sedimentation coefficients of 4.12 and 4.19, respectively (Fig. 4B). The result is consistent with the SAXS data. The data in Fig. 4C demonstrate that the experimental SAXS curves are more in agreement with the heterotetramer theoretical curves than the heterodimer curves (data not shown). The ab initio models were carried out to characterize the Tae4-Tai4 complex shape in solution. The available heterotetramer Tae4-Tai4 structures fit well into their respective SAXS-derived low resolution envelopes (Fig. 4C, right upper). Thus, we can conclude that the Tae4-Tai4 complex is a heterotetramer in solution.
The EcTae4-Tai4 heterotetramer is generated by crystal packing with symmetry-related molecules (symmetry mate x, x-y, -z/H11011). These two complexes from E. cloacae and S. typhimurium showed a high degree of sequence identity (44%). Superposition of these two structures results in an r.m.s.d. value of 3.256 Å (see “Discussion”), suggesting that they share a similar architecture. Consequently, the EcTae4-Tai4 complex is chosen for discussion henceforth, unless otherwise stated.

Dimerization of Tai4—Tai4 is composed of six α-helices, whereas Tai1 displays an all α-fold (7, 9). The Tai4 dimerization occurs mainly through the interaction of β2, β3, and β5 of both monomers (Fig. 5A). The conserved residue Asp-54 is involved in extensive hydrogen-bonding interactions with the main chain atoms of Tyr-96, Gln-97, Ile-98, and Leu-99. These are side chain side chain hydrogen bonds in both monomers between Gln-38 and Gln-38, Ser-48 and Asp-47 (Fig. 5B). In addition, there are indirect interactions of several residues such as Asp-47 and Ser-50, Asp-54 and His-95, Asn-106 and Gln-31, via well ordered water molecules by their side chains. The dimer interface is extensive with a buried surface area of 1446 Å², which is 22.7% of the total surface area per monomer (6346 Å²). In addition, Tai4 behaves as a dimer on the gel filtration column (Fig. 4A), suggesting that Tai4 is a dimer in solution.

When mapping the sequence homology onto the Tai4 structure, the most invariant residues are concentrated at the dimer interface, which means that the dimer interactions are conserved across the Tai4 family (Fig. 5C). The Tai4 model was submitted to the Dali server to search against the PDB for its structural homologues (29). The closest structural homologue (the Z-score is 5.4) is Tel2 from Saccharomyces cerevisiae. This

### TABLE 1
Data collection and structure refinement statistics

Values in parentheses means those for the highest resolution shell.

| Parameters               | EcTae4-Tai4 | StTae4-Tai4 | EcTae4 |
|--------------------------|-------------|-------------|--------|
| Crystal parameters       |             |             |        |
| Wavelength (Å)           | 0.9793      | 0.9793      | 0.9793 |
| Space group              | C121        | P6,22       | P3,21  |
| Unit cell dimensions     | a = 91.34, b = 138.14, c = 64.46 Å β = 127.89° | a = b = 63.90, c = 365.92 Å | a = b = 84.28, c = 43.45 Å |
| Data collection          |             |             |        |
| Resolution (Å)           | 2.10 (2.14–2.10) | 2.40 (2.44–2.40) | 2.00 (2.03–2.00) |
| Number of unique reflections | 36,726 (1834) | 18,525 (850) | 12,215 (593) |
| Completeness (%)         | 100 (100)   | 99.1 (94.3) | 99.1 (98.5) |
| Redundancy               | 7.7 (7.5)   | 17.6 (12.9) | 5.1 (4.8) |
| Mean I/σ (I)             | 34.5 (10.5) | 38.1 (4.0)  | 44.3 (11.1) |
| Molecules in asymmetric unit | 4          | 2           | 1      |
| Rmerge (%)               | 6.2 (23.2)  | 8.2 (43.3)  | 7.0 (14.2) |
| Structure refinement     |             |             |        |
| Resolution range (Å)     | 23.40–2.10  | 38.00–2.40  | 23.29–2.00 |
| Rmerge/Rfree (%)         | 15.6/20.4   | 18.7/22.6   | 20.8/25.3 |
| Number of atoms          |             |             |        |
| Residues                 | 515         | 255         | 159    |
| Protein                  | 3948        | 2004        | 1239   |
| Water                    | 477         | 114         | 98     |
| Average B-factor (Å²)    |             |             |        |
| Main chain (A/B/C/D)     | 15.90/12.09/15.67/11.90 | 42.25/35.94 | 31.63 |
| Side chain (A/B/C/D)     | 18.54/16.32/17.95/16.21 | 45.55/39.95 | 34.46 |
| Water                    | 25.47       | 42.23       | 37.82  |
| Ramachandran statistics (%) | 98.6 | 98.8 | 97.4 |
| Most favored             | 1.4         | 1.2         | 2.6    |
| Allowed                  |             |             |        |
| R.m.s.d.                 |             |             |        |
| Bond lengths (Å)         | 0.007       | 0.009       | 0.008  |
| Bond angles (°)          | 1.051       | 1.079       | 1.120  |

