Diverse type VI secretion phospholipases are functionally plastic antibacterial effectors

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Membranes allow the compartmentalization of biochemical processes and are therefore fundamental to life. The conservation of the cellular membrane, combined with its accessibility to secreted proteins, has made it a common target of factors mediating antagonistic interactions between diverse organisms. Here we report the discovery of a diverse superfamily of bacterial phospholipase enzymes. Within this superfamily, we define enzymes with phospholipase A1 and A2 activity, which are common in host-cell-targeting bacterial toxins and the venoms of certain insects and reptiles. However, we find that the fundamental role of the superfamily is to mediate antagonistic bacterial interactions as effectors of the type VI secretion system (T6SS) translocation apparatus; accordingly, we name these proteins type VI lipase effectors. Our analyses indicate that PldA of Pseudomonas aeruginosa, a eukaryotic-like phospholipase D, is a member of the type VI lipase effector superfamily and the founding substrate of the haemolysin co-regulated protein secretion island II T6SS (H2-T6SS). Although previous studies have specifically implicated PldA and the H2-T6SS in pathogenesis, we uncovered a specific role for the effector and its secretory machinery in intra- and interspecies bacterial interactions. Furthermore, we find that this effector achieves its antibacterial activity by degrading phosphatidylethanolamine, the major component of bacterial membranes. The surprising finding that virulence-associated phospholipases can serve as specific antibacterial effectors suggests that interbacterial interactions are a relevant factor driving the continuing evolution of pathogenesis.

Within proteobacterial genomes, predicted lipases are often encoded adjacent to homologues of the vgrG gene. The VgrG protein is strongly associated with, and functionally important for, the cell-contact-dependent type VI secretion (T6S) protein delivery pathway. This pathway, which is distributed throughout all classes of Proteobacteria, can target both eukaryotic and bacterial cells; however, it is the specificity of its effectors that dictates the consequences of intoxication by the system. Known T6S effectors are few and include enzymes that degrade phospholipids accessible from the periplasm. Within this superfamily, we defined enzymes with phospholipase A1 and A2 activity, which are common in host-cell-targeting bacterial toxins and the venoms of certain insects and reptiles. However, we find that the fundamental role of the superfamily is to mediate antagonistic bacterial interactions as effectors of the type VI secretion system (T6SS) translocation apparatus; accordingly, we name these proteins type VI lipase effectors. Our analyses indicate that PldA of Pseudomonas aeruginosa, a eukaryotic-like phospholipase D, is a member of the type VI lipase effector superfamily and the founding substrate of the haemolysin co-regulated protein secretion island II T6SS (H2-T6SS). Although previous studies have specifically implicated PldA and the H2-T6SS in pathogenesis, we uncovered a specific role for the effector and its secretory machinery in intra- and interspecies bacterial interactions. Furthermore, we find that this effector achieves its antibacterial activity by degrading phosphatidylethanolamine, the major component of bacterial membranes. The surprising finding that virulence-associated phospholipases can serve as specific antibacterial effectors suggests that interbacterial interactions are a relevant factor driving the continuing evolution of pathogenesis.

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and sufficient to restore competitive fitness (Fig. 2b). These data show that Tle1<sub>BT</sub> is an antibacterial effector delivered between cells by T6S, and that I2699, hereafter referred to as type VI secretion lipase immuno-

**Figure 1 | Overview of the Tle superfamily.** a, Evolutionary trees, genetic organization, and phylogenetic distribution of select Tle family members. Genes are coloured by their predicted protein product (blue, Tle proteins with a GXSXG catalytic motif; purple, Tle proteins with dual HXXXXXD catalytic motifs; grey, VgrG proteins; yellow, putative periplasmic immunity proteins). Branch lengths are not proportional to evolutionary distance. Asterisks denote
tle genes without an apparent adjacent vgrG gene. b, Domain organization of a single member of the GXSXG and dual HXXXXXD catalytic classes of Tle proteins. Regions comprising these catalytic motifs are labelled in grey, and positions of all putative catalytic residues are denoted. Sequence logos were generated from alignments of the catalytic motifs from Tle1–4 (GXSXG) and catalytic motifs from Tle5 (HXXXXXD). aa, amino acids; kb, kilobases.

