Identification, Structure, and Function of a Novel Type VI Secretion Peptidoglycan Glycoside Hydrolase Effector-Immunity Pair

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Background: The bacterial type VI secretion system (T6SS) translocates toxic effector proteins into target cells. Novel T6S peptidoglycan glycoside hydrolase effector-immunity families are identified and a representative pair is structurally and functionally characterized. Peptidoglycan glycoside hydrolase effectors are an important component of the T6S effector arsenal. This work expands the repertoire of known T6S substrates and reports the first structure of a peptidoglycan glycoside hydrolase effector.

Bacteria employ type VI secretion systems (T6SSs) to facilitate interactions with prokaryotic and eukaryotic cells. Despite the widespread identification of T6SSs among Gram-negative bacteria, the number of experimentally validated substrate effector proteins mediating these interactions remains small. Here, employing an informatics approach, we define novel families of T6S peptidoglycan glycoside hydrolase effectors. Consistent with the known intercellular self-intoxication exhibited by the T6S pathway, we observe that each effector gene is located adjacent to a hypothetical open reading frame encoding a putative periplasmically localized immunity determinant. To validate our sequence-based approach, we functionally investigate a representative family member from the soil-dwelling bacterium Pseudomonas protegens. We demonstrate that this protein is secreted in a T6SS-dependent manner and that it confers a fitness advantage in growth competition assays with Pseudomonas putida. In addition, we determined the 1.4 Å x-ray crystal structure of this effector in complex with its cognate immunity protein. The structure reveals the effector shares highest overall structural similarity to a glycoside hydrolase family associated with peptidoglycan N-acetylglucosaminidase activity, suggesting that T6S peptidoglycan glycoside hydrolase effector families may comprise significant enzymatic diversity. Our structural analyses also demonstrate that self-intoxication is prevented by the immunity protein through direct occlusion of the effector active site. This work significantly expands our current understanding of T6S effector diversity.

Bacteria utilize specialized protein secretion systems to mediate interactions with organisms in their environment. The bacterial type VI secretion system (T6SS) is a multi-protein apparatus that translocates substrate effector proteins into neighboring Gram-negative bacterial cells. Many of these proteins act as toxins; thus, their delivery grants the donor bacterium fitness in co-culture with susceptible competitors. Although the precise mechanism of effector translocation by the T6SS remains unclear, data indicate that effectors are delivered in single step from the cytoplasm of a donor bacterium to the periplasm of a recipient. Structural similarity between secreted T6S apparatus components and bacteriophage proteins involved in cell puncturing suggest that the translocation process may resemble phage infection.

In recent years, significant progress has been made toward the identification and functional characterization of antibacterial effectors secreted by the T6SS. Bacteria-targeting effectors associated with T6S have been shown to elicit toxicity through the disruption of a variety of cellular structures including nucleic acids, membranes, and the bacterial cell wall. For example, the RhsA (recombination hot spot A) and RhsB proteins of Dickeya dadantii contain C-terminal toxin domains that degrade chromosomal DNA, and Tle (type VI secretion lipase effector) proteins from Vibrio cholerae, Burkholderia thailandensis, and Pseudomonas aeruginosa are lipases that catalyze the breakdown of membrane phospholipids. Cell wall-degrading T6S effectors distribute into two

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6 The abbreviations used are: T6SS, bacterial type VI secretion system; MurNAc, N-acetylmuramic acid; GlcNAc, N-acetylgalactosamine; E-I, effector-immunity; LB-LS, Luria-Bertani low salt; IPTG, isopropyl 1-thio-β-D-galactopyranoside; ITC, isothermal titration calorimetry; G-type, goose-type.
groups: those that act as amidases, cleaving the peptidoglycan molecule within its peptide stems and cross-links and those that act as glycosidases, cleaving the glycan backbone of the molecule. T6S amidase effectors have been studied extensively (3, 9, 10, 12–15). The enzymes are broadly distributed among Proteobacteria and form four phylogenetically distinct families that constitute the Tae (type IV secretion amidase effector) superfamily. Interestingly, the preferred cleavage site within peptidoglycan can vary between Tae families, suggesting the possibility that the effector specificity is dependent on the organism(s) targeted and/or the precise structure of the peptidoglycan found in those organisms.