FIGURE 2. Structural comparisons of Tae4 (yellow) with its two homologues YkfC and Tse1. The α2, α3, and L11 in red represent the regions in Tae4 that differ from the two homologues. Asp-124 of EcTae4 and Tyr-89 of Tae1 are shown in stick representation. A, superposition of Tae4 with the NlpC/P60 domain of YkfC. B, superposition of Tae4 with Tae1.
Crystal and Solution Structures of Tae4-Tai4 Complex

The Interaction between Tae4 and Tai4—The homodimer of Tai4 can be specified as subunit I (green), which interacts with the N-terminal subdomain of Tae4; and subunit II (blue), which interacts with the C-terminal subdomain of Tae4 (Fig. 6, A and B). The total buried surface area at the interface of the Tae4 dimer with one Tae4 monomer is 966 Å². The interactions of two helices (α3 and α5) from Tae4 with α3 and α4 from the Tai4 subunit I account for the majority of the interface (668 Å²). The small interface (298 Å²) is created by loops 10 and 5 from Tae4 with holo-Tai4. The total buried surface area at the interface of the Tai4 dimer with one Tai4 monomer is 752 Å². The interactions of two helices (α3 and α5) from Tai4 with α3 and α4 from the Tae4 subunit I account for the majority of the interface (556 Å²). The small interface (288 Å²) is created by loops 10 and 5 from Tai4 with holo-Tae4. The total buried surface area at the interface of the Tae4 dimer with one Tae4 monomer is 966 Å². The interactions of two helices (α3 and α5) from Tae4 with α3 and α4 from the Tai4 subunit I account for the majority of the interface (668 Å²). The small interface (298 Å²) is created by loops 10 and 5 from Tae4 with holo-Tai4. The total buried surface area at the interface of the Tai4 dimer with one Tai4 monomer is 752 Å². The interactions of two helices (α3 and α5) from Tai4 with α3 and α4 from the Tae4 subunit I account for the majority of the interface (556 Å²). The small interface (288 Å²) is created by loops 10 and 5 from Tai4 with holo-Tae4.

The Mechanism of Tai4 Inhibits Tae4—The structure of Tae4 in Tae4-Tai4 complex (holo-Tae4) is basically identical to the apo-Tae4 structure (Fig. 8A). Structural alignments of apo-Tae4 with holo-EcTae4 and holo-StTae4 structures give an r.m.s.d. of 0.371 and 0.807 Å, respectively. However, there are clear differences in the C-terminal subdomain (Fig. 8B). The first divergent region is the winding loop, which can be characterized as two clips (Fig. 1C). Clip I, which includes residues from 134 to 144, adopts the same structure in the three forms of Tae4. Residues 145–153 form clip II that folds over the catalytic core. The dominant feature of the winding loop in apo-Tae4 is the second clip portion that is shifted by ~1.7 and 6.2 Å compared with holo-EcTae4 and holo-StTae4, respectively (Fig. 8B). As mentioned above, part of this clip could not be traced in apo-Tae4 and holo-StTae4 structures because of the higher B-factor (Fig. 9, A and B). Furthermore, Asn-149 in the missing loop is invariant among Tae4 sequences. There is reason to believe that the conformational flexibility of the loop is connected to enzyme activity.

