Extended Experimental Procedures

Bacterial strain growth, maintenance and construction

Strains were grown on Luria-Bertani media (LB) or LB low salt (LB-LS): 10 g bactopeptone and 5 g yeast per liter) at 37 °C (*P. aeruginosa* and *B. subtilis*) or 30 °C (*P. putida*). *P. putida* strains were grown on media supplemented with 25 μg ml^{-1} irgasan and 50μg ml^{-1} tetracycline as needed. *E. coli* strains were grown on LB at 37 °C or 18°C supplemented with 10μg ml^{-1} tetracycline, 50 μg ml^{-1} kanamycin, or 30 μg ml^{-1} chloramphenicol as needed. *P. aeruginosa* strains bearing deletions of genes encoding the RetS hybrid sensor kinase and Tse1 were constructed as previously described (Mougous et al., 2006; Russell et al., 2011). *E. coli* strains include DH5α for plasmid maintenance and SM10 for conjugal transfer of plasmids into *P. putida*.

The previously-described JMA264 strain of *B. subtilis* JH642 was used. *E. coli* and *P. putida* β-galactosidase-positive strains were constructed using the pminiCTX-plac-lacZ construct from (Vance et al., 2005). This plasmid was used as a replicative plasmid in *E. coli* and as a miniCTX integrant into *P. putida* and *B. thailandensis* for the expression of LacZ.

Tse1 cloning, protein expression, and purification

The full-length Tse1 gene was cloned into the pET29b+ expression vector as previously described (Russell et al., 2011) to create a fusion with the C-terminal six-histidine tag. Shuffle T7 pLysS cells (New England Biolabs) were transformed, and protein expression was induced at an optical density at 600nm (OD_{600}) of 0.6 by the addition of 0.1 mM

IPTG. After 20 h of induction at 18 ^{o}C, cells were harvested and resuspended in 20 mM HEPES pH 7.5, 0.5 M NaCl, 25 mM imidazole, 10% glycerol, and lysed by sonication. Lysate was centrifuged for 1 h at 18,000 x *g*, and Tse1 was purified from the supernatant by elution from a metal-chelating affinity column and size exclusion chromatography (GE Healthcare). Recombinant Tse1 in 0.1 M NaCl, 10 mM HEPES pH 7.5 was concentrated to 10 mg/ml.

Tse1 C30A structure solution

The Tse1 C30A protein was expressed, purified, and crystallized under the same conditions as wild-type Tse1. The C30A structure was determined by molecular replacement and built using the PHENIX software suite (Adams et al., 2010).

Tse1 structure validation

The Tse1 and Tse1 C30A structures were validated by comparison with representative structures in the PDB using PHENIX Polygon (Adams et al., 2010). All values fell within acceptable ranges for their resolution bins with the exception of the Rsym values. However, the Tse1 structure was solved by experimental phasing using the SeMet dataset reported in Table S1 and the model was built using automated methods in PHENIX, suggesting the data are of reasonable quality within the reported resolution range. Moreover, the density-modified experimental map used for phasing Tse1 was consistent with the resolution cut-off utilized. The processed data was further analyzed by PHENIX XTriage, which did not indicate any pathologies such as twinning, pseudo-symmetry, or abnormal intensity statistics.

Ligand model generation

The initial ligand was built in Maestro, including all stereoisomers for *m*DAP. 3D conformations were generated with LigPrep 2.5 (Chen and Foloppe, 2010) using the OPLS2005 force field and neutralized charges. A low-frequency mode conformational search by MacroModel 9.9 was utilized to generate low-energy structures with sufficient searching to switch chiral centers when energetically favorable, over a maximum of 1000 steps. 168 unique conformations were retained.

Computational analysis of docking poses

The docking site was defined by the centroid of the location of bound ligands in other homologs (17.3138, 51.2587, 20.4035) Å (Xu et al., 2010). An outer box of 40x40x40 Å and an inner box of 10x10x10 Å were used to fully search the face of Tse1, within which receptor hydroxyl groups were allowed to move. Up to 20 top poses were saved from the docking results for scoring by the Glide XP function. Our specific protocol was developed to best reflect the interactions between the Tse1 monomer and the flexible substrate. The highest scoring protein-ligand complex was carried forward.

Muropeptide preparation

Following incubation with enzyme, the reaction was stopped by boiling for 10 min. The pH was adjusted to 4.8 and the sample was incubated with 40 µg/ml of muramidase cellosyl (kindly provided by Höchst AG, Frankfurt, Germany) for 16 h at 37°C to convert residual peptidoglycan or solubilized fragments into muropeptides. The sample was

boiled for 10 min and insoluble material was removed by centrifugation. Muropeptides were reduced as described (Glauner, 1988).

Cell lysis measurements

1μM, 0.33μM, and 0.11μM Tse1 was assayed against *B. subtilis*, 0.037 μM, 0.012μM, and 0.004μM against *E. coli*. 0.33μM, 0.11μM, and 0.037μM hen egg white lysozyme (Roche) was tested against *B. subtilis*, and 0.037 μM, 0.012 μM, and 0.004μM against *E. coli*. Tse1(C30A) was tested at 1μM. OD_{600} measurements were taken every 90 s over 10 min 30 s for three biological replicates per concentration. Lysis rate was determined as slope of the best-fit line, and data were analyzed using a two-tailed Student's T-test.

Supplemental References

Adams, P.D., Afonine, P.V., Bunkoczi, G., Chen, V.B., Davis, I.W., Echols, N., Headd, J.J., Hung, L.W., Kapral, G.J., Grosse-Kunstleve, R.W*., et al.* (2010). PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr D Biol Crystallogr *66*, 213-221.

Chen, I.J., and Foloppe, N. (2010). Drug-like bioactive structures and conformational coverage with the LigPrep/ConfGen suite: comparison to programs MOE and catalyst. J Chem Inf Model *50*, 822-839.

Glauner, B. (1988). Separation and quantification of muropeptides with high-performance liquid chromatography. Anal Biochem *172*, 451-464.

Mougous, J.D., Cuff, M.E., Raunser, S., Shen, A., Zhou, M., Gifford, C.A., Goodman, A.L., Joachimiak, G., Ordonez, C.L., Lory, S*., et al.* (2006). A virulence locus of Pseudomonas aeruginosa encodes a protein secretion apparatus. Science *312*, 1526-1530.

Russell, A.B., Hood, R.D., Bui, N.K., LeRoux, M., Vollmer, W., and Mougous, J.D. (2011). Type VI secretion delivers bacteriolytic effectors to target cells. Nature *475*, 343-347.

Vance, R.E., Rietsch, A., and Mekalanos, J.J. (2005). Role of the type III secreted exoenzymes S, T, and Y in systemic spread of Pseudomonas aeruginosa PAO1 in vivo. Infection and immunity *73*, 1706-1713.

Xu, Q., Abdubek, P., Astakhova, T., Axelrod, H.L., Bakolitsa, C., Cai, X., Carlton, D., Chen, C., Chiu, H.J., Chiu, M*., et al.* (2010). Structure of the gamma-D-glutamyl-L-diamino acid endopeptidase YkfC from Bacillus cereus in complex with L-Ala-gamma-D-Glu: insights into substrate recognition by NlpC/P60 cysteine peptidases. Acta Crystallogr Sect F Struct Biol Cryst Commun *66*, 1354-1364.