Acinetobacter baylyi regulates type IV pilus synthesis by employing two extension motors and a motor protein inhibitor

Bacteria use extracellular appendages called type IV pili (T4P) for diverse behaviors including DNA uptake, surface sensing, virulence, protein secretion, and twitching motility. Dynamic extension and retraction of T4P is essential for their function, and T4P extension is thought to occur through the action of a single, highly conserved motor, PilB. Here, we develop Acinetobacter baylyi as a model to study T4P by employing a recently developed pilus labeling method. By contrast to previous studies of other bacterial species, we find that T4P synthesis in A. baylyi is dependent not only on PilB but also on an additional, phylogenetically distinct motor, TfpB. Furthermore, we identify a protein (CpiA) that inhibits T4P extension by specifically binding and inhibiting PilB but not TfpB. These results expand our understanding of T4P regulation and highlight how inhibitors might be exploited to disrupt T4P synthesis.
4P are thin, proteinaceous appendages that are broadly distributed throughout bacteria and archaea\(^3,2\). T4P are composed primarily of major pilin protein subunits that are polymerized or depolymerized through the activity of ATPases to mediate fiber extension and retraction, respectively\(^3\). T4P are subdivided into subcategories (generally T4aP, T4bP, and T4cP) based on protein homology and pilus function, with T4aP being the best characterized. In the T4aP systems found in Gram-negative organisms, polymerization and depolymerization of pilins occurs through interactions of the extension ATPase PilB or retraction ATPase PilT with the integral inner membrane platform protein PilC (Supplementary Fig. 1a). The growing fiber spans an alignment complex from the inner membrane through the periplasm composed of PilNOP to exit through the PilQ outer membrane secretin pore. Dynamic cycles of T4P extension and retraction are critical for the diverse processes that these structures mediate including twitching motility\(^3\), surface sensing\(^3,4\), virulence\(^5,6\), and DNA uptake\(^9,10\).

Acinetobacter species like A. baumannii and A. nosocomialis have emerged to become an urgent medical threat due to their prevalence in hospital-acquired infections and their capacity to acquire antibiotic resistance (AbR) genes; a process that is achieved in part by natural transformation through T4aP-mediated DNA uptake\(^11,12\). A. baumannii is the most naturally transformable species reported to date\(^13\), with up to 50% of cells undergoing natural transformation in laboratory conditions, making it an ideal candidate to study T4P-mediated DNA uptake and natural transformation.

Here, we develop A. baumannii as a new model for studying T4P dynamics. We show that A. baumannii uses two phylogenetically distinct motor proteins to drive T4P extension and a motor protein inhibitor to control T4P synthesis. These findings may be relevant for other T4P and highlight the value of developing new model systems for the study of these broadly conserved surface appendages.

**Results**

**Tn-seq reveals factors important for natural transformation and T4P synthesis.** To study A. baumannii T4P, we applied a recently developed labeling method\(^5,14\) by targeting the major pilin, ComP, for cysteine substitution and subsequent labeling with thiol-reactive maleimide dyes. Maleimide-labeling of the functional \(\text{com}^\text{PT129C}\) strain (Supplementary Fig. 1b, c and Supplementary Table 1) revealed external T4P filaments as seen in other species using this method\(^1,14\). T4P in A. baumannii are much shorter, often appearing as puncta rather than the 1-µm long filaments found in other species like V. cholerae\(^10\) or the 10-µm long filaments found in Legionella pneumophila\(^15\). A. baumannii TFP also localize close together in a line along the long axis of the cell, although the functional consequences of this localization pattern remain unclear (Supplementary Fig. 1c). Recent work has shown that T4P synthesis in A. baumannii is growth-phase dependent\(^16\) but we find here that the T4P of A. baumannii are constitutively made (Supplementary Fig. 2), in line with previous findings that A. baumannii cells are transformable throughout all growth phases\(^17\).

