Supplemental Material

Supplemental Materials and Methods

Bacterial strains, plasmids, and growth conditions. For routine cell cultures, *P. aeruginosa* and *E. coli* strains were grown in lysogeny broth (LB) medium or LB solidified with 1.5% agar. Gentamicin (Gm) was used at 30 μg/ml for *P. aeruginosa* and at 10 μg/ml for *E. coli* selections. Carbenicillin (Cb) was used at 50 μg/ml, kanamycin (Kan) at 50 μg/ml, and nalidixic acid (Nal) at 20 μg/ml for *E. coli*. X-gal was used at 40 μg/ml. For the expression plasmid (pMQ80) harboring the P_{BAD} promoter, arabinose was added to cultures at a 0.2% final concentration. Both M8 and M63 minimal salt media were supplemented with 1mM $MgSO_4$, 0.2% glucose and 0.5% casamino acids (CAA), unless noted otherwise.

Saccharomyces cerevisiae strain InvSc1 (Invitrogen), used for plasmid construction via *in vivo* homologous recombination, was grown with yeast extract-peptone-dextrose (1% Bacto yeast extract, 2% Bacto peptone, and 2% dextrose). Selections with *S. cerevisiae* InvSc1 were performed using synthetic defined agar-uracil (catalog no. 4813-065; Qbiogene).

The strains, plasmids and oligonucleotide sequences used in this study are listed in Table S2. All gene deletion mutants are in-frame deletions, and constructed via allelic exchange as previously described (1). Briefly, DNA fragments upstream and downstream of the gene of interest were PCR amplified from *P. aeruginosa* PA14 genomic DNA using primers KO-1 with KO-2, and KO-3 with KO-4, respectively. These 1-kb

fragments were cloned into pMQ30 via homologous recombination in yeast *S. cerevisiae* InvSci. The resulting pMQ30-gene KO plasmids were used to transform *E. coli* S17 and introduced into *P. aeruginosa* strains via conjugation. Integrants were selected with Gm and Nal, followed by sucrose (10%) counter-selection. The resolved integrants were confirmed by PCR using primers KO-check-1 and KO-check-2.

The P_{pilY1}-*lacZ* transcriptional reporter strains were constructed using vector pUC18-mini-Tn7T-Gm-*lacZ* as previously described (2). Briefly, the *pilY1* promoter region, including a 214 bp nucleotide sequence upstream of the *fimU* start codon, was PCR amplified from *P. aeruginosa* PA14 genomic DNA using primers P_{pilY1}-F and P_{pilY1}-R (Table S2), and cloned into vector pUC18-mini-Tn7T-Gm-*lacZ*. The resulting plasmid integrated into the *attTn7* site in strain *P. aeruginosa* PA14 chromosome by co-transformation with a helper plasmid pTns2 harboring a transposase.

The *pilY1* expressing strains were constructed by transforming pMQ80-*pilY1* vector (pPilY1), or the empty vector pMQ80, into *P. aeruginosa* strains via electroporation. The transformants were selected with Gm.

The cAMP reporter strains were constructed by transforming pR-*lacZ* (empty vector control) or pR-P_1-*lacZ* (referred as P_1-*lacZ*) into *P. aeruginosa* strains via electroporation. The transformants were selected with Gm.

The P_{pilY1}-*lacZ* and P_{fimS}-*lacZ* constructs used in the PAK strains were constructed by cloning promoter-containing DNA fragments upstream of the *lacZ* gene in plasmid mini-CTX-*lacZ* (3). The promoter fragment for *pilY1* (523 bp) was amplified with oligonucleotides 5'PpilY1rep (5'-AGAGGGCTGGCTGCTTGCGGAAGAC-3') and 3'PpilY1rep (5'-CGCATTGAGTTCCTCGGCGGCGCTCTG-3'). The promoter region

of *fimS* (441 bp) was amplified with primers 5'PfimSrep (5'-GCGGTAGAGACGCTTGTCGAAGTC-3') and 3'PfimSrep (5'-AGCAGCACCAGGGTGAACAGGGC-3'). The 5' and 3' oligonucleotides were tailed with EcoRI and BamHI restriction sites, respectively. After amplification from strain PAK genomic DNA, the promoter fragments were digested with EcoRI and BamHI, and ligated into the corresponding restriction sites of mini-CTX-*lacZ*. The resulting plasmids were used to move the promoter-*lacZ* fusions onto the chromosome of the wild type and mutant PAK strains as described (3). All PAK deletion alleles and complementation vectors used in this study were previously described (4, 5).

