Supplementary Information for

Endogenous membrane stress induces T6SS activity in *Pseudomonas aeruginosa*

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Other supplementary materials for this manuscript include the following:
- Dataset S1
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Supplementary methods

Strain construction

dcas9 from S. pyogenes (sequence from 44249, Addgene) was codon optimized for P. aeruginosa and ordered as a gblock from Integrated DNA technologies. The gblock was inserted into XmaI and EcoRI linearized pSW196 using Gibson assembly (1). Subsequently, pSW196 containing dcas9 was integrated into the attB site and the vector backbone was excised using pFLP (2). To generate a smaller vector for the guide RNA vector, lacI was deleted from pPSV37. For this, an in-frame deletion of lacI was performed using a splice overlap extension (SOE) approach with the primers oAS022-oAS025. The promoter and sg-RNA sequence (sequence from 44251, Addgene) was amplified using oAS034 and oAS035 and inserted into a DraIII and HindIII linearized ppSV37 ΔlacI using Gibson assembly. New sgRNAs were designed as previously described (3). Briefly, an inverse PCR using a forward primer containing the targeting sequence and overlap with the vector backbone and oAS058 was performed, gel purified, Gibson assembled, dialyzed and electroporated into DH5α. Sanger sequence confirmed guides were then electroporated into the according PAO1 attB::dcas9 and selected using 30 µg/mL gentamicin. Mutations in the PAM site used for knockdown of bamA were mutated using an SOE approach. In frame mutation of the PAM site were generated with the primers oAS384, oAS385, oAS413 and oAS414 and Gibson assembled into EcoRI and HindIII linearized pEXG2 vector and transformed into DH5α. Sanger sequencing confirmed plasmids were transformed into SM10λ.pir and conjugated into the according PAO1 attB::dcas9 strain. Allelic exchange was achieved by homologous recombination and subsequent sucrose selection. PAM mutation was confirmed with the primers oAS388-oAS396. To generate in frame deletions of tagT, vipA and fusion of clpV-GFP, SM10λ.pir strains with the according pEXG2 vector were conjugated into the according PAO1 background and sucrose selected (4). Correct clones were confirmed using Sanger sequencing. Primers used in this study are listed in Table S2.
Bacterial competition assays

*P. aeruginosa* and *V. cholerae* overnight cultures were diluted to an OD$_{600}$ of 0.05 in appropriate antibiotics. *P. aeruginosa* cultures were complemented with 0.1 % arabinose. Bacteria were grown to an OD$_{600}$ ranging from 0.7 to 0.85. Harvested bacteria were resuspended to an OD$_{600}$ of 10 in LB, 0.2 % arabinose. *V. cholerae* and *P. aeruginosa* were then mixed in ratios of either 1:1, 1:10, as specified and 5 μL of the mixed cultures were spotted onto an LB agar, 0.2 % arabinose plate and incubated at 37 °C for either one or two hours, respectively. The spot of the plate was then cut out, resuspended into 1 mL LB and serially diluted. The sample was then plated on LB agar plates containing either 5 μg/mL irgasan (to select for *P. aeruginosa*) or 100 μg/mL streptomycin (to select for *V. cholerae*). Plates were incubated at 37 °C overnight and colonies were counted. The log10 (CFU) values were calculated in Microsoft Excel v16.27 and graphs were generated in GraphPad Prism v7.0b.

qRT-PCRs

RNA was extracted from cultures grown to mid log. Harvested bacteria from 1 ml culture were resuspended in 1 ml Trizol (Invitrogen) and incubated at RT for 5 min. 200 μl of chloroform was added, vortexed and incubated for 5 min at RT. After a 10 min centrifugation step at 8000 x g at 4 °C, the aqueous solution was added to 400 μl ethanol and transferred to Purelink RNA Mini columns (Invitrogen). RNA was washed, dried and eluted following manufacturer’s instructions. RNA was DNAse treated following manufacturer’s instructions. (Invitrogen, TURBO DNA-free Kit,). The concentration of the RNA was measured using a Thermo Scientific NanoDrop 1000. qRT-PCR was performed using the KAPA SYBR FAST One-Step Universal kit (KAPA Biosystems) on the Eppendorf Mastercycle RealPlex 2 system, following manufacturer’s instructions. Primers for gene amplification are listed in Table S2. The mean of the three technical replicates was analyzed in Microsoft Excel v16.27, normalizing to the housekeeping gene *rpsL*. Relative gene expression was calculated after Livak (5). Three independent experiments were performed. Graphs were generated using GraphPad Prism v7.0b.
Hcp secretion assays