In contrast to the amidases, there are few identified glycoside hydrolase cell wall-targeting effectors. Moreover, the general utilization of this effector activity by T6SS+ organisms remains uncertain. *P. aeruginosa* Tse3, the sole biochemically characterized glycoside hydrolase effector, acts as a muramidase, cleaving the β-(1,4) linkage between *N*-acetylglucosamine (MurNAc) and *N*-acyethylglucosamine (GlcNAc) (3). The only other glycoside hydrolase effector identified to date is VgrG-3 from *V. cholerae*. This protein carries a C-terminal lysozyme-like effector domain that degrades peptidoglycan (3, 11, 16).

A commonality among all identified effectors with antibacterial activity is that their corresponding open reading frames are found adjacent to genes encoding cognate immunity proteins. These proteins confer immunity by specifically binding to and inactivating their associated effector (3, 13–15). As effectors do not access the donor cell periplasm in transit to the recipient cell, the immunity proteins of cell wall-targeting effectors, which reside in the periplasm, serve the exclusive purpose of preventing intercellular self-intoxication.

The sequence divergence and sporadic distribution of T6S effectors present a challenge for the identification of these important mediators of interbacterial interactions. Owing to their frequent horizontal inheritance, bacteria related at the genus, or even the species level, often contain a completely unique repertoire of these proteins. For example, the plant commensal bacterium *Pseudomonas protegens* does not contain homologs of the three established effectors of the *P. aeruginosa* Hcp secretion island I-encoded T6S (H1–T6SS), Tse1–3; however, this organism possesses a T6SS orthologous to the H1–T6SS (17).

One way in which the challenge of identifying T6SS effectors has been overcome is by exploiting the tendency of their corresponding genes to reside within or in close proximity to T6SS-encoding gene clusters. This approach was used for the identification of a representative Tae from *P. protegens* (12). Alternatively, mass spectrometry-based methodologies have been successful in the identification of T6S effectors from *P. aeruginosa*, *Burkholderia thailandensis*, and *S. marcescens* (2, 9, 18). Finally, our group utilized a sequence homology-independent informatic search based on common properties found within effector-immunity (E-I) pairs to identify the Tae superfamily (9). These properties, applied independently to the candidate effector and immunity protein, included size, isoelectric point, predicted subcellular localization, and the presence of a cysteine-histidine catalytic dyad.

In this study, we performed an informatic search for T6SS substrates and found previously unidentified families of peptidoglycan glycosidase hydrolase effectors, herein named Tge proteins (type VI secretion glycoside hydrolase effectors). Characterization of a representative Tge from *P. protegens* showed that the protein displays periplasmic toxicity, is secreted in a T6-dependent manner, and confers a fitness advantage when *P. protegens* is grown in competition against *P. putida*, a co-occurring soil bacterium. Additionally, we solved the 1.4 Å crystal structure of *P. protegens* Tge in complex with its cognate immunity protein. Together, our findings show a broader distribution of T6S glycoside hydrolase effectors than was previously appreciated and offer insights into the molecular basis for glycoside hydrolase activity and inhibition.

**EXPERIMENTAL PROCEDURES**

**Bioinformatic Screen**—Putative effector-immunity candidates were identified using a similar informatic search protocol as described previously (9). Briefly, a custom Perl script was used to search 115 T6SS+ genomes for bicistronic genes with the following criteria for the encoded effector protein: 1) no predicted signal sequence, 2) a predicted pl greater than 8.0, and 3) fewer than 200 amino acids. The criteria for the immunity protein included the presence of a predicted signal sequence and fewer than 200 amino acids. Protein sequences obtained from this screen were submitted in batch mode to the Phyre2 server and examined manually for the presence of lysozyme-like folds (19). Candidate peptidoglycan glycosidase hydrolases and associated immunity proteins were then used as Blastp search queries to identify all unique family members in the NCBI database.