Microscopy and single cell tracking. Microscopy and bacterial cell tracking analysis using flow cells were performed as previously described, with modification (6, 7). Briefly, cells from overnight culture in regular M63 minimal salt medium were subcultured in the same medium and grown to an OD_{600} of 0.4. The cell culture was then diluted 1:100 and injected into a flow cell. The medium used for flow cell was M63 minimal salt medium supplemented with 1mM $MgSO_4$, 0.05% glucose and 0.125% CAA. The medium flow rate was 3 ml/h. Movies containing 24,000-48,000 images were collected at 20 frames per minute for 20-40 hrs using the Olympus IX81 microscope with an Andor iXon EMCCD camera. The image size is 67 μm × 67 μm (1,024 × 1,024 pixels). The aspect ratio of each cell was calculated as projected length/projected width. The probability density is obtained from the histogram of aspect ratios, that is, at each aspect ratio, the probability density is calculated as the proportion of cells appearing in the bin centered at that particular aspect ratio, divided by the bin size (set to 0.1).

Transposon mutagenesis. *Mariner* transposon mutagenesis was used to screen gene mutations that affect P_{pilY1}-*lacZ* expression. The donor strain *E. coli* S17-1 λ *pir* carrying the pBT20 plasmid (*mariner* transposon) and the recipient strain *P. aeruginosa* harboring the P_{pilY1}-*lacZ* reporter (SMC 6610, unmarked) were conjugated as previously described (8). Mutant strains on the library plate were screened for either intensified or weakened blue color compared to the control strain (SMC6610) on LB plates containing X-gal. The candidate colonies were patched on fresh LB X-gal plates twice to confirm the color phenotypes. LacZ activities in the candidate mutants were further quantified using the β-galactosidase assay. Mutant strains showing at least 1.5 fold changes in LacZ activity compared to the control strain were further analyzed and their transposon insertion sites were mapped using arbitrary PCR as previously described (9, 10). PCR products were sequenced at the Molecular Biology and Proteomics Core at Dartmouth College. The resulting DNA sequences were aligned to the PA14 genomic sequence using the NCBI BLAST program. The transposon mutagenesis assay was performed ten times and was completed when no additional mutations can be identified in this approach. ~60,000 transposon mutants were screened, over 300 mutants were tested using β-galactosidase assays and 32 candidate genes were identified. This screen approached but did not reach saturation.

β-galactosidase activity assay. The β-galactosidase activity assay of strains harboring *lacZ* fusions were performed as previously described (11), except that the reactions were

performed at room temperature and the absorbance was measured in 96-well flat bottom plates using SpectraMax M2 microplate reader (Molecular Devices).

For the time lapse β-galactosidase assay with the P_1-*lacZ* fusion, overnight LB-grown cultures were diluted 1:100 in regular M8 broth, and grown to OD_{600} of 0.4 (mid-log phase) at 37 °C with vigorous shaking. Bacterial cultures were split in two parts, one continued to grow in M8 broth in glass test tubes with shaking, the other was spread (200 µl per plate) on M8 agar (1% agar), and incubated at 37 °C. Cells were harvested from liquid broth or scraped from agar surface every hour, and β-galactosidase activity assays were performed as described above.

PilY1 cellular localization, SDS-PAGE and Western blotting. Strains of interest were streaked in a grid-like pattern on M8 agar plate (1% agar), and incubated for 16 h at 37 °C. Cells were harvested by gently scraping with a plastic cover slip into microcentrifuge tubes. Cells were gently washed twice by resuspending in 5 ml of 1x PBS (pH 7.4), and centrifuged at 4,500 x *g* for 5 min. The washed cell pellets were resuspended in 1 ml 1x PBS, and adjusted to an OD_{600} of 50. Cell suspension was vigorously vortexed for 30 s to release surface-associated proteins, and 10 µl of cell suspension were saved for whole cell lysate prep below. Cells in the rest of the suspension (1 ml) were pelleted by centrifugation at 16,000 x *g* for 10 min at room temperature. The supernatants were transferred to fresh microcentrifuge tubes and recentrifuged at 16,000 x *g* for 20 min at room temperature to remove the remaining cells. The resulting supernatant was designated as the cell surface (CS) protein fraction, which was either used directly or further concentrated with trichloroacetic acid (TCA) precipitation. TCA was added to the

cell surface fraction to a final concentration of 20%. Proteins were allowed to precipitate on ice overnight and collected by centrifugation at 16,000 x *g* for 20 min at 4 °C. The protein pellets were washed with 1 ml ice-cold acetone three times, and solubilized in 50 µl 1x SDS buffer containing 100mM dithiothreitol (DTT).