Overnight cultures were diluted to an OD$_{600}$ of 0.1 in 20 mL LB complemented with the according antibiotics and 0, 0.05, 0.1 or 0.2 % arabinose in stationary Erlenmeyer flasks to avoid shear stress. Samples were grown for approximately 5 hours and 3 mL of the cultures were harvested, washed in 1 mL LB with their respective arabinose concentrations and antibiotics and resuspended in 1 mL LB with their respective arabinose concentrations. Cultures were then incubated without shaking for 15 min at 37°C. Samples were centrifuged for 5 min at 8000 rcf and 500 µL supernatants were collected. The pellet was resuspended to an OD$_{600}$ of 10 in Lämmli buffer. Ice-cold trichloroacetic acid was added to the supernatant to a concentration of 10 % and kept at -20 °C overnight. Precipitates were centrifuged for 20 min at 20000 x g at 4 °C. The supernatant was discarded and the pellet was washed with 500 µL ice-cold acetone. Samples were centrifuged for 20 min at 12700 rpm at 4 °C. The pellet was dried at 50 °C and then resuspended matching the OD$_{600}$ of 10 in Lämmli buffer and boiled for 15 min at 95 °C. SDS-PAGE was performed using Invitrogen’s BoltTM 10% Bis-Tris Plus 12-well gels in an Invitrogen Mini Gel Tank with 1X MES Running Buffer. 7 µL protein sample were loaded onto the gels and separated for 10 min at 100 V and then 35 min at 170 V for a total of 45 min. Gels were then rinsed and transferred onto a blot using Invitrogen iBlot2 NC Mini Stacks and an Invitrogen iBlot2 Gel Transfer Device. Blots were blocked for 1 h at RT using a 1:1 PBS:Li-Cor Odyssey Blocking Buffer solution and subsequently incubated in primary antibody solution of 1:10,000 anti-PA0085 (Hcp) rabbit antibodies (custom made from Genscript) and 1:10,000 anti-RNAP mouse antibody (Thermo Fisher Scientific, #MA1-25425) in 1:1 PBS:Li-Cor Odyssey Blocking Buffer overnight, rocking at 4 °C. After rinsing three times for 5 min blots were incubated with secondary antibody solution of 1:20,000 Li-Cor IRDye 800CW Goat anti-Rabbit antibody, 1:20,000 Li-Cor IRDye 680RD Goat anti-Mouse antibody in 1:1 PBS:Li-Cor Odyssey Blocking Buffer for 1 h. Blots were visualized using a Li-Cor Odyssey CLx set to detect light in the 700 nm and 800 nm channels. Images were generated and quantified using Li-Cor’s ImageStudio v4.0.21.
Knockdown of essential genes leads to growth defects. Guides against different essential genes were designed. Bacteria were grown in the presence of inducer and growth was compared to sg-CTRL. OD$_{600}$ measurements were taken every 30 min. Graphs display mean ± SD of three independent experiments.
Knockdown of essential genes (except for secY (C)) results in uptake of PI. Strains were grown in the presence of 0.2 % arabinose and grown for the indicated time. 20 µl of each sample was added to 1 ml DPBS containing PI (1 µg/ml) and incubated for 15 min. Incorporation of PI was measured using flow cytometry. Graphs display mean ± SD of three independent experiments.
Volcano plots were generated to demonstrate transcriptional changes between sg-bamA (A), sg-lptD (B) and sg-ftsH (C) to sg-CTRL. Dashed lines indicate significance intervals of genes that are upregulated (log2 $\geq 2$, -log10 (p-value) $\geq 2$) or downregulated (log2 $\leq -2$, -log10 (p-value) $\geq 2$). Green dots indicate the target gene of the CRISPRi knockdown. Red dots indicate genes of the H1-T6SS cluster. The genes with the highest log2 fold changes and -log10 (p-value) where labeled with the gene name. D) PCA plots of sg-bamA, sg-lptD, sg-ftsH and sg-CTRL RNAseq datasets.
Fig. S4: Transcriptional profiling of essential gene knockdowns

Heat map of H2-T6SS (A) and H3-T6SS (B) cluster transcript level measured by RNA-seq of sg-bamA, sg-lptD or sg-ftsH knockdown and sg-CTRL as a control knockdown (each group n=3).

Fig. S5: CRISPRi sg-tolB, sg-bamA and sg-lptD knockdowns result in increased T6SS gene expression

Knockdown of tolB, bamA and lptD results in increase in T6SS gene expression. Expression of indicated genes were analyzed in qRT-PCRs after 3h (A) and 5h (B). Log2 fold change to PAO1 CRISPRi sg-CTRL (induced) is displayed. Graphs display mean ± SD of three independent experiments.
Fig. S6: Mutation in PAM region for bamA CRISPRi knockdown restores bamA expression and results in normal growth.

A) Expression of dcas9 and bamA were analyzed in qRT-PCRs in a combination of different background strains (wt vipA or ΔvipA, wt clpV or clpV-gfp, wt PAM or mutated PAM). Log2 fold change to PAO1 CRISPRi sg-CTRL (induced) is displayed. Graphs display mean ± SD of three independent experiments. B) Strains with mutated PAM site in PAO1 CRISPRi background were analyzed in their growth behavior with sg-CTRL or sg-bamA guide. Bacteria were grown in the presence of inducer. OD$_{600}$ measurements were taken every 30 min. Graphs display mean ± SD of three independent experiments.
Fig. S7: Knockdown of bamA causes dynamic T6SS activation