Having demonstrated that members of two GXSXG Tle families function as antibacterial T6S effectors, we next sought to investigate their biochemical activity. To characterize Tle1<sub>BT</sub> and Tle2<sub>VC</sub>, we purified the proteins and catalytic nucleophile substitution mutant (His6-MBP), which we found to be necessary to generate and maintain solubility (Supplementary Figs 8 and 9). Notably, Tle1–4 possess a Ser-Asp-His catalytic triad used by a diversity of esterase enzymes, including thioesterases, acetylesterases and assorted lipase and phospholipases<sup>1</sup>. Given this wide range of potential activities, we initially confirmed general esterase activity of His6-MBP–Tle1BT and His6-

**Table 1**

| tle | Gene | Species |
|-----|------|---------|
| tle1 | Gene | Species |
| tle2 | Gene | Species |
| tle3 | Gene | Species |
| tle4 | Gene | Species |
| tle5 | Gene | Species |

**Figure 2** | Linking effector activity to antibacterial phenotype. a, Consistent with previous observations, Tle5<sub>PA</sub> catalyses the release of choline from phosphatidylcholine, in a manner dependent on a predicted catalytic histidine residue (His 855) (Fig. 3a and Supplementary Fig. 11). Under similar conditions neither Tle1<sub>BT</sub> nor Tle2<sub>VC</sub> showed appreciable activity in this assay, underscoring the diverse substrate specificity within the Tle superfamily. A candidate Tle5<sub>PA</sub> periplasmic immunity protein is not readily apparent, as the adjacent gene, PA3488, is predicted to encode a cytoplasmic protein. However, expression of PA3488 from a second, upstream, predicted start site yields a periplasmically localized protein, hereafter referred to as Tli5<sub>PA</sub>, that binds specifically to Tle5<sub>PA</sub> (Supplementary Fig. 13). To probe the role of Tle5<sub>PA</sub> and Tli5<sub>PA</sub> in interbacterial interactions, we generated a lysis reporter strain bearing a deletion of the tle5<sub>PA</sub> tli5<sub>PA</sub> bicistron. Lysis of this strain was greatly increased when co-cultured with a wild-type, but not a Δtle5<sub>PA</sub> donor, strain (Fig. 3b). Furthermore, expression of tli5<sub>PA</sub> in the recipient was sufficient to protect from Tle5<sub>PA</sub>-dependent lysis. Together, these data
Figure 2  |  Tle GXSXG-type proteins are antibacterial phospholipase effectors delivered by the T6SS. a. Outcome of growth competitions between the indicated V. cholerae strains and E. coli. The AssSM strain is inactive for T6S. Asterisks denote competitive outcomes significantly different than those obtained with wild type (P < 0.05, n = 3). b. Growth competition assays between the indicated B. thailandensis donor and recipient strains. The ΔclpV1 strain is inactivated for T6SS-1, which is required for Tle1BT export. The parental strain is ΔI2697–2703. Asterisks denote competitive outcomes significantly different between indicated recipient strains (left) or indicated donor strains (right) (P < 0.05, n = 3). c,d, Enzymatic activity of the designated effectors delivered by the T6SS. a.u., arbitrary units; MH, His6-MBP. c, Enzymatic activity of His6-MBP–Tle1BT. d, Enzymatic activity of the designated proteins against vesicles containing phospholipid derivatives with fluorescent moieties at the steremochemical numbering (sn) positions sn-1 and sn-2 (n = 4). e, Enzymatic activity of His6-MBP–Tle1BT demonstrate that Tle5PA acts as an antibacterial toxin and that Tle5PA is its cognate immunity determinant.

The P. aeruginosa genome encodes three T6SSs, the H1–3-T6SSs. The H1-T6SS is the only system with known substrates and a demonstrated role in interbacterial interactions. To define the T6SS involved in Tle5PA transport, we constructed strains bearing individual in-frame deletions of the crucial ATPas genes, clpV1–3, associated with the H1–3 systems, respectively. Specific inactivation of the H2-T6SS in a donor strain abrogated Tle5PA-dependent toxicity, indicating that this system is responsible for Tle5PA delivery (Fig. 3b).

The finding that Tle5PA transits the H2-T6SS pathway is interesting in light of data that implicate this T6SS as a virulence factor in plant, mammalian cell culture, worm, and mouse models of infection. To more thoroughly explore the role of Tle5PA and the H2-T6SS in interbacterial interactions, we measured their influence on competition outcomes between P. aeruginosa and a model T6S target, Pseudoomonas putida. Our results showed that both Tle5PA and the H2-T6SS significantly contribute to the fitness of P. aeruginosa in interspecies competition under T6S-conducive conditions (Fig. 3c and Supplementary Fig. 14). These findings show that Tle5PA is a potent antibacterial effector delivered by the H2-T6SS.