**Bacterial Strains and Growth Conditions**—All *P. protegens* strains generated in this study were derived from the sequenced strain Pf-5 (20). *P. protegens* strains were grown in Luria-Bertani (LB) media at 30 °C supplemented with 15 μg ml⁻¹ gentamicin and 25 μg ml⁻¹ isergan where appropriate. The pEXG2 suicide vector was used for in-frame chromosomal deletions in *P. protegens* as described previously for *P. aeruginosa* (21). Similar to *P. aeruginosa*, deletion of retS is required for activation of T6S in *P. protegens* (22, 23). Locus tags for retS, tge2pp, tgi2pp, and tgiV are PFL_0664, PFL_3037, PFL_3036, and PFL_6093, respectively. The *P. putida* strain used for competition assays was derived from the sequenced strain KT2440 (24) and grown in LB media at 30 °C. *E. coli* strains used included DH5α for cloning, SM10 for conjugal transfer of plasmids into *P. protegens*, BL21 pLysS for toxicity assays, growth curves, and phase contrast microscopy, and Shuffle® T7 Express lysY (New England Biolabs) for expression of proteins for purification. *E. coli* strains were either grown in LB or LB low salt (LB-LS) at 37 °C supplemented with 50 μg ml⁻¹ kanamycin, 150 μg ml⁻¹ carbenicillin, 30 μg ml⁻¹ chloramphenicol, 200 μg ml⁻¹ trimethoprim, 0.1% (w/v) l-rhamnose and the indicated concentrations of IPTG as required.

**E. coli Toxicity Assays**—Tge2pp was cloned into pET-29b(+) and pET-22b(+) using the BamHI/HindIII and NdeI/HindIII restriction sites, respectively. Tgi2pp and tgiVPA (PA3485) were cloned into pSCrhaB2-CV using the NdeI/XbaI restriction sites. The tge2pp E69Q mutation was introduced using SOE.
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PCR. Overnight cultures of *E. coli* BL21 pLysS pET-29b(+), pET-29b(+)::tge2pp, pET-22b(+), pET-22b(+)::tge2pp, pET-22b(+)::tge2pp E69Q, pET-22b(+) + pSCrhaB2-CV, pET-22b(+)::tge2pp + pSCrhaB2-CV, pET-22b(+)::tge2pp + pSCrhab2-CV::tgi1PA were diluted 10^5 in 10-fold increments and stamp plated onto LB-LS 3% agar plates containing the appropriate antibiotics. For comparison of cytoplasmic versus periplasmic toxicity of Tge2pp, cells were induced with 100 µM IPTG. For immunity rescue experiments, expression was induced with 40 µM IPTG for Tge2pp and 0.1% (w/v) l-rhamnose for Tgi2pp/Tgi1PA. Western blot analysis of Tge2pp and Tge2pp E69Q expression levels was performed using an anti-His HRP conjugate according to the manufacturer’s instructions (Qiagen). All protein constructs were expressed in *Shuf-286*

Interbacterial Competitions—Competition experiments were performed in a similar manner as described previously for *B. thailandensis* (9). Briefly, *P. protegens* and *P. putida* strains were mixed in a 1:1 ratio, and 10 µl of the mixture was spotted on 0.2-µm nitrocellulose membrane overlaid on LB-LS 3% agar plates. Plate counts were taken of the initial inoculum and after 18 h of competition at 30 °C. The recipient *P. putida* strain contained a GFP expression construct integrated into the attTn7 site allowing for differentiation of the donor and recipient cells using fluorescence imaging as described previously (1, 2, 26). Statistical analyses were carried out using a two-tailed Student’s t test.