Time-lapse imaging of ClpV-GFP in *P. aeruginosa* strains expressing sg-bamA (A) or sg-CTRL (B) grown in the presence of 0.1 % arabinose. Images were taken every 10 s. Scale bar: 10 µm. Arrows indicate dynamic firing.
Fig. S8: Knockdown of lptD and tolB causes dynamic T6SS activation

Time-lapse imaging of ClpV-GFP in *P. aeruginosa* strains expressing sg-lptD (A) or sg-tolB (B) grown in the presence of 0.1 % arabinose. Images were taken every 10 s. Scale bar: 10 µm. Arrow heads indicate dynamic firing.
Fig S9: Competition between V. cholerae and P. aeruginosa

Time-lapse imaging of T6SS- V. cholerae 2740-80 ClpV-mCherry (1:10 ratio) in mixture with P. aeruginosa ClpV-GFP
A) sg-CTRL, and B) ΔvipA sg-bamA. Scale bar: 10 µm.
Fig. S10: Knockdown of essential genes does not lead to increased *V. cholerae* killing

A) *P. aeruginosa* with knockdowns in different essential genes was challenged with T6SS+ *V. cholerae* for 1 h in a 1:1 ratio, serial diluted and plated on 100 µg/ml Streptomycin plates to recover *V. cholerae* (left) or 5µg/ml Irgasan plates to recover *P. aeruginosa* (right). B) *P. aeruginosa* with knockdowns in different essential genes was challenged with T6SS- *V. cholerae* for 2 h in a 10:1 ratio, serial diluted and plated on 100 µg/ml Streptomycin plates to recover *V. cholerae* (left) or 5µg/ml Irgasan plates to recover *P. aeruginosa* (right).

Supplementary Tables
## Table S1: List of strains

| Strain number | Description | Reference |
|---------------|-------------|-----------|
| AS151         | PAO1 attB: dcas9 | This study |
| AS218         | PAO1 attB: dcas9 sq-fsH | This study |
| AS303         | PAO1 attB: dcas9 sq-tolB | This study |
| AS305         | PAO1 attB: dcas9 sq-bamA | This study |
| AS323         | PAO1 attB: dcas9 sq-lpD | This study |
| AS329         | PAO1 attB: dcas9 sq-secY | This study |
| AS330         | PAO1 attB: dcas9 sq-loIC | This study |
| AS338         | PAO1 attB: dcas9 sq-CTRL | This study |
|               | PAO1 clpV-gfp, ΔvipA | (6) |
| AS598         | PAO1 attB: dcas9, clpV-gfp, ΔvipA, sq-bamA | This study |
| AS608         | PAO1 attB: dcas9, clpV-gfp, ΔvipA sq-CTRL | This study |
|               | PAO1 clpV-gfp | (7) |
| AS631         | PAO1 attB: dcas9, clpV-gfp, sq-bamA | This study |
| AS632         | PAO1 attB: dcas9, clpV-gfp, sq-secY | This study |
| AS613         | PAO1 attB: dcas9, clpV-gfp, sq-tolB | This study |
| AS634         | PAO1 attB: dcas9, clpV-gfp, sq-loIC | This study |
| AS635         | PAO1 attB: dcas9, clpV-gfp, sq-lpD | This study |
| AS637         | PAO1 attB: dcas9, clpV-gfp, sq-fsH | This study |
| AS619         | PAO1 attB: dcas9, clpV-gfp, sq-CTRL | This study |
| AS663         | PAO1 attB: dcas9, mut PAM, sg-CTRL | This study |
| AS664         | PAO1 attB: dcas9, mut PAM, sq-bamA | This study |
| AS665         | PAO1 attB: dcas9, clpV-gfp, mut PAM, sg-CTRL | This study |
| AS666         | PAO1 attB: dcas9, clpV-gfp, mut PAM, sq-bamA | This study |
|               | 274080 clpV-mCherry | (8) |
|               | 274080 ΔvipA, clpV-mCherry | (8) |
|               | PAO1 ΔtagT1 | (9) |
| AS668         | PAO1 attB: dcas9, clpV-gfp, ΔtagT1, sg-CTRL | This study |
| AS669         | PAO1 attB: dcas9, clpV-gfp, ΔtagT1, sq-bamA | This study |