Incubation of cells with fluorescently labeled DNA resulted in co-localization of DNA with T4P, which is consistent with the essential role of T4P-DNA binding during natural transformation in Vibrio cholerae\(^10\) (Fig. 1a). We reasoned that natural transformation could be used to screen for other factors that regulate T4P synthesis in A. baumannii. To that end, we performed a high-throughput transposon-sequencing screen (Tn-seq)\(^18\) to identify genes required for natural transformation as done previously in other species\(^15,19,20\). In this screen, loss of known T4P-related genes resulted in negative selection, indicating that

![Image](https://example.com/image.png)

**Fig. 1 Tn-seq reveals factors important for natural transformation and T4P synthesis.** a Representative images of cells with AF488-mal-labeled pili incubated with fluorescently labeled DNA. b Representative images of pilT-intact strains from natural transformation assays shown in d labeled with AF488-mal. Zoomed-in images of representative single cells from each strain are outlined in dashed boxes and shown below. Scale bars, 2 µm. c Visual representation of Tn-seq screen showing the relative abundance of each gene in the “total pool” of transposon mutants compared to the “transformant pool” recovered after natural transformation. Known T4P structural/regulatory genes are outlined in red. tfpB is outlined in green, and cpiR is outlined in cyan. d Natural transformation assays of indicated strains. Each data point represents a biological replicate \((n = 4)\) and bar graphs indicate the mean ± SD. LD limit of detection. Source data are provided as a Source Data file.
they were critical for natural transformation as expected (Fig. 1c, Supplementary Data 1, and Supplementary Table 1). To validate our Tn-seq results, we made in-frame deletions of representative genes from each T4P-encoding operon, as well as genes known to be essential for natural transformation that act downstream of T4P. Mutations in the T4P platform protein gene pilC, the pilus regulatory gene pilY1, the outer membrane secretin gene pilQ, and the retraction ATPase gene pilT all showed a marked reduction in natural transformation, with most mutants exhibiting transformation rates below our limit of detection (Fig. 1d). T4P labeling of pilC, pilY1, and pilQ mutants revealed no visible T4P fibers (Fig. 1b). Mutations in genes that act downstream of T4P, including the periplasmic DNA-binding protein gene comEC, the inner membrane DNA-transporter protein gene comEA, and the DNA-recombination helicase gene comM likewise resulted in a reduction in transformation frequency; however, these strains still produced T4P fibers as expected (Fig. 1b, d).

**TfpB is a distinct PilB homolog required for efficient T4P extension.** We next sought to determine whether any of the previously uncharacterized genes from our Tn-seq screen reduced natural transformation in *A. baylyi* by affecting T4P synthesis. Tn-seq revealed that mutations in pilB, the canonical extension ATPase gene that is typically co-transcribed with pilC and the pre-pilin peptidase, pilZ, resulted in reduced natural transformation (Fig. 1c). We also found an additional pilB homolog that likewise exhibited lower rates of transformation (Fig. 1c). We named this new PilB homolog TfpB for “type four pilus PilB-like protein” because although it has homology to PilB, it does not exhibit the same gene synergy as pilB, which is typically co-transcribed with pilC, the gene encoding the cognate inner membrane platform protein (Supplementary Fig. 3). Surprisingly, deletion of the canonical pilB did not ablate transformation as would be expected based on homology to other T4P9 (Fig. 2a). Mutation of *tpfB* reduced transformation rates by two orders of magnitude, similar to the *pilB* mutant; however, natural transformation was undetectable in the *pilB tpfB* double mutant (Fig. 2a).

T4P labeling in *pilB*, *tpfB*, and *pilB tpfB* mutants revealed that none of these three mutants produced visible pili while *pilT* was intact (Fig. 2a). Because T4P are required for natural transformation, we hypothesized that *pilB* or *tpfB* mutants, which are still highly transformable, may either (1) make short pili that cannot be resolved by our fluorescence microscopy-based approach or (2) exhibit reduced rates of pili dynamic activity that result in a reduction in detectable surface pili in static images. To test the latter hypothesis, deletion of *pilT* prevents T4P retraction2 and can thus reveal more subtle effects on pili extension since all T4P made by a cell remain in an assembled state. In a *pilT* deletion background, *pilB* mutant populations had similar numbers of cells producing T4P compared to the Δ*pilT* parent while, surprisingly, *tpfB* mutants were highly defective in T4P synthesis (Fig. 2b, c). The *pilB* *tpfB* *pilT* triple mutant produced no detectable T4P fibers (Fig. 2b, c). These data suggest that PilB and TfpB may play distinct roles in T4P extension. Because *tpfB* *pilT* mutants make very few T4P, but *pilB* *pilT* mutants make similar levels of surface pili compared to the Δ*pilT* parent, we hypothesize that TfpB plays a larger role in initiating T4P extension, while PilB plays a larger role in driving processive T4P extension. In this model, each motor must still be able to partially compensate for the loss of the other to achieve some degree of T4P extension, which would account for the observed similarities in natural transformation frequencies when comparing *pilB* and *tpfB* single mutants. While T4P labeling in *pilT* mutant backgrounds supports this model (Fig. 2b, c), it does not rule out the possibility that *tpfB* *pilT* mutants produce many short T4P that cannot be resolved by our pilus labeling approach. To test this, we incubated extension motor mutants with fluorescently labeled DNA, reasoning that DNA binding would reveal short T4P synthesized in *tpfB* *pilT* mutants. Fluorescent DNA did not bind the *tpfB* *pilT* mutant above the background level observed in the *pilB* *tpfB* *pilT* mutant except where T4P were visible (Supplementary Fig. 4), suggesting that short pili are not produced in the *tpfB* *pilT* strain, which further supports the