The whole cell lysate was prepared as follows. The 10 µl of cell suspension saved from the procedure above was mixed with 60 µl of H2O, 10 µl of Popculture reagent (EMD Chemicals Millipore), 10 µl of 50 mg/ml lysozyme, 1 µl of DNase I (25U,NEB), and 10 µl of 10 x DNase I buffer, and incubated at room temperature for 20 min. The resulting lysate is designated as whole cell lysate, and was mixed with 100 µl of 2X SDS loading buffer containing 200mM DTT prior to SDS-PAGE.

All protein samples in SDS loading buffers were boiled for 10 min, and resolved by SDS-PAGE using 7.5% polyacrylamide gels (Bio-rad). Proteins were transferred to a nitrocellulose membrane using Trans-Blot Turbo Transfer system (Bio-rad) and probed with PilY1 antiserum to detect PilY1 protein (12), with FliC antiserum to detect flagellin, or with Cas3 antiserum to detect the cytoplasmic protein Cas3 (13). Western blots were developed with the Western Lightning ECL detection kit (Perkin-Elmer, Boston, MA) according to the manufacturer's instructions.

Quantification of cellular c-di-GMP. Quantification of the cellular pool of c-di-GMP was performed as previously described with modification (14, 15). Briefly, overnight LB-grown cells (2.5 µl) were spotted onto the surface of swarm agar plates and incubated for 16 h at 37 °C. Swarm cells were scraped from the agar surface and resuspended in 1ml 1x M8 salt buffer. The cell suspensions were filtered through a 40 µm cell strainer to remove

agar bits, and centrifuged for 1 min at 16, 000 x *g*. Supernatants were removed and cell pellets were resuspended in nucleotide extraction buffer. Subsequent extraction nucleotide was performed as previously described (15), and the quantitative analysis of c-di-GMP by liquid chromatography-mass spectrometry (LC-MS) was performed at the Mass Spectrometry Facility at Michigan State University.

Bacterial adenylate cyclase two-hybrid (BATCH) assay. Protein-protein interactions were identified by the BACTH assay (Euromedex) as previously described (16). The full-length genes (*pilJ*, *fimS*, *pilX*, *pilW*, *pilY1* or *sadC*) were cloned into pKNT25 and pUT18 plasmids, generating C-terminal protein fusions with T25 and T18 fragments, respectively. These protein fusions were chosen in order to preserve the N-terminal signal peptides and to allow proper localization in the membrane. The recombinant pKNT25 and pUT18 vectors were co-transformed into *E. coli* BTH101 cells. The transformants were serial diluted 10-fold, spotted (2.5 µl) on LB agar plates supplemented with Cb, Kan and X-gal, and incubated for 40 h at 30 °C. The protein interaction efficiencies were quantified by β-galactosidase activities with cells grown in LB liquid cultures supplemented with Cb and Kan for 14 h at 30 °C. We systematically tested proteins for pairwise interactions, that is, each protein was fused to either the T25 or the T18 fragment, and tested versus every other protein for interaction. The tested pairs included PilJ-FimS, PilJ-PilX, PilJ-PilX, PilJ-PilW, PilJ-PilY1, FimS-PilX, FimS-PilW, FimS-PilY1, PilX-PilW, PilX-PilY1, PilW-PilY1, PilX-SadC and PilY1-SadC. The BACTH positive controls were performed with T25-zip and T18-zip plasmids, containing DNA sequences coding for leucine zipper motifs. Negative controls were performed with the

two empty T18 and T25 plasmids, as well as each protein of interest against the empty T18 or T25 plasmids.

Growth curves. Fresh single colonies were first grown in LB broth to OD_{600} of 0.4, diluted 1:100 in M8 minimal salt medium, and inoculated in sterile 96-well flat-bottomed polystyrene microtiter plates (Corning, USA) with a total inoculum of 100 µl. The plates were incubated at 37°C with shaking. Cell growth was measured spectrophotometrically (OD_{600}) using a SpectraMax M2 microplate reader (Molecular Devices). Each test was performed with biological triplicates and repeated twice.

Swarming and biofilm assays. Swarming was performed as previously described (17). All swarming assays were performed with regular M8 medium supplemented with 0.5% agar. Biofilm formation in 96-well microtiter plates was assayed and quantified as previously described (18, 19). All biofilm assays were performed using regular M63 minimal salt medium.

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