Although our data thus far show that Tle1BT, Tle2VC and Tle5PA possess phospholipase activity in vitro, this did not allow us to assign definitively the toxic consequences of these effectors to membrane destruction. The phospholipase activity of the effectors could be accessory to a second toxicity mechanism found in these large, multidomain proteins. To resolve this remaining ambiguity concerning the Tle function, we focused our studies on Tle5PA. Because a mixture of healthy and intoxicated cells could complicate our measurements, we decided to assay Tle5PA effects in self-intoxicating monocultures of Δtli5PA, in which each cell serves both as a donor and a sensitive recipient. As expected, this strain exhibited increased membrane permeability in a manner dependent on an active H2-T6SS and Tle5PA (Supplementary Fig. 15).

Under conditions promoting intercellular delivery of Tle5PA, we extracted lipids from both non-intoxicated (wild type) and intoxicated (Δtli5PA) cells, and quantified their phospholipid composition using mass spectrometry. This analysis showed that the unchecked action of Tle5PA leads to a severely perturbed membrane phospholipid composition. Notably, phosphatidic acid, a product of PLD activity and a minor constituent of wild-type membranes (0.17%), was present at 8.1% in Δtli5PA—a 48-fold enrichment (Fig. 3d and Supplementary Table 1). The increased phosphatidic acid seemed to derive primarily from phosphatidylethanolamine, as it underwent a concomitant decrease in sn-2-labelled phospholipids as measured in c after the addition of the indicated immunoprecipitate (arrow) (n = 5).

Donor recipient
(Δte1BT ΔI2699–I2700)

Control Tli1BT Δte1BT ΔI2701–I2703

Donor 

Recipient

Figure 3 | Tle GXSXG-type proteins are antibacterial phospholipase effectors delivered by the T6SS. a. Outcome of growth competitions between the indicated V. cholerae strains and E. coli. The AssSM strain is inactive for T6S. Asterisks denote competitive outcomes significantly different than those obtained with wild type (P < 0.05, n = 3). b. Growth competition assays between the indicated B. thailandensis donor and recipient strains. The ΔclpV1 strain is inactivated for T6SS-1, which is required for Tle1BT export. The parental strain is ΔI2697–2703. Asterisks denote competitive outcomes significantly different between indicated recipient strains (left) or indicated donor strains (right) (P < 0.05, n = 3). c,d, Enzymatic activity of the designated effectors delivered by the T6SS. a.u., arbitrary units; MH, His6-MBP. c, Enzymatic activity of His6-MBP–Tle1BT. d, Enzymatic activity of the designated proteins against vesicles containing phospholipid derivatives with fluorescent moieties at the steremochemical numbering (sn) positions sn-1 and sn-2 (n = 4). e, Enzymatic activity of His6-MBP–Tle1BT demonstrate that Tle5PA acts as an antibacterial toxin and that Tle5PA is its cognate immunity determinant.

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charged character of the molecule is likely to have a detrimental effect on both integral and peripheral membrane-associated proteins. It is known that phosphatidic acid induces membrane curvature that can promote fusion and fission events\(^1\); therefore, Tle5PA activity might also lead to generalized membrane destabilization, membrane blebbing and depolarization. Interestingly, the in vivo specificity of Tle5PA for phosphatidylethanolamine, the major phospholipid constituent of most bacterial membranes, affords \(P.\ aeruginosa\) the capacity to use this enzyme against a vast array of competitors.

The discovery of T6SS-delivered phospholipase effectors has many implications. Crucially, their biochemical activity does not intrinsically limit their toxicity to bacterial cells (Fig. 3e). Indeed, two specificities now ascribed to Tle superfamily members, PLD and PLA2, are both highly represented in host cell-targeting bacterial toxins\(^2\). As these effectors are found in numerous established and emerging opportunistic pathogens, our work highlights the need to understand the biochemical, genetic and evolutionary basis of interdomain targeting by the T6SS. Such knowledge may ultimately become a component of a larger strategy to develop predictive algorithms for the evolution of bacterial pathogens. In addition, our findings add a new dimension to our understanding of the mechanisms used during bacterial competition. On the basis of our data it appears that membrane targeting evolved independently on several occasions as an antibacterial strategy. This convergent evolution underscores the susceptibility of the bacterial membrane to attack, a theme mirrored by the previous observation that bacteriolytic T6S effectors likewise degrade an essential, conserved bacterial structure\(^3\). The continued discovery of antibacterial effectors promises to illuminate further vulnerabilities of the bacterial cell, and thus may aid our efforts to define promising therapeutic targets.