Structural alignment of the three Tae4 structures shows that the most prominent difference between apo- and holo-structures is the lid loop (loop 10). Evidently, the lid loop adopts well ordered in the holo form (Fig. 7). Furthermore, alignment of these regions results in a higher r.m.s.d. value. These residues involved in the α3 of Tae4 and Tai4 interface are nonconserved (Figs. 1B and 5C). Taken together, the insertions tend to correlate with the species specificity during the Tae4 inhibition by Tai4.

Notably, the residues from Leu-63 to Asn-67 located in α3 and the following loop of Tai4 subunit I form a number of hydrogen bonds with Tae4. The amino group in the main chain of Lys-83 in the α5 from Tae4 directly interacts with hydroxy groups of Glu-64–Asn-67 of Tai4 subunit I (Fig. 6CII). Similarly, the conserved residue Arg-81 of Tae4 forms hydrogen bonds with the main chain atoms of Leu-63 and Leu-68 in Tai4. Additional interactions are found between the strictly conserved residues Val-82 and Glu-64. Substitutions S66A, N67A, and L68A exhibit affinity to Tae4 equal to that of the WtTai4 protein. However, the E64A variant causes a ~10-fold reduction in affinity, and the Kₐ value is affected significantly (~140-fold reduction) by the alanine substitution for leucine at residue 63. This observation indicates that these two residues are important for Tai4-Tae4 recognition. Intriguingly, the equivalent helix of Tsi2 is a crucial segment responsible for binding Tse2 (4). Along with the sequence conservation of these residues in Tae4 and Tai4 (Figs. 1B and 5C), these observations support the idea that the α3 of Tai4 is a major determinant of the Tae4 interaction.

A protruding loop (loop 4) in the Tai4 subunit II also contributes to the hydrogen-bonding network, thereby stabilizing the heterotetramer. The residues Leu-123 and Asp-124 in loop 10, and Ser-151 in the winding loop from Tae4 make direct interactions with Gly-89, Thr-91, and Gly-90 of the protruding loop, respectively (Fig. 6CII). In addition, Gly-89 interacts indirectly with Gly-121 in loop 10 via a water molecule (W145). Because loops 10 and 11 are involved in the formation of the Tae4 catalytic sites, the protruding loop of Tai4 may be closely associated with inhibiting Tae4 enzyme activity.

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The helix α3 of Tae4 is oriented toward subunit I and interacts with Arg-40 and Glu-74 through hydrogen and salt bonds (Figs. 1C and 6CII). To validate the interactions at the interface, we introduced point mutations and used SPR to identify the residues required for stable interactions (Table 2 and supplemental Fig. 1). Substitutions R40A and E74A disrupted binding affinity compared with the wild-type Tae4 (WtTae4). The results are in agreement with extensive interactions between the α3 of Tae4 and Tai4. As shown in Fig. 2, A and B, two insertions (a2 and a3) protrude from the N-terminal subdomain compared with Tae1 and YkFC. Although these helices in the apo-Tae4 structure have a high B-factor value, they are

protein is involved in chromatin remodeling and PIKK stabilization (35). However, significant differences between these two proteins were observed, such as the oligomeric state, molecular mass, and the arrangement of the helix-turn-helix repeats.

Despite low sequence identities, the arrangement of Tai4 is similar to Tsi2 (Fig. 5D). We used a mutagenesis approach to identify the residues important for the dimerization. Like Tsi2, the Tai4 mutant variants have no effects on the dimerization or lead to protein precipitation (4, 5). We postulate that the dimerization is important for the physiological functions, such as inhibition of Tae4.

The Interaction between Tae4 and Tai4—The homodimer of Tai4 can be specified as subunit I (green), which interacts with the N-terminal subdomain of Tae4; and subunit II (blue), which interacts with the C-terminal subdomain of Tae4 (Fig. 6, A and B). The total buried surface area at the interface of the Tae4 dimer with one Tae4 monomer is 966 Å². The interactions of two helices (α3 and α5) from Tae4 with α3 and α4 from the Tai4 subunit I account for the majority of the interface (668 Å²). The small interface (298 Å²) is created by loops 10 and 11 from Tae4 interacting with loop 4 from the neighboring Tai4 molecule (subunit II). Moreover, there are up to five well ordered water molecules located at the interface which further stabilize the complex.