## Table S2: List of primers

| Oligo number | Sequence | Information |
|--------------|----------|-------------|
| oAS009       | CGAGTTGTTTAAGGCAGCGGTCTTGA | att-F Mini CTX sequencing Primer |
| oAS10        | AGTTCGGCCTTAGGAAACAACTCG | att-R Mini CTX sequencing Primer |
| oAS22        | CCTCGGTGCTCGGCAGAGAC | fw ppSV37 Δ lacl fragment 1 |
| oAS23        | TCCTCTAGTGACCTCGAG | rev ppSV37 Δ lacl fragment 2 |
| oAS24        | CTCACATATTACCGTTGCGCATTCCACCCCTGAATTGACT | fw ppSV37 Δ lacl fragment 2 |
| oAS25        | AGTCATCTCAGGTGTGAATTGCCGCAAGCAATTAATGGAG | rev ppSV37 Δ lacl fragment 1 |
| oAS034       | agggtgaaacgcaaaaaagcaccacctcAGATTCTAAGATCTTGGACACGCTAGCT | fw sg-RNA pPSV37 Δ lacl |
| oAS035       | ccgtttagggccggaggggttgtgctaaGGGGCCAAACCTGAAAAG | rev sg-RNA pPSV37 Δ lacl |
| oAS058       | ACTAGTTATATACCTAGGACTGAGCTGACGTTGAATGACATTTAGT | Reverse primer design new sg-RNA |
| oAS073       | gcctagaaacttagggcagaagcagctatagaaaaggtgactgctccagcattctctctttgcagagtctag | rev dcas9 into pPSW196 |
| oAS088       | Accctaggctatttgggtcgctcctagcactgagagatatacat | fw dcas9 into pPSW196 |
| oAS109       | TGA AGG TCA CAA CCT GCA AGA GCA | fw qRT-PCR rpsL |
| oAS110       | AAC GAC CCT GCT TAC GGT CCT TGA | rev qRT-PCR rpsL |
| oAS111       | CAGTCTCCTCTTGAAGAGATGAC | fw qRT-PCR dcas9 |
| oAS112       | CTTCCTCGGACGGGACATATTAC | rev qRT-PCR dcas9 |
| oAS117       | GTCCCTAGGAATACCTAGTCTCGGGGCGAGAAATTGTTCATCAGATTGAGCTAGATAATAATAGAGCG | fw guide ftsH |
| oAS125       | GGCTCACAAGGCTGCATCATT | fw qRT-PCR ftsH |
| oAS126 | GACAGGCTAGCGATCTTC | rev qRT-PCR rtsH |
| oAS143 | GTCCAGGTATATAACTAGTGGCAAGCGGATCGAACTACAGTTGGACTGGAATA | fw sg-loI |
| oAS145 | GTCCAGGTATATAACTAGTGGCAAGCGGATCGAACTACAGTTGGACTGGAATA | sg-g | ftsH |
| oAS163 | GTCCAGGTATATAACTAGTGGCAAGCGGATCGAACTACAGTTGGACTGGAATA | sg-g |
| oAS165 | GTCCAGGTATATAACTAGTGGCAAGCGGATCGAACTACAGTTGGACTGGAATA | sg-mreB |
| oAS169 | GTCCAGGTATATAACTAGTGGCAAGCGGATCGAACTACAGTTGGACTGGAATA | sg-secY |
| oAS170 | GTCCAGGTATATAACTAGTGGCAAGCGGATCGAACTACAGTTGGACTGGAATA | sg-loI |
| oAS179 | GTCCAGGTATATAACTAGTGGCAAGCGGATCGAACTACAGTTGGACTGGAATA | sg-CTRL |
| oAS196 | AGACGGTACGCCTATGAAGA | fw qRT-PCR bamA (PA3648) |
| oAS197 | GATCAGGATGTTACCACCGAAG | rev qRT-PCR bamA (PA3648) |
| oAS384 | cccgtggaatattatgagttacgTCCCGATCCCATCGCCTC | fw bamA PAM mutation P to G fragment 1 |
| oAS387 | gccggaagctaatgtaaagcaCGGTACTACGTACAAATCAGAA | rev bamA PAM mutation P to G fragment 2 |
| oAS388 | GGAACGGGGGTCAACCTGCAGC | bamA sequencing primer |
| oAS389 | CTGGATGGCTGCGGAAGGCC | bamA sequencing primer |
| oAS390 | GGCTCTGCTGCTATCTGCTT | bamA sequencing primer |
| oAS391 | CGCCACCTGCAAGCGTGCCG | bamA sequencing primer |
| oAS392 | CTACACCTTCCGCAACGTCAA | bamA sequencing primer |
| oAS393 | CTCGACGTCGACGTCGCCAGC | bamA sequencing primer |
| oAS394 | GATCCGAAGCTTCGGTGCT | bamA sequencing primer |
| oAS395 | AGCCGCATGATGGCTGCGT | bamA sequencing primer |
| oAS396 | CGAGCAGCGCGCATCTGGT | bamA sequencing primer |
| oAS413 | CCATGAAACGCTTTCTCTGAAAGCGCTGCTGTCGCCTGAT | fw bamA PAM mutation P to G fragment 2 |
| oAS414 | ATCAGCCGCTGACAGCGCTTCTGGAGCGGCGTTTGTACATG | rev bamA PAM mutation P to G fragment 1 |
| oJT_702 | AGGTGCGTTGCGGTAACAGAAG | fw qRT-PCR for PA0083 (vipA) |
| oJT_703 | AGGTGCGTTGCGGTAACAGAAG | rev qRT-PCR for PA0083 (vipA) |
| oJT_706 | TTCGCCTTCGAGGAAGAAC | fw qRT-PCR for PA0084 (vipB) |
| oJT_707 | GAAGGAGCGTTGATGGGA | rev qRT-PCR for PA0084 (vipB) |
| oJT_710 | GACTCACCGCAAGGAATTC | fw qRT-PCR for PA0085 (hcrp) |
| oJT_711 | ATGTAAGGCTGTAACGGAAGG | rev qRT-PCR for PA0085 (hcrp) |
| oJT_726 | CTGGTGGATGGAATTGAAGAAG | fw qRT-PCR for PA0090 (clpV) |
| oJT_727 | GGGTGTCTGGAAGCGATCGA | rev qRT-PCR for PA0090 (clpV) |
| Illumina Sequencing Stats | sg-ftsH | sg-bamA | sg-lptD | sg-CTRL |
|---------------------------|---------|---------|---------|---------|
| Total reads passed quality filter | r1: 7368897 | r1: 5170586 | r1: 3118001 | r1: 3853529 |
| sg-ftsH | r2: 3392153 | r2: 512720 | r2: 3396558 | r2: 4014763 |
| sg-bamA | r3: 3548829 | r3: 3095422 | r3: 6076093 | r3: 8174960 |
| sg-lptD | r1: 6917838 | r1: 3920845 | r1: 179482 | r1: 3546723 |
| sg-CTRL | r2: 372413 | r2: 3380738 | r2: 1317970 | r2: 1751635 |
| NC_002516 | r3: 3216681 | r3: 2959442 | r3: 5870799 | r3: 1381814 |
| Reds mapped to PAO1 NC_002516 | r1: 6917838 | r1: 3920845 | r1: 179482 | r1: 3546723 |
| Percentage of total reads mapped to PAO1 NC_002516 | r2: 372413 | r2: 3380738 | r2: 1317970 | r2: 1751635 |
| Reads not mapped to PAO1 NC_002516 | r3: 3216681 | r3: 2959442 | r3: 5870799 | r3: 1381814 |
Table S4: Expression levels and differential expression of CRISPRi targets