**METHODS SUMMARY**

\(B.\ thailandensis, V.\ cholerae\) and \(P.\ aeruginosa\) strains used in this study were derived from the strains E264, A1552 and PAO1, respectively\(^4\). All deletions were in-frame, unmarked, and generated by allelic exchange. All protein and nucleotide sequences were obtained from the NCBI GenBank database. Informatic analysis of Tle protein identity, distribution, alignment and phylogeny used a combination of structural prediction, homology, subcellular localization and tree-building algorithms as detailed in the Methods. Tle proteins were purified as fusions to both MBP and a His\(_6\) tag. Lipase activity was measured using fluorescent reporters as detailed in the Methods. Bacterial competition experiments were performed on Luria–Bertani (\(V.\ cholerae\) and \(B.\ thailandensis\)) or synthetic cystic fibrosis sputum media (CFM)\(^5\). Primers as detailed in the Methods. \(P.\ aeruginosa\) lysis assays were performed as previously described on competitions grown at 23 °C on 1.5% (w/v) agar CFM plates\(^6\). \(P.\ aeruginosa\) propidium iodide staining experiments were performed on mononucleotides grown at 23 °C on 1.5% (w/v) agar CFM plates. Real-time single-cell quantification of propidium iodide staining under competition conditions used a modification of previously described custom software for the analysis of labelled bacteria visualized by fluorescence microscopy\(^7\). Lipodinomic studies were performed by the Kansas State University Lipodinomics Research Center.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions A.B.R., M.L., P.A.W., S.N.W. and J.D.M. conceived and designed experiments. A.B.R., M.L., K.H., D.M.A., T.I. and J.D.M. conducted experiments. A.B.R. and J.D.M. wrote the paper.

Author Information GenBank accession numbers for all Tle proteins identified in this study are found in Supplementary Figs 1–5. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to J.D.M. (mougous@u.washington.edu).
METHODS

Bacterial strains and growth conditions. *B. thailandensis* strains used in this study were derived from the sequenced strain E264 (ref. 16). *B. thailandensis* NCTC 4259 was grown on either Luria–Bertani (LB) medium, or the equivalent saltless LB medium supplemented with 10% (w/v) sucrose at 37 °C to an attenuation (D) of 800 μm D600nm of 0.5, with 0.0002% (w/v) arabinose and then collected at a D600nm of 1.0. Cells were grown in LB medium or LB with 0.1% (w/v) sucrose at 30 °C to an attenuation (D) of 800 μm D600nm of 0.5. Both initial and final counts of *B. thailandensis* were performed by washing four times with immunoprecipitation wash buffer (20 mM Tris–Cl, pH 7.5, 50 mM KCl, 8.0% (v/v) glycerol, 1.0% (v/v) Triton, supplemented with DNase I (Roche), lysozyme (Roche) and 200 μM phenylmethylsulphonyl fluoride (PMSF)). Cells were disrupted by sonication and the solution clarified by centrifugation. A sample of supernatant was then taken for analysis of total protein. The remainder of the supernatant was incubated with anti-VSV-G agarose beads (Sigma) for 1 h at 4 °C. Beads were washed four times with immunoprecipitation wash buffer (100 mM NaCl, 25 mM KCl, 0.1% (v/v) Triton, 20 mM Tris–Cl, pH 7.5, and 2% (v/v) glycerol). Proteins were removed from beads with SDS-loading buffer (125 mM Tris, pH 6.8, 2% (w/v) 2-mercaptoethanol, 20% (v/v) glycerol, 0.001% (w/v) bromophenol blue and 4% (w/v) SDS), and analysed by western blot.