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**Fig. 2 TfpB is a phylogenetically distinct PilB homolog that is required for efficient T4P extension in *A. baylyi*.** A Natural transformation assays of indicated strains. Each data point represents a biological replicate (n = 4) and bar graphs indicate the mean ± SD. LD limit of detection. ComP is the major pilin in *A. baylyi*, and the mutant is used here as a negative control. B Percent of pilated cells in *pilT* mutant populations of indicated strains. Each data point represents an independent, biological replicate (n = 3) and bar graphs indicate the mean ± SD. For each biological replicate, a minimum of 70 total cells were assessed. ND no pili detected. C Representative images of indicated strains labeled with AF488-mal with background fluorescence subtracted. Scale bars, 2 μm. D A rooted phylogeny of TfpB homologues found among Gammaproteobacteria. Branches are colored according to protein annotations in IMG, and nodes with bootstrap values greater than or equal to 70% are indicated by black circles. Black stars are at the tips of branches representing *A. baylyi* proteins with indicated protein names. Source data are provided as a Source Data file.
A. baylyi

PilB and TfpB are essential for optimal T4P production in (Supplementary Fig. 5). Together, these results indicate that both among Gammaproteobacteria1,22, which include members of the of transcriptional repressors that is critical for natural transformation (Figs. 1c and 3a). Deletion of this regulator, named cpiR for competence pilus inhibition repressor, resulted in a 100-fold reduction in transformation (Fig. 3b). We hypothesized that CpiR repressed a factor that inhibits natural transformation. Transcriptional repressors are often transcribed immediately adjacent to the genes they repress.23,24 We thus deleted the upstream gene, named cpiA for competence pilus inhibition actuator (Fig. 3a), and found that transformation frequency was restored in the cpiR cpiA double mutant background (Fig. 3b). Expression of cpiA under the control of an IPTG-inducible promoter (P_IPTG) was sufficient to reduce transformation frequency even in the presence of cpiR, further suggesting that CpiR represses cpiA transcription (Fig. 3b). Furthermore, we found that CpiR was sufficient to repress the cpiA promoter (using a P_flac-GFP reporter) in the heterologous host Vibrio cholerae, which suggests that CpiR is a direct repressor of P_cpiA (Fig. 3c). Finally, we found that CpiA protein is only produced in a cpiR mutant (Fig. 3d). These results demonstrate that under lab conditions, CpiR represses cpiA transcription to allow for high rates of natural transformation; however, in the absence of CpiR, CpiA is produced and natural transformation is inhibited ~100-fold.

CpiA lacks primary sequence or structural homology to any proteins or domains of known function.25,26 So we next sought to determine the mechanism of transformation inhibition by CpiA. Labeling of T4P in a cpiR mutant (i.e., when CpiA is expressed) revealed that cells were deficient in T4P synthesis (Fig. 4a). This defect was dependent on cpiA because pilation was restored in the cpiR cpiA double mutant (Fig. 4a). Deletion of pilT restored T4P synthesis in the cpiR mutant (i.e., the cpiR pilT double mutant) (Fig. 4a, b). This phenotype was reminiscent of the restoration of pilation observed in the pilB pilT double mutant, so we hypothesized that CpiA may regulate natural transformation by inhibiting PilB.