The helix α3 of Tae4 is oriented toward subunit I and interacts with Arg-40 and Glu-74 through hydrogen and salt bonds (Figs. 1C and 6CII). To validate the interactions at the interface, we introduced point mutations and used SPR to identify the residues required for stable interactions (Table 2 and supplemental Fig. 1). Substitutions R40A and E74A disrupted binding affinity compared with the wild-type Tae4 (WtTae4). The results are in agreement with extensive interactions between the α3 of Tae4 and Tai4. As shown in Fig. 2, A and B, two insertions (a2 and a3) protrude from the N-terminal subdomain compared with Tae1 and YkFC. Although these helices in the apo-Tae4 structure have a high B-factor value, they are
different conformations in the apo- and holo-Tae4 structures (Fig. 8A). In Tae1, Tyr-89 is hypothesized to regulate substrate recognition (9, 10). Asp-124, which is located in the lid loop of Tae4, lies in a similar orientation to Tyr-89 of Tae1, and the carboxyl group of Asp-124 is positioned at the entrance of the substrate binding cleft. However, the D124A variant has no significant effect on cell viability (Fig. 8B).

It is conceivable that the inhibition of Tae4 by Tai4 is not through binding Asp-124 but through a conformational change in the lid loop. Compared with the apo-Tae4 structure, the active pocket is covered by the closed lid loop in both holo structures. Conversely, a positively charged patch in the protruding loop of Tai4 extends from Lys-87 to Thr-91, and could shield the negatively charged patch of the active sites of Tae4. A variant (Δ86–91) lacking 6 residues (Gln-86–Thr-91) of Tai4 was created to investigate its influence on the inhibition of Tae4. In the assays examining cell viability, cells can be rescued by the induced expression of Tai4. As expected, the variant Δ86–91 is not capable of inhibiting amidase activity (Fig. 8C).

To further understand the nature of Tae4 inhibition by Tai4, we

**FIGURE 4. Solution behavior of Ec- and StTae4-Tai4 complexes.** A, purified EcTae4 (red), EcTai4 (green), and Tae4-Tai4 (blue) complex eluted from gel filtration chromatogram (Superdex™ 200 10/300 GL) at 18.5, 17.0, and 15.0 ml, respectively. B, sedimentation coefficient distributions of Ec- (upper) and St- (lower) Tae4-Tai4 complexes. C, solution conformation of Ec- (left) and St- (right) Tai4-Tae4 complex by SAXS analysis. Curve 1, experimental data; curve 2, scattering patterns computed from the GASBOR model. Inserts: lower left, P(r) function; upper right, GASBOR models overlapping with heterotetramer crystal structures. The experimental data compare well with the theoretical curves of crystal structure in both Ec- and St- (generated with a symmetry-related molecule) Tai4-Tae4 complex.
performed an in vitro His tag pulldown assay. Our results showed that the deletion mutant can still bind to Tae4 (Fig. 8D), excluding the possibility that the heterotetramer disruption impedes the inhibition of Tae4 by Tai4. Further, the tip of the protruding loop is located near the catalytic triad and can make contact with the active site histidine via a water molecule (Fig. 6, A and B). In summary, the inhibition is achieved through insertion of the protruding loop into the pocket and induction of the closed conformation of the lid loop. As a result, the access to the catalytic sites is blocked.

**DISCUSSION**

The bacteria utilize T6SS and effectors to kill the rival cells. The effector Tae4 is present in the periplasmic space and catalyzes the hydrolysis of PG. Tae4 adopts a canonical papain-like \( \alpha/\beta \) fold and belongs to the N1pC/P60 superfamily of cell wall cysteine peptidases, which are associated with the cell wall hydrolysis and recycling (36, 37). These cell wall peptidases, widely distributed in bacteria, are often characterized by the presence of the auxiliary domain, which functions mainly as a scaffold mediating protein localization (26, 30). The \( \alpha \)-glutamyl-\( \lambda \)-diamino acid endopeptidase YkFC, for instance, contains two additional SH3b domains (30). However, a single domain is present in Tae1 and Tae4. Gel filtration experiments also revealed that Tae4 behaves as a monomer in solution (Fig. 4A). The characteristic configuration endows Tae1 and Tae4 with extensive and accessible substrate binding surface (Figs. 7, A and B, and 8B) (13). In addition, the effectors can switch one target to another quickly and efficiently due to the loss of the localization process. As a result,
both Tae1 and Tae4 show a higher potency with respect to the PG degradation (6, 8).