Bacterial competition experiments. *Burkholderia* competition experiments were performed as described previously28. Anti-RNA polymerase, anti-VSV-G, anti-β-lactamase, anti-His and anti-CAMP receptor protein (CRP) western blot analyses were performed using previously defined methods35,36. To analyse the expression of epitope-tagged Tle2^VC^ and Tle2^Ser371Ala^ in *V. cholerae*, cells were grown in LB medium at 37 °C to an attenuation (D) of 800 μm D600nm of 0.5, with 0.0002% (w/v) arabinose, and then collected at a final D600nm of 2.0. To analyse the expression of epitope-tagged Tle5^PA^, Tle5^PA^H167R and Tle5^PA^H855R, in *P. aerugi- nosa* complementation in *B. thailandensis* BTH I2698 was grown on LB supplemented with 0.1% (w/v) sucrose at 37 °C and collected at a D600nm of 1.0. Subcellular localization of epitope-tagged Tle5^PA^ and Tle5^PA^ in *P. aeruginosa* was performed identically to previous localization studies of Tsi1 and Tsi3 (refs 9, 39). For immunoprecipitation experiments, BL21(DE3) pRξ cells co-expressing periplasmic His-tagged Tle5^PA^ from a PET22b^+^ vector and VSV-G-tagged immunity proteins from pSCrhaB2 vectors were pelleted and resuspended in lysis buffer (20 mM Tris–Cl, pH 7.5, 50 mM KCl, 8.0% (v/v) glycerol, 1.0% (v/v) Triton, supplemented with DNase I (Roche), lysozyme (Roche) and 200 μM phenylmethylsulphonyl fluoride (PMSF)). Cells were disrupted by sonication and the solution clarified by centrifugation. A sample of supernatant was then taken for analysis of total protein. The remainder of the supernatant was incubated with anti-VSV-G agarose beads (Sigma) for 1 h at 4 °C. Beads were washed four times with immunoprecipitation wash buffer (100 mM NaCl, 25 mM KCl, 0.1% (v/v) Triton, 20 mM Tris–Cl, pH 7.5, and 2% (v/v) glycerol). Proteins were removed from beads with SDS-loading buffer (125 mM Tris, pH 6.8, 2% (w/v) 2-mercaptoethanol, 20% (v/v) glycerol, 0.001% (w/v) bromophenol blue and 4% (w/v) SDS), and analysed by western blot.

Informatic identification of Tle proteins. All sequences were obtained from the NCBI, and GenBank accession numbers for all Tle proteins identified in this study are found in Supplementary Figs 1–5. BTH I2698 from *B. thailandensis* E264, PA0260, PA1510 and PA3487, and PA5089 from *P. aeruginosa* PA01, and VC1418 from *V. cholerae* V52, all encoded adjacent to vgrG genes, were identified as putative lipases using the PHYRE 2 structural prediction server. Using the amino acid sequences of these predicted lipases, blast analysis were performed against the non-redundant protein database (ftp://ftp.ncbi.nih.gov/blast/db/) to identify conserved domains of their homologues. Homologues identified by the blast search were used to compare these proteins into five distinct Tle families. Each family was aligned using the MUSCLE algorithm and phylogenetic trees were generated using the PhyML 3.0 method with bootstrap analysis of 1,000 replicates34.35. Proteins encoded by the genes shown in Fig. 1a were analysed for subcellular localization using the SignalP 3.0 and TMHMM 2.0 servers, and VgrG proteins were identified using blast36.37. Regions depicted in Fig. 1a were extracted based on boundaries defined by the presence of a tle, tle or vgrG gene. Figure 1b catalytic residues were determined by both PHYRE 2 structural alignment with known lipase enzymes and conservation of those residues within the Tle family alignments. Sequence logos were generated from a manual alignment of conserved catalytic motifs using Geneious software.