CpiA inhibits PilB activity. To test if CpiA functions by inhibiting PilB, we made pilB cpiR and tfpB cpiR double mutants. T4P synthesis was unaffected in pilB cpiR pilT (where PilB is the sole extension ATPase present), while no T4P were detected in tfpB cpiR pilT (where PilB is the sole extension ATPase present) (Fig. 4a, b). The defect in T4P synthesis in the tfpB cpiR pilT strain was restored when CpiA was deleted in this background.
demonstrating that CpiA acts as a PilB inhibitor to control natural transformation (Fig. 4a, b). Transformation frequency assays corroborated these results by demonstrating that deletion of cpiR only inhibited transformation in the tfpB mutant background (where PilB is the sole extension ATPase) and not the pilB mutant background (where TfpB is the sole extension ATPase) (Fig. 4c). Immunoblotting revealed that CpiA does not affect PilB production or stability (Fig. 3d), and we thus hypothesized that CpiA may interact with PilB to disrupt its function. Coimmunoprecipitation experiments with functional fusion proteins (Supplementary Figs. 7 and 8) revealed that CpiA and PilB interact with each other, and that their interaction is not dependent on the pilus machinery proteins PilMNOPQ (Fig. 4d and Supplementary Fig. 8), ruling out indirect binding of CpiA to PilB through other machinery components required for T4P synthesis. CpiA and TfpB do not interact (Supplementary Fig. 8), consistent with our genetic evidence that CpiA specifically inhibits PilB and not TfpB (Fig. 4a–c). Together, these data suggest that CpiA directly binds to and inhibits PilB.

**Discussion**

Environmental conditions play an integral role in regulating how bacteria respond to and interact with their surroundings. The mechanisms of T4P synthesis regulation identified here likely reflect how environmental conditions influence bacterial physiology. The acquisition of the additional extension motor TfpB and the PilB-specific inhibitory protein CpiA likely resulted from a need to modulate T4P extension under different environmental conditions. Bacteria generally limit T4P synthesis to environments where they provide a selective advantage. For example, in *V. cholerae*, the production of competence T4aP requires chitin and quorum sensing, which are environmental conditions that cells experience when they are likely to encounter other bacterial cells for horizontal gene transfer, while the toxin-coregulated T4bP required for intestinal colonization are produced in response to host-specific cues. In *A. baylyi*, both PilB and TfpB are required for optimal T4P extension and, consequently, efficient natural transformation. While CpiA is not expressed under laboratory conditions (due to CpiR repression), it is possible that...
different environmental conditions derepress cpiA to decrease pilus activity.

The discovery that two extension motors drive T4P synthesis in A. baylyi raises an interesting biological question. How might two extension motors engage with the machinery during extension? Previous work has shown that multiple retraction motors can work together to drive forceful T4P retraction\textsuperscript{21,30}, and while the nature of how these motors are able to simultaneously interact with the machinery remains unknown, a similar mechanism may exist for PilB and TfpB. Alternatively, each motor may play temporally distinct roles during extension that allow each motor complex to interact with the machinery separately. Our data show that tfpB mutants make substantially fewer pili than pilB mutants in the absence of retraction, suggesting that TfpB may play a role in initiating T4P extension while PilB may promote processive pilus extension. In this model, each motor possesses a distinct function that could be accomplished by temporally segregated machinery interactions. Interestingly, our phylogenetic analysis demonstrated that 15 of the 16 species that contained a TfpB motor homolog also encoded a PilB motor homolog (Supplementary Table 2). The exception to this observation, Pseudomonas fluorescens A506, possesses T4P machinery components, but lacks a pilB homolog and instead only harbors a tfpB homolog (Supplementary Fig. 3). This could suggest that TfpB is capable of acting as the sole extension motor in this species, though data are needed to test this hypothesis.

Secretion ATPases like PilB belong to a broadly distributed class of proteins that play essential roles in diverse microbial behaviors, yet there are few known inhibitors of these proteins. The ones that are characterized are encoded by phages that use T4P to infect cells, and it is speculated that PilB inhibition by these proteins prevents superinfection by other T4P-dependent phages\textsuperscript{31,32}. CpiA is poorly modeled by structural prediction algorithms like Phyre2\textsuperscript{25}. However, it is predicted that CpiA has a secondary structure rich in alpha helices, similar to a recently described phage-encoded PilB inhibitor, Aqs1\textsuperscript{32}. Although Aqs1 and CpiA are dissimilar at the sequence level, it is possible that they share structural similarities that enable PilB binding and inhibition. In Acinetobacter baylyi, cpiA is encoded on the chromosome, and it does not appear to be found in other sequenced species of Acinetobacter. Thus, it is tempting to speculate that CpiA was acquired after phage infection of an ancestral strain and co-opted for regulation of T4P activity. While expression of phage-encoded PilB-inhibitors like Aqs1 is not repressed in the same manner as CpiA, it could be that the CpiR repressor was acquired after CpiA to regulate its effects on T4P extension.