| Locus tag | sg-bamA TPM | log2 ratio | p-value | sg-lpdA TPM | log2 ratio | p-value | sg-ftsh TPM | log2 ratio | p-value | sg-CTRL TPM |
|-----------|-------------|------------|---------|-------------|------------|---------|-------------|------------|---------|-------------|
| bamA PA3648 | 17.6        | -3.56      | 9.28E-50 | 843.70      | -0.23      | 3.04E-01 | 525.87      | -1.13      | 4.75E-05 | 1110.8      |
| lpdA PA0595 | 567.07      | -0.84      | 7.45E-04 | 96.37       | -3.36      | 2.57E-38 | 421.10      | -1.60      | 2.23E-08 | 1230.5      |
| ftsh PA4751 | 437.93      | -0.02      | 9.23E-01 | 510.17      | 0.07       | 7.62E-01 | 68.00       | -3.17      | 1.51E-19 | 534.87      |

Table S5: Expression levels and differential expression of H1-T6SS

| Locus tag | sg-bamA TPM | log2 ratio | p-value | sg-lpdA TPM | log2 ratio | p-value | sg-ftsh TPM | log2 ratio | p-value | sg-CTRL TPM |
|-----------|-------------|------------|---------|-------------|------------|---------|-------------|------------|---------|-------------|
| PA0070    | 8829.67     | 1.33       | 3.90E-07 | 9567.33     | 1.28       | 6.99E-07 | 1004.50     | -2.11      | 7.21E-13 | 4123.33     |
| PA0071    | 1398.87     | 1.41       | 3.21E-09 | 1195.10     | 1.04       | 9.56E-06 | 668.37      | 0.01       | 9.84E-01 | 636.47      |
| PA0072    | 78.9        | 1.78       | 3.86E-04 | 56.47       | 1.08       | 1.67E-02 | 14.07       | -0.79      | 1.89E-01 | 26.6        |
| PA0073    | 45.87       | 0.81       | 2.04E-01 | 36.27       | 0.36       | 4.95E-01 | 25.00       | -0.67      | 3.30E-01 | 30.2        |
| PA0074    | 165.23      | 1.19       | 2.39E-06 | 133.17      | 0.74       | 2.94E-03 | 33.50       | -1.55      | 1.33E-06 | 86.93       |
| PA0075    | 134.00      | 1.02       | 1.27E-02 | 168.13      | 1.12       | 2.48E-03 | 20.30       | -1.81      | 5.14E-04 | 78.80       |
| PA0076    | 90.83       | 0.63       | 1.90E-01 | 81.70       | 0.35       | 4.15E-01 | 15.07       | -2.10      | 1.90E-04 | 70.07       |
| PA0077    | 112.10      | 0.95       | 5.79E-04 | 88.73       | 0.49       | 7.63E-02 | 31.23       | -1.31      | 5.83E-05 | 70.23       |
| PA0078    | 241.77      | 0.57       | 3.38E-02 | 236.07      | 0.40       | 1.41E-01 | 47.93       | -1.99      | 6.21E-10 | 198.53      |
| PA0079    | 102.80      | 1.25       | 2.23E-03 | 77.17       | 0.69       | 5.60E-02 | 32.57       | -0.86      | 5.14E-02 | 50.60       |
| PA0080    | 77.03       | 1.04       | 8.45E-02 | 53.03       | 0.32       | 5.73E-01 | 26.07       | -1.01      | 1.36E-01 | 44.00       |
| PA0081    | 130.97      | -0.01      | 9.76E-01 | 190.50      | 0.39       | 1.37E-01 | 156.97      | -0.10      | 7.35E-01 | 159.97      |
| PA0082    | 320.17      | 1.23       | 5.29E-05 | 359.33      | 1.23       | 3.24E-05 | 164.50      | -0.04      | 9.12E-01 | 164.97      |
| PA0083    | 1304.10     | 2.00       | 4.30E-15 | 1051.23     | 1.52       | 1.82E-09 | 110.33      | -1.70      | 2.30E-06 | 393.77      |
| PA0084    | 868.80      | 1.86       | 1.18E-17 | 755.93      | 1.51       | 3.86E-11 | 121.80      | -1.35      | 5.36E-07 | 285.47      |
| PA0085    | 1306.80     | 2.17       | 5.92E-16 | 815.60      | 1.34       | 6.42E-07 | 155.60      | -1.04      | 4.90E-03 | 344.37      |
| PA0086    | 153.90      | 1.78       | 1.14E-05 | 112.43      | 1.14       | 3.69E-03 | 58.10       | 0.13       | 7.72E-01 | 53.23       |
| PA0087    | 220.57      | 1.86       | 2.99E-05 | 169.13      | 1.24       | 3.56E-03 | 50.03       | -0.58      | 2.79E-01 | 72.77       |
| PA0088    | 140.67      | 1.34       | 6.78E-05 | 165.80      | 1.39       | 7.12E-06 | 29.07       | -1.21      | 2.58E-03 | 65.57       |
| PA0089    | 310.00      | 1.71       | 5.67E-08 | 232.67      | 1.13       | 4.55E-04 | 32.73       | -1.70      | 5.48E-05 | 112.40      |
| PA0090    | 314.53      | 1.84       | 7.36E-16 | 235.63      | 1.27       | 3.81E-08 | 50.67       | -1.21      | 4.49E-05 | 105.77      |
Table S6: Expression levels and differential expression of H2-T6SS