Western blot analyses. Whole-cell fractions were prepared as described previously38. Anti-RNA polymerase, anti-VSV-G, anti-β-lactamase, anti-His and anti-CAMP receptor protein (CRP) western blot analyses were performed using previously defined methods35,36. To analyse the expression of epitope-tagged Tle2^VC^ and Tle2^Ser371Ala^ in *V. cholerae*, cells were grown in LB medium at 37 °C to an attenuation (D) of 800 μm D600nm of 0.5, with 0.0002% (w/v) arabinose, and then collected at a final D600nm of 2.0. To analyse the expression of epitope-tagged Tle5^PA^, Tle5^PA^H167R and Tle5^PA^H855R, in *P. aeruginosa* complementation in *B. thailandensis* BTH I2698 was grown on LB supplemented with 0.1% (w/v) sucrose at 37 °C and collected at a D600nm of 1.0. Subcellular localization of epitope-tagged Tle5^PA^ and Tle5^PA^ in *P. aeruginosa* was performed identically to previous localization studies of Tsi1 and Tsi3 (refs 9, 39). For immunoprecipitation experiments, BL21(DE3) pRξ cells co-expressing periplasmic His-tagged Tle5^PA^ from a PET22b^+^ vector and VSV-G-tagged immunity proteins from pSCrhaB2 vectors were pelleted and resuspended in lysis buffer (20 mM Tris–Cl, pH 7.5, 50 mM KCl, 8.0% (v/v) glycerol, 1.0% (v/v) Triton, supplemented with DNase I (Roche), lysozyme (Roche) and 200 μM phenylmethylsulphonyl fluoride (PMSF)). Cells were disrupted by sonication and the solution clarified by centrifugation. A sample of supernatant was then taken for analysis of total protein. The remainder of the supernatant was incubated with anti-VSV-G agarose beads (Sigma) for 1 h at 4 °C. Beads were washed four times with immunoprecipitation wash buffer (100 mM NaCl, 25 mM KCl, 0.1% (v/v) Triton, 20 mM Tris–Cl, pH 7.5, and 2% (v/v) glycerol). Proteins were removed from beads with SDS-loading buffer (125 mM Tris, pH 6.8, 2% (w/v) 2-mercaptoethanol, 20% (v/v) glycerol, 0.001% (w/v) bromophenol blue and 4% (w/v) SDS), and analysed by western blot.

Bacterial competition experiments. *Burkholderia* competition experiments were performed as described previously28. Recipient strains (Fig. 2b, left) or donor strains (Fig. 2b, right) were labelled with a GFP-expression construct integrated into the attTn7 site, allowing the disambiguation of donor and recipient colonies through fluorescence imaging. For *V. cholerae* competition experiments with *B. thailandensis*, both strains were grown to a D600nm of 0.5 in LB medium before being mixed 1:1 by volume. This mixture was then spotted on a nitrocellulose membrane on a 1.5% (w/v) agar LB plate containing 300 mM NaCl and 0.002% (w/v) arabinose. Colonies were incubated for 5 h at 37 °C. Cells were then collected and competitions analysed. Initial and final colony-forming units of *V. cholerae* and *B. thailandensis* were enumerated on LB plates supplemented with rifampin and streptomycin, respectively. For *P. aeruginosa* competitions with *P. putida*, strains were grown overnight on solid LB medium at 37 °C (*P. aeruginosa*) or 30 °C (*P. putida*) and mixed 1:1 by volume. This mixture was then spotted onto a 1.5% (w/v) agar SCFM media plate or inoculated into liquid media of the same. After 23 h of incubation at 23 °C, a temperature previously demonstrated conducive to H2-T6SS and Tle5^PA^ expression under in vitro conditions3,41, cells were collected and related numbers of bacteria determined. Both initial and final counts of *P. aeruginosa* and *P. putida* were determined by plate counts. *P. aeruginosa* self-toxication assays were performed under identical conditions to solid media competition assays, save for the addition of 1 mM IPTG. After 23 h of growth, cells were stained with 5 μg ml−1 propidium iodide in PBS, pH 7.0, for 10 min and washed before fluorescence measurements at an excitation/emission of 535/617 nm with a BFP2000 microscope with a 485 nm excitation filter and 535 nm long pass filter. Competition results for *B. thailandensis* and *P. aeruginosa* are the change in ratio of donor cells to recipient cells, competition results from *V. cholerae* represent the final ratio alone. Data from all competitions were analysed by a two-tailed Student’s t-test, and data from monoculture experiments were analyzed by Student’s t-test.
were analysed by a one-tailed Student’s t-test for a significant increase in propidium iodide staining.