The molecular mechanism of CpiA-dependent inhibition of PilB remains unclear. We demonstrate that CpiA binds to PilB. Thus, it is possible that (1) CpiA sequesters PilB to prevent it from interacting with machinery components, (2) that CpiA binds PilB to inhibit its ATPase activity, or (3) that CpiA modifies PilB to disrupt its function. Testing these hypotheses will be the focus of future work. A better understanding of secretion ATPase function and the evolution of mechanisms by which they can be inhibited may enable the development of tools to control their activity. Precise control of T4P synthesis may provide a means to manipulate behaviors that require T4P, like biofilm formation, virulence, and natural transformation, which are clinically relevant in diverse pathogens.

Methods

Bacterial strains and culture conditions. A. baylyi strain ADP1 was used throughout this study. For a list of strains used throughout, see Supplementary Table 3. A. baylyi cultures were grown at 30 °C in Miller lysogeny broth (LB) medium and on agar supplemented with kanamycin (50 µg/mL), spectinomycin (60 µg/mL), gentamicin (30 µg/mL), and/or chloramphenicol (30 µg/mL), rifampicin (30 µg/mL), azlocillin (100 µg/mL), apramycin (30 µg/mL), and/or streptomycin (10 µg/mL) as appropriate.

Construction of mutant strains. Mutants in A. baylyi were made using natural transformation as described previously\textsuperscript{21}. Briefly, mutant constructs were made by splicing-overlap (SOE) PCR to stich (1) ~3 kb of the homologous region upstream of the gene of interest, (2) the mutation where appropriate (for deletion by allelic replacement with an AbR cassette, or the fusion protein), and (3) ~3 kb of the homologous downstream region. For a list of primers used to generate mutants please see the Supplementary Table 4. The upstream DNA was amplified using F1 + R1 primers, and the downstream region was amplified using F2 + R2 primers. All AbR cassettes were amplified with AB123 (ATTCCGGGAGATCCGTGAC) and AB124 (TGTAGGGTGGATGTGGTC). Fusion proteins were amplified using the primers indicated in Supplementary Table 4. In-frame deletions were achieved by first amplifying the upstream region using F1 and R1 primers, and R2 + F2 primers to amplify the downstream region with ~20 bp homology to the remaining region of the downstream region built into the R1 primer and ~20 bp homology to the upstream region built into the F2 primer. SOE PCR reactions were performed using a mixture of the upstream and downstream regions, and middle region where appropriate using F1 + R2 primers. SOE PCR products were added with 50 µL of overnight-grown culture to 450 µL of LB in 2-mL round-bottom microcentrifuge tubes (USA Scientific) and grown at 30 °C rotating on a roller drum for 1–3 h. For AbR-constructs, transformants were serially diluted and plated on LB and LB + antibiotic. For in-frame deletions and protein fusion constructs, overnight cultures were incubated, cells were incubated with 10 µL of DNase I (New England Biolabs) for 5 min at room temperature before 450 µL of LB was added. Transformations were grown for an additional hour before cells were diluted and 100 µL of 10^-6 dilution was plated on LB plates. In-frame deletions were confirmed by PCR using primers ~150 bp up- and downstream of the introduced mutation, and fusion constructs were confirmed by sequencing. Complementation strains were constructed by placing the gene of interest under a constitutive P~tac~ promoter at the vanAB locus, which has previously been established as a site for chromosomal expression\textsuperscript{37}. First, the vanAB genes were replaced with a kanR cassette as described above. Then, a P~tac~ promoter was introduced using primers CE317 + CE600 to amplify the upstream region, CE261 + CE336 to amplify the P~tac~ promoter, CE1205 + CE1206 to amplify pilB or CE1207 + CE1208 to amplify tfpB, and CE406 + CE176 to amplify the downstream region. SOE PCRs and natural transformation were then performed exactly as described above.