TPM values and Differential Log2 Ratio (compared to sg-CTRL) of H2.

| Locus tag | sg-bamA TPM | log2 ratio | p-value | sg-lptD TPM | log2 ratio | p-value | sg-ftsH TPM | log2 ratio | p-value | sg-CTRL TPM |
|-----------|--------------|------------|---------|--------------|------------|---------|--------------|------------|---------|------------|
| PA1656    | 238.90       | 0.44       | 1.46E-01| 324.10       | 0.71       | 8.85E-03| 117.63       | -0.86     | 9.17E-03| 210.27     |
| PA1657    | 910.77       | 1.08       | 1.26E-03| 889.90       | 0.88       | 7.02E-03| 200.03       | -1.43     | 1.92E-04| 494.23     |
| PA1658    | 430.73       | 1.46       | 9.20E-08| 416.40       | 1.24       | 4.15E-06| 109.40       | -0.88     | 5.74E-03| 183.27     |
| PA1659    | 214.17       | 1.06       | 3.50E-02| 275.47       | 1.18       | 3.86E-03| 55.53        | -0.85     | 1.36E-01| 115.13     |
| PA1660    | 45.13        | -0.08      | 8.46E-01| 39.00        | -0.36      | 3.59E-01| 18.77        | -1.42     | 2.46E-03| 57.03      |
| PA1661    | 52.70        | 0.32       | 5.10E-01| 43.00        | -0.04      | 9.34E-01| 34.70        | -0.84     | 1.13E-01| 51.20      |
| PA1662    | 96.10        | 0.64       | 1.17E-01| 100.40       | 0.54       | 1.49E-01| 25.50        | -1.43     | 1.73E-03| 71.27      |
| PA1663    | 36.37        | 0.39       | 4.22E-01| 47.30        | 0.59       | 1.35E-01| 8.40         | -1.67     | 2.27E-03| 32.80      |
| PA1664    | 75.80        | 0.88       | 3.26E-01| 218.33       | 1.74       | 1.99E-03| 30.17        | -0.41     | 6.17E-01| 42.17      |
| PA1665    | 137.53       | 0.75       | 4.91E-02| 176.07       | 0.91       | 1.01E-02| 38.70        | -1.46     | 7.72E-04| 95.50      |
| PA1666    | 155.37       | 0.50       | 2.95E-01| 176.20       | 0.49       | 2.53E-01| 26.87        | -1.95     | 2.21E-04| 126.80     |
| PA1667    | 56.20        | 0.38       | 3.88E-01| 79.07        | 0.69       | 5.47E-02| 20.53        | -1.26     | 5.90E-03| 51.93      |
| PA1668    | 44.43        | -0.05      | 9.29E-01| 73.30        | 0.45       | 3.05E-01| 17.93        | -1.74     | 2.20E-03| 54.13      |
| PA1669    | 89.27        | 0.34       | 3.14E-01| 119.57       | 0.60       | 5.35E-02| 31.87        | -1.48     | 3.29E-05| 83.20      |
| PA1670    | 1.60         | -2.41      | 1.16E-02| 11.73        | -0.53      | 3.66E-01| 4.93         | -1.39     | 6.69E-02| 23.80      |
| PA1671    | 17.40        | 0.31       | 6.77E-01| 13.33        | -0.13      | 8.20E-01| 30.77        | 0.43      | 4.97E-01| 16.93      |
| PA1511    | 36.13        | 0.32       | 4.31E-01| 47.50        | 0.55       | 1.06E-01| 54.07        | 0.54      | 1.24E-01| 35.37      |
| PA1512    | 25.30        | 0.43       | 6.01E-01| 18.77        | -0.03      | 9.58E-01| 40.17        | 0.72      | 2.78E-01| 21.00      |
Table S7: Expression levels and differential expression of H3-T6SS