Although the Tae4 structure is broadly similar to Tae1 structure, Tae4 exhibits two unique features. The first difference is a winding loop, which is involved in substrate binding and hydrolysis. The cleavage of the PG is independent of the third catalytic residue Cys-110, which is buried in the interior of Tae1. However, Asp-139, located in Tae4 winding loop, is solvent-exposed and makes close contacts with His-128 (≈ 2.7 and 3.0 Å). Asp-139 can be available to stabilize the oxyanion hole. Consistent with these, we found that Asp-139 is an indispensable catalytic residue for the enzyme activity (Fig. 3B). Furthermore, the intact catalytic triad confers higher activities on Tae4 (6, 8). Two helix insertions are the other characteristic profile. Based on structural analysis, the insertions are responsible for defining the species specificity of Tai4 binding to Tae4. In total, the structural variability in Tae1 and Tae4 results from the evolution and renders divergence in substrate recognition.
Tai1 is an all β-sheet protein, whereas Tai4 adopts a superhelical conformation and shows remarkable similarity to the immunity protein Tsi2 from *P. aeruginosa* (4, 5). The Tae1-Tai1 complex is a heterodimer, but the Tae4-Tai4 complex forms a compact heterotrimer that comprises one Tai4 homodimer binding two Tae4 molecules. The inhibitory mechanism for Tae1 by Tai1 includes a localization event where five β-β loops (βc-βd, βe-βf, βh-βi, βj-βk, and βl-βm) of Tai1 interact with Tae1 (PDB ID code 3VPJ) (Fig. 9A). Previous studies have shown that the insertion of βh-βi loop into the catalytic cleft is critical for inhibition (7, 9). The substrate binding sites are occupied by the βh-βi loop, Ser-107 of which shows a remarkable binding affinity to Tae1. Apart from these, Ser-109 lactated in the βh-βi loop makes direct interaction with the active site His-91 (Fig. 9B). Although both Tae1 and Tae4 are D,L-endopeptidases, their immunity proteins work differently from each other. These β-β loops facilitate accurate positioning of Tai1 and match well with the narrow and extended cleft of Tae1. However, the helices of one Tai4 subunit are involved in the Tai4 orientation, and the deep V-shaped groove of Tae4 is not fully occupied by the protruding loop of the other Tai4 subunit. The lid loop of Tae4 serves as a molecular switch to control the PG access to the catalytic pocket. When the protruding loop of Tai4 gets stuck in Tae4, the lid loop is closed, and amidase activity is suppressed. Conversely, the lid loop adopts an open conformation, and Tae4 can reactivate in the absence of the protruding loop. It is worthy to note that the protruding loop of Tai4 participates in both the interaction with the lid loop of Tae4 and the formation of the Tai4 dimer (Figs. 5B and 6C). This protruding loop further contributes to the Tai4 dimer interface through the interaction between Gln-97, Tyr-96, and Asp-54 of both subunits. In other words, the dimerization of Tai4 is required for the protruding loop orientation. As mentioned above, the interaction between Tae4 and Tai4 subunit I is primarily responsible for their interaction. We conclude that the inhibition requires both Tai4 monomers. The
distinct inhibitory modes indicate the evolutionary divergence between these two amidase families. It is also suggested that the organisms carrying the Tae1-Tai1 pair compete with that harboring the Tae4-Tai4 pair in nature (6). These hypotheses should be tested in the following studies.

Our studies reveal a novel mechanism of the inhibition of the amidase effector by its cognate immunity protein. To our knowledge, this inhibition mode is first discovered in effector-immunity pairs. These findings will improve our understanding of the T6SS role in interspecies competition and toxin-antitoxin interactions. The significant toxicity of Tae4 and the close relationship between Gram-negative bacteria and pathogenicity make it an attractive target for the antipathogen therapy. We can also develop some small molecule inhibitors or small peptides that bind to Tai4 and inhibit the binding of Ta4 to Tae4 competitively. Without the protection of immunity proteins, the bacteria would suffer toxic effects. The chemical modification of the substrate may be another way for drug discovery.

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