Mutants in V. cholerae were made by chitin-induced natural transformation exactly as previously described\textsuperscript{35}. SOE products were generated exactly as described above. The chromosomally integrated P~tac~ ectopic expression construct is described in detail in ref. 38.

Natural transformation assays. Assays were performed exactly as previously described\textsuperscript{37}. Briefly, strains were grown overnight in LB broth at 30 °C rolling. Then, ~10^6 cells were subcultured into fresh LB medium and 100 ng of tDNA was added. In this study, a ΔAC1AD1511::Spec\textsuperscript{38} or a ΔAC1AD1511::Apyramycin\textsuperscript{35} PCR product was used for transformation. Transformants were grown for an additional hour before cells were diluted and 100 µL of 10^-6 dilution was plated on LB plates. In-frame deletions were confirmed by PCR using primers ~150 bp up- and downstream of the introduced mutation, and fusion constructs were confirmed by sequencing. Complementation strains were constructed by placing the gene of interest under a constitutive promoter at the vanAB locus, which has previously been established as a site for chromosomal expression\textsuperscript{37}. First, the vanAB genes were replaced with a kanR cassette as described above. Then, a P~tac~ promoter was introduced using primers CE317 + CE600 to amplify the upstream region, CE261 + CE336 to amplify the P~tac~ promoter, CE1205 + CE1206 to amplify pilB or CE1207 + CE1208 to amplify tfpB, and CE406 + CE176 to amplify the downstream region. SOE PCRs and natural transformation were then performed exactly as described above.

ΔAC1AD1511::Spec\textsuperscript{38} or ΔAC1AD1511::Apyramycin\textsuperscript{35} PCR product, an Rpsl. K43R (Sm\textsuperscript{45}) PCR product, or an RpoB PCR product from a spontaneous rifampicin-resistant mutant. Following the 5-h incubation with tDNA, a small portion of each transformation reaction was diluted and plated for quantitative culture on selective media (i.e., containing the appropriate antibiotic to select for transformants) to assess the number of transformants in each reaction. The remainder of the reactions were split and outgrown in selective medium overnight (i.e., with the appropriate antibiotic added to select for transformants = "transformant pool") or grown overnight in nonselective medium (i.e., in plain LB medium = "total pool"). There were ~10^7 total transformants obtained for each transformation reaction.

Tn-seq analysis. Transposon mutant libraries of A. baylyi were generated by electroporation of cells with pDL1093, a vector that allows for Kan\textsuperscript{44} mini-Tn10 mutagenesis\textsuperscript{38,39}, and plating on kanamycin plates. Approximately 100,000 Tn mutant colonies were scraped off of plates and pooled to generate the input transposon mutant library. Subsequent sequencing (as described below) revealed that this input library contained 62,799 unique Tn insertions. This mutant library was then subected to natural transformation in the absence of selective pressure directly as described above using three different sources of tDNA (1000 ng of each): a ΔAC1AD1511::Spec\textsuperscript{38} PCR product, an Rpsl. K43R (Sm\textsuperscript{45}) PCR product, or an RpoB PCR product from a spontaneous rifampicin-resistant mutant. Following the 5-h incubation with tDNA, a small portion of each transformation reaction was diluted and plated for quantitative culture on selective media (i.e., containing the appropriate antibiotic to select for transformants) to assess the number of transformants in each reaction. The remainder of the reactions were split and outgrown in selective medium overnight (i.e., with the appropriate antibiotic added to select for transformants = "transformant pool") or grown overnight in nonselective medium (i.e., in plain LB medium = "total pool"). There were ~10^7 total transformants obtained for each transformation reaction.

Sequencing libraries of the transposon-genomic junctions were generated for the illumina platform using HTM-PCR exactly as previously described\textsuperscript{38,40}. All sequencing data analysis was performed on the Galaxy platform\textsuperscript{45} essentially as previously described\textsuperscript{35}. Reads were mapped to the A. baylyi ADP1 genome\textsuperscript{42} using Bowtie with its default settings. Then, mapped reads were
further analyzed using Tufts TUCF genomics custom scripts that determine (1) the total number of unique reads that map to each gene (i.e., the number of unique Tn insertions in each gene), and (2) the total number of reads that map to each gene (i.e., the relative abundance of Tn mutants in each gene in the overall cell population for a given sample). The total number of reads that map to each gene (i.e., the relative abundance of Tn insertions within a gene within the sample) is then normalized based on the relative size of each gene and the total number of mapped reads for the sample (number of reads that mapped to gene X / the total number of mapped reads for the sample)/(size of gene X/size of genome). Thus, the expected normalized abundance for a “neutral” gene based on this standardization (i.e., a gene whose inactivation is not selected for or against) is 1.0.