Enzymatic assays of lipase activity. The hydrolysis of polysorbate 20 was measured as described previously41. These experiments were performed at 28 °C at a final enzyme concentration of 60 nM in a buffer consisting of 20 mM Tris-Cl, pH 7.2, 100 mM NaCl, 3 mM CaCl₂, and 2% (v/v) polysorbate 20. Fluorescence assays for phospholipase A activity were performed using PED-A1 (sn-1-labelled) and PED6 (sn-2-labelled) fluorescent substrates according to manufacturer’s directions (Invitrogen). At least three Tle1BT and Tle2VC on these substrates was measured at an enzyme concentration of 300 nM (Tle1BT) or 30 nM (Tle2VC) at 28 °C. For Tle1BT-inhibition assays, immunoprecipitate was obtained as detailed under western blots from E. coli DH5x bearing pSCraB2:thi-1BT-V expression construct or the equivalent empty vector control, with the modification that proteins were eluted from anti-VSV-G agarose beads by the addition of VSV-G peptide at a concentration of 100 μg ml⁻¹ and no PMSF was used. After the addition of immunoprecipitate to Tle1BT enzymatic reactions, samples were incubated for 4 min, after which the first reading was normalized to the measurement immediately before treatment. Fluorescence assays for phospholipase D activity were performed by measuring the production of peroxide by choline oxidase through the generation of the fluorescent molecule resorufin from Amplex red reagent (Invitrogen) according to the manufacturer’s instructions with the following modifications: reactions were performed in a buffer consisting of 50 mM Tris-Cl, pH 7.2, 100 mM NaCl, 5 mM CaCl₂, and 2 mM MgCl₂, and vesicles consisting of equal amounts dioleoylphosphatidylcholine and dioleoylphosphatidylglycerol were used as a substrate at a final reaction concentration of 16.7 μM for each lipid species. Activity was measured at an enzyme concentration of 130 nM at 28 °C. In all assays fluorescent values were corrected for fluorescence as measured in a buffer-only control.

Competitive lipase assay. The lysis of P. aeruginosa reporter strains was determined by the relative partitioning of LacZ to the supernatant. Lysis reporter strains were generated by the chromosomal integration of a previously described miniCTX vector containing lacZ under the expression of a constitutive promoter41. Lysis reporter strains and unmatched donor strains were grown overnight on solid LB medium at 37 °C and then resuspended in water to a D₆₀₀ of 0.3. Donor and recipient strains were mixed 1:1 and spotted on 1.5% (w/v) agar SCFM plates supplemented with 1 mM IPTG and incubated at 23 °C for 23 h. Relative levels of supernatant LacZ activity as compared to total LacZ activity were then determined as previously described25. Data were analysed using a two-tailed Student’s t-test.

Microscopic analyses of interbacterial competitions. Time-lapse fluorescence microscopy sequences were acquired with a Nikon Ti-E inverted microscope fitted with a ×60 oil objective, automated focusing (Perfect Focus System, Nikon), a Xenon light source (Sutter Instruments), a CCD camera (Clara series, Andor), and a custom environmental chamber. NIS Elements (Nikon) was used for automated image acquisition. Overnight cultures of recipient (B. thailandensis ABTH_I2698-I2703 attTn7-gfp) and donor (either B. thailandensis wild-type, ABTH_I2698 ABTH_I2701-3, or ABTH_I2598) strains were mixed 1:1 and diluted twofold with LB medium. The resulting bacterial suspension (~2 μl) was spotted onto growth pads made with LB medium, 2.5% (w/v) agarose, 0.2% (w/v) sodium nitrate, and 2.5 μg ml⁻¹ propidium iodide. Automated image acquisition was performed at 5-min intervals for 6–8 h at 30 °C. Cell identification, cell linking, and donor contact analyses were performed using customized Matlab-based software (2012a, Mathworks) as described previously21. Donor (unlabelled) and recipient (GFP-labelled) populations were identified using an empirically determined green fluorescence gate. A propidium iodide uptake event was defined as the first frame in which a cell achieved an empirically determined mean red fluorescence intensity threshold. Counting error was calculated as the square root of measurable events. Results represent two fields of view from a single experiment; each experiment was independently repeated at least three times. Videos generated from cropped regions of the three growth competition experiments depicted are provided (Supplementary Videos 1–3).