*P. protegens* Secretion Assay and Western Blot Analysis—Tge2pp, including its native ribosome binding site, were cloned into pPSV35-CV using the Sacl/Xbal restriction sites. The resulting construct encodes Tge2pp fused to a C-terminal vesicular stomatitis virus G (VSV-G) epitope tag to facilitate detection by immunoblotting. Overnight cultures of *P. protegens* ΔretS and ΔretSΔIpV harboring pPSV35-CV::tge2pp were subcultured 1:500 into LB containing 100 µM IPTG and grown to mid-log phase. Cell and supernatant fractions were separated and analyzed for the presence of Tge2pp by Western blot using an anti-VSV-G antibody as described previously (21).

Growth Curves—Overnight cultures of *E. coli* BL21 pLysS containing pET-22b(+), pET-22b(+)::tge2pp and pET-22b(+)::tge2pp E69Q were subcultured to an optical density at 600 nm (A_600) of 0.01 in LB-LS and grown at 37 °C. A_600 measurements were taken every 30 min until the cultures reached an A_600 of ~0.2, at which point protein expression was induced with 100 µM IPTG. Following IPTG induction, A_600 measurements were taken every 15 min for the remainder of the growth curve.

Protein Expression and Purification—For the isothermal titration calorimetry (ITC) experiment, full-length tge2pp and tgi2pp lacking its encoded signal sequence (Tgi2pp[24–156]) were cloned into pET-28b(+) and pET-15b using the Ndel/Xhol and Ndel/BamHI restriction sites, respectively, to generate N-terminal His tags on both proteins. For crystallization, full-length tge2pp was cloned into pET-24a(+) using the Ndel/Xhol restriction sites to generate an untagged construct suitable for coexpression with the Tgi2pp[24–156] construct described above. All protein constructs were expressed in Shuflle® T7 Express lysY cells, which allow for cytoplasmic disulfide bond formation, and grown in LB broth supplemented with the appropriate antibiotics. For each construct, cells were grown at 37 °C to an A_600 of 0.6 before protein expression was induced with 1 mM IPTG at room temperature for 18 h. For the protein samples used in the ITC experiment, cells were resuspended in 50 mM sodium phosphate, pH 7.2, 300 mM NaCl, 5 mM imidazole, and lysed by sonication. Following centrifugation, the cleared cell lysates were purified by Ni^{2+}-nitrilotriacetic acid affinity chromatography using a linear gradient of 5–400 mM imidazole. The purified proteins were then dialyzed against 20 mM sodium phosphate, pH 7.2, 150 mM NaCl prior to use in downstream experiments. For crystallization, cells containing coexpressed Tge2pp/Tgi2pp[24–156] were resuspended in 50 mM Heps, pH 7.5, 300 mM NaCl, 5 mM imidazole, and lysed by sonication. The complex was then purified using Ni^{2+}-nitrilotriacetic acid affinity chromatography in 20 mM Heps, pH 7.5, 300 mM NaCl, 5–400 mM imidazole followed by size exclusion chromatography in 20 mM Heps, pH 7.5, 150 mM NaCl.

Isothermal Titration Calorimetry—Purified Tge2pp and Tgi2pp[24–156] were degassed before experimentation. ITC measurements were performed with a VP-ITC microcalorimeter (MicroCal Inc., Northampton, MA). Titrations were carried out with 250 µM Tgi2pp[24–156] in the syringe and 16 µM Tge2pp in the cell. The titration experiment consisted of one 2-µl injection followed by 29 5-µl injections with 300-s intervals between each injection. The ITC data were analyzed using the Origin software (version 5.0, MicroCal, Inc.) and fit using a single-site binding model.

Phase Contrast Microscopy—Phase contrast microscopy images were acquired with a Nikon Ti-E inverted microscope fitted with a 60× oil objective, a xenon light source (Sutter Instruments), and a CCD camera (Clara series, Andor). Overnight cultures of *E. coli* BL21 pLysS pET-22b(+)::tge2pp and pET-22b(+)::tge2pp E69Q were subcultured 1:300 into LB broth containing the appropriate antibiotics and grown to an A_600 of 0.2. IPTG was then added to a concentration of 200 µM, and after a 45-min incubation, the bacterial suspension (1 µl) was spotted onto growth pads made with LB-LS medium and 1% (w/v) agarose, and cells were imaged immediately.