Contact analysis for Tn-seq were performed treating the “transformant pool” as the output and the “total pool” as the input to determine genes that were over- and under-represented following selection for transformants. Gene fitness was only assessed if a gene contained at least 1 transposon insertion in at least two out of the three replicates of the “total pool” samples. Also, the normalized abundance of insertions within a gene had to be greater than 0.01 in the “total pool” when averaged across all replicates. These cutoffs allowed us to assess the phenotype of ~83% of the genes (2747/3310) in the A. baylyi ADP1 genome. For visualization, the relative abundance of Tn insertions within each gene is plotted from the “transformant pool” relative to the “total pool.” See Supplementary Data 1 for the relative abundance of Tn insertions in each gene for all of the Tn-seq datasets analyzed for this manuscript. Also, the raw sequencing data for these Tn-seq experiments has been uploaded to the Sequence Read Archive (www.ncbi.nlm.nih.gov/sra); PRJNA716813.

Pilin labeling, imaging, and quantification. Pilin labeling was performed as described previously with some changes14. Briefly, 100 µL of overnight-grown cultures was added to 900 µL of LB in 1.5 mL microcentrifuge tube, and cells were grown to mid-log, then 20 µL of 1% CaCl2 rotating at a roller drum for 30 min. Cells were then centrifuged at 18,000 x g for 1 min, and then resuspended in 50 µL of LB before labeling with 25 µg/mL of AlexaFluor 488 C5-maleimide (AF488-mal) (ThermoFisher) for 15 min at room temperature. Labeled cells were centrifuged, washed once with 100 µL of LB without disrupting the pellet, and resuspended in 5–20 µL LB. Cell bodies were imaged using phase-contrast microscopy while labeled pilis were imaged using fluorescence microscopy using a Nikon TiE microscope using a Plan Apo 100X objective, a GFP filter cube for pilis with a Hamamatsu ORCAFlash4.0 camera, and Nikon NIS Elements Imaging Software. Cell numbers and the percent of cells making pili were quantified manually using ImageJ15. All imaging was performed under 1% UltraPure agarose (Invitrogen) pads made with phosphate-buffered saline (PBS) solution. To image pilis at different growth phases, 50 µL of overnight-grown cultures was only assessed if a gene contained at least 1 transposon insertion in at least two out of the three replicates of the “total pool” samples. Also, the normalized abundance of insertions within a gene had to be greater than 0.01 in the “total pool” when averaged across all replicates. These cutoffs allowed us to assess the phenotype of ~83% of the genes (2747/3310) in the A. baylyi ADP1 genome. For visualization, the relative abundance of Tn insertions within each gene is plotted from the “transformant pool” relative to the “total pool.” See Supplementary Data 1 for the relative abundance of Tn insertions in each gene for all of the Tn-seq datasets analyzed for this manuscript. Also, the raw sequencing data for these Tn-seq experiments has been uploaded to the Sequence Read Archive (www.ncbi.nlm.nih.gov/sra); PRJNA716813.

Western blotting. Approximately 106 cells from overnight cultures were concentrated into a pellet by centrifugation, and the culture supernatant was removed. Cell pellets were resuspended in 50 µL PBS and then mixed with an equal volume of 2x SDS-PAGE sample buffer (125 mM Tris, pH 6.8, 20% glycerol, 4% SDS, 0.4% bromophenol blue, and 10% β-mercaptoethanol) and boiled using a heat block set to 100 °C for 15 min. Proteins were separated on a 4–20% pre-cast polyacrylamide gel (Biorad) by SDS electrophoresis, electrophoretically transferred to a nitrocellulose membrane, and probed with 1:5000 dilution of mouse monoclonal α-FLAG antibodies (Sigma), 1:2500 dilution of mouse monoclonal α-GFP antibody (Roche), and/or 1:12,000 dilution of mouse monoclonal α-RpoA (BioLegend) primary antibodies. Blots were then incubated with 1:10,000 dilution of α-mouse IRDye secondary antibodies (Licor) and imaged using a Licor imaging system.