Protein purification. For purification, Tle proteins were purified from pET28a:His6–MBP–TEV–His6, in Shuffle T7 plLysY Express cells (New England Biolabs). Proteins were purified to homogeneity using nickel chromatography followed by size-exclusion chromatography using previously reported methods, with the modification that reducing agents were excluded41. Lipidomic analyses. Wild-type and iliC mutant P. aeruginosa strains were grown as 20 individual 10-ml spots on 1.5% (w/v) agar SCFM plates for 23 h at 23 °C. These spots were transferred to PBE plates and grown for 3 days. Bligh–Dyer method42. Purified lipid samples were analysed for phosphatidylethanolamine, phosphatidylcholine, phosphatidylglycerol and phosphatidic acid content by the Kansas State University Lipidomics Research Center. An automated electrospray ionization-tandem mass spectrometry approach was used, and data acquisition and analysis were carried out as described previously43, with modifications. The lipid samples were dissolved in 1 ml chloroform. An aliquot of 50 μl of extract in chloroform was used. Precise amounts of internal standards, obtained and quantified as previously described44, were added in the following quantities (with some small variation in amounts in different batches of internal standards): 0.6 nmol didodecylphosphatidylcholine (di12:0-PC), 0.6 nmol di24:1-phosphatidylcholine (PC), 0.6 nmol 13:0-lipoPC, 0.6 nmol 19:0-lipoPC, 0.3 nmol di12:0-phosphatidyl ethanolamine (PE), 0.3 nmol di20:3-PE, 0.3 nmol 14:0-lipoPE, 0.3 nmol 18:0-lipoPE, 0.3 nmol di14:0-phosphatidylglycerol (PG), 0.3 nmol di20:4(phytanyloyl)-PG, 0.3 nmol di14:0-phosphatidic acid (PA), and 0.3 nmol di20:0(phytanyloyl)-PA. The sample and internal standard mixture was combined with solvents, such that the ratio of chloroform:methanol:300-mM ammonium acetate in water was 300:665:35, and the final volume was 1.4 ml. Unfractionated lipid extracts were introduced by continuous infusion into the ESI source on a triple quadrupole tandem mass spectrometer (MS/MS; 4000Q Trap, Applied Biosystems). Samples were introduced using an autosampler (LC Mini PAL, CTC Analytics AG) fitted with the required injection loop for the acquisition time and presented to the electrospray ionization (ESI) needle at 30 μl min⁻¹. Sequential precursor and neutral loss scans of the extracts produce a series of spectra with each spectrum revealing a set of lipid species containing a common headgroup. Lipid species were detected with the following scans: phosphatidylcholine and lipoPC, [M + H]⁺ ions in positive ion mode with precursor of 184.1 (Pre 184.1); phosphatidylethanolamine and lipoPE, [M + H]⁺ ions in positive ion mode with neutral loss of 141.0 (NL 141.0); phosphatidylglycerol, [M + NH₄]⁺ in positive ion mode with NL 189.0 for phosphatidylglycerol; and phosphatidic acid, [M + NH₄]⁺ in positive ion mode with NL 115.0. The collision gas pressure was set at 2 (arbitrary units). The collision energies, with nitrogen in the collision cell, were +28 V for phosphatidylethanolamine, +40 V for phosphatidylcholine, +25 V for phosphatidic acid, and +20 V for phosphatidylglycerol. Declustering potentials were +100 V for all lipids. Entrance potentials were +15 V for phosphatidylethanolamine and +14 V for phosphatidylcholine, phosphatidic acid and phosphatidylglycerol. Exit potentials were +11 V for phosphatidylethanolamine and +14 V for phosphatidylcholine, phosphatidic acid and phosphatidylglycerol. The scan speed was 50 or 100 μs⁻¹. The mass analyzers were adjusted to a resolution of 0.7 U full-width at half height. For each spectrum, 9–150 continuous scans were averaged in multiple channel analyser mode. The source temperature (heated nebulizer) was 100 °C, the interface heater was on, +5.5 kV were applied to the electrospray capillary, the curtain gas was set at 20 (arbitrary units), and the two ion source gases were set at 45 (arbitrary units). The background of each spectrum was subtracted, the data were smoothed, and peak areas integrated using a custom script and Applied Biosystems Analyst software, and the data were isotopically deconvoluted. The first set of mass spectra was acquired on the internal standard mixture only. Peak areas corresponding to the target lipids in these spectra were identified and molar amounts calculated in comparison to the two internal standards on the same lipid class. To correct for chemical or instrumental noise in the samples, the molar amount of each lipid metabolite detected in the ‘internal standards only’ spectra was subtracted from the molar amount of each metabolite calculated in each set of sample spectra. The data from each ‘internal standards only’ set of spectra was used to correct the data. Values expressed are the percentage of the total polar lipid signal detected. Statistical significance analysed by a two-tailed Student’s t-test.

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