Crystallization and Structure Determination—Selenomethionyl-incorporated Tge2pp-Tgi2pp[24–156] complex was concentrated to 10 mg ml^{-1} by spin ultrafiltration (10 kDa molecular mass cut-off, Millipore) and screened against commercially available sparse matrix crystal screens (Crystal Screens 1 and 2, Hampton Research). Crystal trials were setup in 48-well plates using the sitting drop vapor diffusion technique. Protein and crystallization solutions were mixed in a 1:1 ratio with a final drop size of 2 µl suspended over 250 µl of crystallization solution and stored at room temperature.

After 3–4 days, diffraction quality crystals grew in 0.1 M Tris-HCl, pH 8.5, 0.2 M CaCl_2, and 30% (w/v) PEG 4000 and were flash frozen without any added cryoprotectant. X-ray diffraction data were collected on beamline 5.0.3 at the Lawrence Berkeley National Laboratory Advanced Light Source (27). The data were merged, integrated, and scaled using the xia2 system (28). Experimental phases were obtained using the Phenix AutoSol Wizard (29) resulting in density modified selenium single-wavelength anomalous diffraction phased maps of
**RESULTS**

Identification of Peptidoglycan Glycoside Hydrolase Effector/Immunity Families—Previously, we reported the development of a heuristic search method that was successfully employed for the identification of a widespread type VI peptidoglycan amidasere effector superfamily (9). Using a customized Perl script, T6SS+ genomes were searched for bicistrons that encode protein products with a defined set of properties commonly associated with T6S effector-immunity pairs. For the effector protein, this included the absence of a signal peptide and primary sequence length limitation (<200 amino acids). These selection criteria yielded 418 total candidate E-I pairs, within which amidasereeffectors were identified by structure prediction algorithms and subsequent direct experimentation (19). To adapt this pipeline for the identification of glycoside hydrolase effectors, we excluded the histidine and cysteine constraints, generating a list of 831 candidate E-I pairs from 115 T6SS+ genomes. These were then examined by structure prediction servers to identify lysozyme-like folds and manually curated to remove systematic false-positives (see “Experimental Procedures”). This approach yielded two phylogenetically distinct families, which combine with *P. aeruginosa* Tse3 to form the basis for three distinct type VI peptidoglycan glycoside hydrolase effector and immunity groups (Tge1–3 and Tgi1–3). Although it likely functions as a muramidase, we have opted not to include *V. cholerae* VgrG-3 in this reclassification of T6S glycoside hydrolase effectors. This reclassification of type VI peptidoglycan-glycoside hydrolases universally employ a conserved glutamate, acting as a catalytic acid, to initiate cleavage of the glycosidic bond (33). Consistent with our hypothesis that Tge2 and Tge3 proteins such as Tse3 function as peptidoglycan glycoside hydrolases, we identified a strictly conserved Glu within a putative active motif in both enzyme families.

**Tge2PP-Tgi2PP Are an Effector-immunity Pair Secreted by the T6SS—Tge1 from *P. aeruginosa* (Tge1PP, formerly Tse3) has been demonstrated previously to function as a peptidoglycan glycoside hydrolase effector. To validate a new family identified by our informatics search, we chose to characterize a Tge2-Tgi2 E-I pair from the soil-dwelling bacterium *P. protegens* (Tge2PP-Tgi2PP). To confirm that Tge2PP functions as a periplasmic effector, we performed toxicity assays in *E. coli*. As expected for a peptidoglycan-degrading enzyme, we observed a significant decrease in *E. coli* viability and optical density when Tge2PP was artificially targeted to the periplasm via a sec-dependent leader sequence (peri-Tge2PP, Fig. 2, A and B). Furthermore, these effects of Tge2PP periplasmic toxicity were specific to Tge2PP catalytic activity, as a conservative mutation of its predicted catalytic glutamate (E69Q) abolished toxicity. Consistent with its proposed role as a peptidoglycan glycoside hydrolase effector, peri-Tge2PP induced cell rounding and lysis compared with the catalytic point mutant (Fig. 2C). Importantly, Western blot analysis confirmed that the observed reduction in toxicity and cell rounding was not due to decreased expression levels of the E69Q protein relative to wild-type (Fig. 2D). Despite our ability to observe Tge2PP activity in a cellular context, we were unable to detect significant degradation of purified peptidoglycan sacculi by Tge2PP over a range of conditions, raising the intriguing
possibility that the effector may require a yet unidentified periplasmic cofactor or binding partner.