TPM values and Differential Log2 Ratio (compared to sg-CTRL) of H3.

| Locus tag | TPM  | log2 ratio | p-value | TPM  | log2 ratio | p-value | TPM  | log2 ratio | p-value | TPM  |
|-----------|------|------------|---------|------|------------|---------|------|------------|---------|------|
| PA2359    | 67.67| 1.92       | 1.75E-04| 21.00| 0.19       | 7.20E-01| 21.67| 0.02       | 9.71E-01| 20.07|
| PA2360    | 11.90| 0.78       | 3.52E-01| 4.97 | -0.23      | 7.04E-01| 8.17 | -0.19      | 8.05E-01| 7.47 |
| PA2361    | 2.47 | -1.52      | 2.80E-02| 4.43 | -0.79      | 1.12E-01| 4.63 | -1.17      | 3.54E-02| 9.77 |
| PA2362    | 0.00 | -0.59      | 4.76E-01| 1.23 | -0.20      | 6.13E-01| 0.00 | -0.38      | 5.35E-01| 2.40 |
| PA2363    | 4.30 | -0.43      | 6.29E-01| 4.63 | -0.33      | 5.82E-01| 5.80 | -0.32      | 6.73E-01| 7.80 |
| PA2364    | 20.63| 0.46       | 6.21E-01| 14.93| 0.05       | 9.39E-01| 17.93| 0.10       | 8.99E-01| 15.67|
| PA2365    | 12.10| 0.58       | 5.65E-01| 2.37 | -0.44      | 4.02E-01| 7.83 | 0.12       | 8.83E-01| 7.23 |
| PA2366    | 11.27| -0.01      | 9.85E-01| 11.73| -0.09      | 8.69E-01| 13.10| -0.21      | 7.45E-01| 13.90|
| PA2367    | 8.63 | -0.79      | 3.93E-01| 1.87 | -1.28      | 3.31E-02| 16.30| -0.55      | 4.81E-01| 21.63|
| PA2368    | 3.33 | 0.42       | 6.08E-01| 0.00 | NA         | NA      | 4.10 | 0.08       | 8.82E-01| 0.00 |
| PA2369    | 2.73 | -0.33      | 7.37E-01| 1.10 | -0.64      | 2.59E-01| 4.53 | -0.40      | 6.19E-01| 4.63 |
| PA2370    | 2.23 | -0.44      | 6.71E-01| 2.87 | -0.22      | 7.01E-01| 1.47 | -0.73      | 3.71E-01| 5.17 |
| PA2371    | 9.67 | 0.15       | 8.12E-01| 7.80 | -0.23      | 6.68E-01| 12.63| 0.06       | 9.18E-01| 10.77|

Table S8: Upregulated pathways in sg-bamA knockdown vs sg-CTRL

Significantly upregulated pathways (p-value ≤ 0.001)

| Category | Description                                                                 | Full set | In subset | Expected in subset | Observed - expected | p-value  |
|----------|-----------------------------------------------------------------------------|----------|-----------|--------------------|---------------------|----------|
| 1901760  | beta-L-Ara4N-lipid A biosynthetic process (PMID:17928292 [ISS])              | 7        | 7         | 1                  | 6                   | 6.61E-06 |
| 8300     | isoprenoid catabolic process (PMID:15345388 [IMPI])                          | 6        | 6         | 1                  | 5                   | 3.65E-05 |
| 8152     | metabolic process (PMID:24451626 [ISM] InterPro:PF00884)                    | 305      | 81        | 56                 | 25                  | 1.02E-04 |
| 6552     | leucine catabolic process (PMID:16820476 [IMPI])                            | 7        | 6         | 1                  | 5                   | 2.16E-04 |
| 55114    | oxidation-reduction process (PMID:24451626 [ISM] InterPro:PIRSF004750)      | 442      | 107       | 81                 | 26                  | 4.36E-04 |
| 32885    | regulation of polysaccharide biosynthetic process (PMID:14982774 [IMPI])    | 6        | 5         | 1                  | 4                   | 1.02E-03 |
| 43436    | oxoacid metabolic process (PMID:3416875 [ISS])                              | 4        | 4         | 1                  | 3                   | 1.11E-03 |
| 45226    | extracellular polysaccharide biosynthetic process (PMID:22176658 [IMPI])    | 20       | 10        | 4                  | 6                   | 1.22E-03 |
| 42128    | nitrate assimilation (PMID:24451626 [ISM] InterPro:G3DSA:3.90.420.10)       | 9        | 6         | 2                  | 4                   | 1.85E-03 |
| 44550    | secondary metabolite biosynthetic process (PMID:11728716 [ISS])             | 7        | 5         | 1                  | 4                   | 3.05E-03 |
| 44010    | single-species biofilm formation (PMID:19778968 [IMPI])                     | 41       | 15        | 7                  | 8                   | 4.09E-03 |
| 33103    | protein secretion by the type VI secretion system (PMID:18524912 [IAGC])     | 8        | 5         | 1                  | 4                   | 6.91E-03 |
| 8272     | sulfate transport (PMID:24451626 [ISM] InterPro:TIGR00969)                  | 8        | 5         | 1                  | 4                   | 6.91E-03 |
Table S9: Downregulated pathways in sg-bamA knockdown vs sg-CTRL