Commmunoprecipitation (pulldown) experiments. Overnight cultures of cells grown in tubes in LB medium were diluted by 1/10 into fresh LB for a total volume of 30 or 50 mL in 125- or 250-mL volume flasks, respectively. Then, 30–50 mL cultures were grown to exponential growth phase by shaking for 1.5 h at 30 °C. The total culture volume was then harvested at 10,000 x g for 10 min at room temperature, and the supernatant was removed. Cell pellets were resuspended in 2 mL of Buffer 1 (50 mM Tris–Cl pH 7.4, 150 mM NaCl, 1 mM EDTA) and transferred to 2 mL volume microcentrifuge tubes and centrifuged at 18,000 x g for 1 min. Cells were washed once more with 2 mL of Buffer 1, and washed pellets were resuspended in 1 mL of Buffer 2 (50 mM Tris–Cl pH 7.4, 150 mM NaCl, 1 mM EDTA, 10 mM MgCl2, 0.1% Triton X-100, 2% glycerol). To lyse cells, 400 units of Ready-Lyse lysosome (Sigma), 30 units of Dnase I (New England Biolabs), and 10 µL of concentrated protease inhibitor cocktail (Sigma) (one pellet dissolved in 500 µL of Buffer 1) were added to cell suspensions and incubated at room temperature for 45 min. Cell debris was removed by centrifugation at 10,000 x g for 5 min at 4 °C. Then, the cell lysate was used as an “input” sample; 50 µL aliquots of α-FLAG magnetic bead slurry (Sigma) or α-GFP magnetic bead slurry (MBL biotech) in 1.5 mL microcentrifuge tubes were washed three times with 1 mL of Buffer 2 using a magnetic collection stand; and 1 mL of cell lysates was added to washed magnetic beads and subjected to end-over-end rotation at 4 °C for 2 h. Western blotting

Measuring GFP fluorescence in reporter strains. V. cholerae reporter strains harboring Ppil::GFP were grown to late log in LB medium with or without 0.2% arabinose as indicated. Cells were then washed once in instant ocean medium (7 g/L; Aquarium Systems) and transferred to a 96-well plate. Fluorescence was then determined on a Biotech H11M plate reader with monochromator set to 500 nm for excitation and 540 nm for emission.

Fluorescent DNA binding/uptake. A ~7 kb PCR product was fluorescently labeled as described previously16 using the Cy3 LabelIt kit (Mirus Biosciences) as per the manufacturer’s recommendations. For the parent strain, 1 µL (100 ng) of Cy3-DNA was added to 900 µL of LB in a 1.5 mL microcentrifuge tube along with 100 µL of overnight-grown cultures, and cells were grown at 30 °C rotating on a roller drum for 70 min. Pili were then labeled as described above. To visualize DNA binding in pilT mutants, cells were grown as described above, but 100 ng (300 ng of Cy3-DNA) was added to cells along with AF488-mal and incubated for 15–25 min before washing. Cells were imaged using the same microscopy setup described above, using a dSRed filter cube to image Cy3-DNA.

Phylogenetic analysis. TfpB homologues were identified by BLAST using 43 manually selected Gamma proteobacteria genomes17 through Integrated Microbial Genomes and Microbiomes online resources ( IMG) resulting in 239 protein sequences. The TfpB protein from A. baylyi ADP1 was added manually and used as an outgroup. Through the NGPhylogeny server46, sequences were aligned using the default parameters of MAFFT47, and phylogenetic tree construction was performed on the aligned sequences using the default parameters of FastTree software48 to set to perform 100 bootstraps48. The resulting tree was visualized using the Interactive Tree of Life visualization software41, from which trees were exported for publication.

Statistics and reproducibility. All experiments were repeated a minimum of two times, and all attempts at replication were successful. Data in Figs. 1a, 3d, and 4d, and Supplementary Figs. 4 and 8, are representative of two independent experiments. Data in Figs. 1b, 2c, and 4a are representative of three independent experiments. Data in Supplementary Fig. 1c are representative of dozens of independent experiments.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Tn-seq data are available on Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) under accession code PRJNA716813. Source Data are provided with this paper.

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