Next, we examined whether Tgi2PP could inhibit the toxicity exhibited by peri-Tge2PP. Tgi2PP co-expressed with peri-Tge2PP significantly rescued Tge2PP-dependent toxicity, indicating that Tgi2PP functions as an immunity protein (Fig. 2E).

As predicted by its lack of sequence homology to Tgi2PP, co-expression of P. aeruginosa Tgi1 (Tgi1PA, formerly Tsi3) was not able to rescue E. coli viability. Next, we investigated the mechanism of inhibition of Tge2PP activity by Tgi2PP. To probe for a direct binding mechanism, we conducted ITC measurements on purified Tge2PP and Tgi2PP. The binding isotherm obtained by titration of the effector with Tgi2PP fit a single-site binding model with a stoichiometry of 1:1 ($K_d = 0.26 \pm 0.04 \text{ nM}$) and a dissociation constant of 0.26 ± 0.04 nM (Fig. 2F). Titration of Tgi2PP into buffer and buffer into Tge2PP confirmed that the observed saturation of the heats of injection was due to the Tge2PP-Tgi2PP interaction and not between either of the proteins and a component of the buffer solution (supplemental Fig. S3). The tight binding observed between Tge2PP and Tgi2PP is consistent with the affinities measured for other T6SS E-I pairs, including P. aeruginosa Tae1-Tai1, Enterobacter cloacae Tae4-Tai4, and two Tae4-Tai4 family members from S. marcescens, which all bind with low nanomolar to high picomolar dissociation constants (12, 13, 15).

P. aeruginosa possesses three functionally non-redundant T6SSs, encoded by HSI-I-III. Of these, the H1-encoded system is post-transcriptionally activated by the Gac/Rsm pathway (23). P. protegens possesses only a single T6SS. This system is orthologous to the H1-T6SS, and is also regulated by the Gac/
test the hypothesis that Tge2PP is a T6SS substrate. Robust insight into Tge2PP function and its mode of inhibition by secretion of Tge2PP was observed in the supernatant fraction of competitive fitness against with a non-functional T6SS display a 1000-fold decrease in Tge2PP, and residues 24–26, 155–156, and the N-terminal His6 tag of Tgi2PP. The final model was refined to an Rwork of 14.9% and an Rfree of 17.5%, respectively.

Tge2PP Resembles Family 73 Glycoside Hydrolases—Tge2PP adopts a lysozyme-like fold consisting of large and small lobes that are positioned such that a substrate-binding groove is formed between them (Fig. 3A). The large lobe is made up of a short 310 helix, a1–a2 and a4–a6, whereas the small lobe consists of a3 and a β-hairpin formed by β1–β2. The termini are covalently linked by a Cys32–Cys177 disulfide bond, which connects the N-terminal 310 helix to the C-terminal loop. As determined by DALI (39), the overall structure resembles closely that of glycoside hydrolase family 73 members, which includes the

![Structure of Tge2PP](image)

**FIGURE 3. Overall structure of Tge2PP in complex with Tgi2PP.** A, Tge2PP adopts a lysozyme-like fold. Ribbon (left) and surface (right) representations of Tge2PP shown at two orthogonal orientations. Secondary structure elements and the catalytic acid, Glu69, are labeled. Tgi2PP is omitted for clarity. B, structural overlay of Tge2PP (blue), Auto (orange), and FlgJ (gray) shown as schematic representations (left panel). Superposition of the Tge2PP, Auto and FlgJ active site residues are shown as stick representations (right panel). The labels indicate the residue numbering of Tge2PP followed by Auto and FlgJ. C, structure of the Tge2PP–Tgi2PP complex. Tge2PP and Tgi2PP are shown as surface and ribbon representations, respectively. Secondary structure elements of Tgi2PP are labeled, and Glu69 of Tge2PP is colored yellow. D, a protruding loop of Tgi2PP occludes the active site of Tge2PP. Shown is a close-up view of the β2–β3 loop of Tgi2PP interacting with the active site of Tge2PP. Invariant residues Gly38 and Ala66 of Tgi2PP interact with His69 and Tyr147 in the active site cleft of Tge2PP.
peptidoglycan hydrolase FlgJ from Sphingomonas sp. strain A1 (Z-score, 16.4; Cα root mean square deviation of 1.6 Å over 124 equivalent positions) and Auto, a peptidoglycan N-acetylglucosaminidase from Listeria monocytogenes (Z-score, 13.9; Cα root mean square deviation of 2.6 Å over 126 equivalent positions) (Fig. 3B) (40, 41). It is proposed that members of this family catalyze the hydrolysis of peptidoglycan using a single displacement mechanism similar to that originally demonstrated for G-type egg white lysozyme (GEWL). In this mechanism, a catalytic glutamate donates a proton to the scissile glycosidic bond, resulting in its cleavage and formation of an oxocarbenium intermediate. This short-lived intermediate then undergoes nucleophilic attack by a water molecule, resulting in the hydrolyzed product with inverted stereochemistry at the anomeric position (34). In agreement with our functional studies, the catalytic glutamate in Tge2PP corresponds to Glu69, inferred from its nearly identical position to the corresponding glutamate residue in both FlgJ and Auto (Fig. 3B). Glu69 protrudes from the end of α2 and lies deep within the substrate-binding groove. In addition, the hydroxyl group of Tyr147 forms a hydrogen bond with Glu69, in a manner similar to the aforementioned peptidoglycan glucosaminidase and G-type lysozyme enzymes, perhaps to orient it for catalysis.

_Tgi2PP Inhibits Tge2PP by Protruding into the Substrate-binding Groove—_The structure of Tgi2PP consists of a short N-terminal 31α helix and a central five-stranded β-sheet flanked by three α-helices (Fig. 3C). A Cys117-Cys152 disulfide bond connects β5 to the C-terminal loop, anchoring α3 to the β-sheet core. Tgi2PP does not share strong structural similarity to other proteins of known structure; however, it does contain a similar topology to the periplasmic E. coli colicin M immunity protein (Z-score, 3.5; Cα root mean square deviation of 3.7 Å over 62 equivalent positions) (42). Colicin M is a bacteriocin with phosphatase activity that targets the undecaprenyl phosphate-linked peptidoglycan precursors lipid I and lipid II in the periplasm of recipient bacteria (43). The observation that the colicin M immunity protein resembles Tgi2PP suggests that these immunity proteins may have undergone diversifying selection to acquire effector specificity.

The interaction between Tge2PP and Tgi2PP involves the insertion of the β-sheet core of Tgi2PP into the substrate-binding groove of Tge2PP. Most notably, the elongated loop connecting β2 and β3 protrudes into the active site in an orientation predicted to prevent the catalytic glutamate from accessing its peptidoglycan substrate. Overall, the Tge2PP-Tgi2PP complex consists of a 1237 Å2 interface stabilized predominantly by hydrogen bonding and hydrophobic interactions. Conservation mapping of Tgi2PP homologs suggests that invariant residues Gly94 and Ala95, which are located in the β2-β3 loop, are important for direct interaction with the active site of Tge2PP (Fig. 3D). Binding site analysis using PDBePISA indicates that the amide nitrogen and oxygen atoms of Gly94 form hydrogen bonding interactions with the main chain of Tyr147 and His88 of Tge2PP, respectively, whereas the side chain of Ala95 becomes desolvated upon complex formation (44). Additional conserved interfacing residues include Tyr56, Asp69, Leu69, His78, Tyr88, Pro91, Trp93, Gly100, Leu101, Glu113, Cys117, Gly149, Leu150, Gly151, Cys152, and Asp153. The inhibition mode observed in the Tge2PP-Tgi2PP complex is reminiscent of bacterial proteinaeous inhibitors of eukaryotic lysozymes. The _E. coli_ inhibitor of vertebrate lysozyme (Ivy), _P. aeruginosa_ membrane-bound lysozyme inhibitor of _C_-type lysozyme (MiIC), and _E. coli_ PliG (periplasmic lysozyme inhibitor of _G_-type lysozyme) all use active site occlusion as a mechanism of inhibition (Fig. 4) (45–47). Moreover, in each case, the region involved in inhibition consists of a loop region connecting two β-strands. However, it is interesting to note that there is little fold similarity between these lysozyme inhibitors, suggesting they arose through convergent evolution.

**DISCUSSION**

In this work, we identified two families of T6S peptidoglycan glycoside hydrolase effectors using informatic methods. Previously, we used homology-independent search criteria to identify a T6S peptidoglycan amidase effector superfamily. The identification of the peptidoglycan glycosyl hydrolase families described herein further exemplifies the utility of this method as a means to identify novel effector-immunity pairs. Prior characterization of Tge1PA has shown that this enzyme possesses β-(1,4)-N-acetylglucosaminidase (lysozyme) activity. Furthermore, Tge1PA contains a GLXQ motif found in both GEWL and _E. coli_ soluble lytic transglycosylase (Slt70) in addition to its catalytic glutamate, suggesting it is likely structurally similar to these enzymes (3, 48). Interestingly, Tge3 family members contain a conserved Glu-Asp-Thr catalytic triad reminiscent of phage T4 lysozyme. Thus, similar to Tge1, we expect this family to exhibit β-(1,4)-N-acetylglucosaminidase activity. In contrast, we find that Tge2PP most closely resembles members of a peptidoglycan hydrolase family with ascribed _N_-acetylglucosaminidase activity. Members of this family contain a catalytic glutamate residue embedded in an AAXE(S/T) motif.
This observation suggests that Tge2 enzymes could target a different chemical bond in the peptidoglycan backbone to facilitate breakdown of the cell wall in recipient bacteria. Bacteria are known to chemically modify the glycan moieties of peptidoglycan. As this can affect their susceptibility to cell wall-targeting enzymes, it is intriguing to speculate that the different peptidoglycan glycoside hydrolase effectors delivered by the T6SS could harbor specificity for the presence or absence of these modifications. Modifications influencing muramidase activity include MurNAc and GlcNAc de-N-acetylation, as well as MurNAc O-acetylation (49). These generally result in lysozyme resistance, whereas N-acetylgalactosaminidase enzymes can be unaffected by the acetylation state of peptidoglycan (50). Future studies will be needed to address the specificity of the Tge families, as well as the extent to which particular cell wall modifications influence their activity.

In light of the peptidoglycan glycoside hydrolases identified in this study, it is now apparent that the pathogenic bacteria secretes multiple T6 effectors targeting peptidoglycan. For example, in addition to Tge2 peptidoglycan glycoside hydrolases, Salmonella Typhi and P. protegens also possess Tae2 and Tae3 peptidoglycan amidases, respectively (9). The reason for secreting a multitude of peptidoglycan-targeting T6S effectors may be myriad. It is conceivable that the T6 donor bacterium requires complete digestion of recipient peptidoglycan sacculi to utilize it as a carbon source after lysis occurs.

A non-mutually exclusive explanation may be the existence of enzymatic synergy between amidase and muramidase effectors, wherein the breakdown product of one enzyme may make the substrate of the second more accessible. The importance of this type of enzymatic synergy was recently demonstrated for cellulose-degrading enzymes (51). Alternatively, the various E-I loci within a given organism may be subject to differential regulation, such that the effector most beneficial in a particular environmental condition is expressed. The magnitude of this disproportionate benefit could be dependent upon the target organism(s) present, the physiological state of the target(s) (e.g., growing versus sessile), and the osmolarity of the surrounding milieu.

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