| Category | Description                                                                 | Full set | In subset | Expected in subset | Observed - expected | p-value   |
|----------|-----------------------------------------------------------------------------|----------|-----------|---------------------|----------------------|-----------|
| 30254    | protein secretion by the type III secretion system (PMID:24451626 [ISM] InterPro:TIGR01026) | 29       | 13        | 4                   | 9                    | 9.44E-05  |
| 15886    | heme transport (PMID:10658665 [IMP])                                        | 7        | 5         | 1                   | 4                    | 1.12E-03  |
| 43683    | type IV pilus biogenesis (PMID:8899718 [IMP])                               | 13       | 6         | 2                   | 4                    | 6.85E-03  |
| 15074    | DNA integration (PMID:24451626 [ISM] InterPro:PS50994)                     | 10       | 5         | 1                   | 4                    | 9.12E-03  |

Table S10: Upregulated pathways in sg-ftsH knockdown vs sg-CTRL

| Category | Description                                                                 | Full set | In subset | Expected in subset | Observed - expected | p-value   |
|----------|-----------------------------------------------------------------------------|----------|-----------|---------------------|----------------------|-----------|
| 6355     | regulation of transcription, DNA-dependent (PMID:0 [IDA])                   | 382      | 308       | 205                 | 103                  | 0         |
| 8033     | tRNA processing (PMID:24451626 [ISM] InterPro:PIRSF004976)                  | 16       | 15        | 9                   | 6                    | 7E-04     |
| 162      | tryptophan biosynthetic process (PMID:0 [ISS])                              | 14       | 13        | 8                   | 5                    | 2.18E-03  |
| 9073     | aromatic amino acid family biosynthetic process (PMID:24451626 [ISM] InterPro:TIGR01358) | 9        | 9         | 5                   | 4                    | 3.71E-03  |

Table S11: Downregulated pathways in sg-ftsH knockdown vs sg-CTRL

| Category | Description                                                                 | Full set | In subset | Expected in subset | Observed - expected | p-value   |
|----------|-----------------------------------------------------------------------------|----------|-----------|---------------------|----------------------|-----------|
| 6935     | chemotaxis (PMID:24451626 [ISM] InterPro:PR00260)                          | 42       | 9         | 3                   | 6                    | 6.61E-04  |
| 6810     | transport (PMID:24451626 [ISM] InterPro:TIGR01726)                         | 321      | 32        | 19                  | 13                   | 2.18E-03  |
| 9697     | salicylic acid biosynthetic process (PMID:7500944 [IDA])                   | 2        | 2         | 0                   | 2                    | 3.59E-03  |

Table S12: Upregulated pathways in sg-lptD knockdown vs sg-CTRL

| Category | Description                                                                 | Full set | In subset | Expected in subset | Observed - expected | p-value   |
|----------|-----------------------------------------------------------------------------|----------|-----------|---------------------|----------------------|-----------|
| 8300     | isoprenoid catabolic process (PMID:15345388 [IMP])                          | 6        | 6         | 1                   | 5                    | 6.01E-05  |
| 1901760  | beta-L-Ara4N-lipid A biosynthetic process (PMID:17928292 [ISS])             | 7        | 6         | 1                   | 5                    | 3.5E-04   |
| 8152     | metabolic process (PMID:24451626 [ISM] InterPro:PF00884)                    | 305      | 82        | 61                  | 21                   | 1.05E-03  |
| 32885    | regulation of polysaccharide biosynthetic process (PMID:14982774 [IMP])     | 6        | 5         | 1                   | 4                    | 1.53E-03  |
| 43436    | oxoacid metabolic process (PMID:3416875 [ISS])                              | 4        | 4         | 1                   | 3                    | 1.54E-03  |
| 44550    | secondary metabolite biosynthetic process (PMID:11728716 [ISS])             | 7        | 5         | 1                   | 4                    | 4.47E-03  |
| 6552     | leucine catabolic process (PMID:16820476 [IMP])                             | 7        | 5         | 1                   | 4                    | 4.47E-03  |
### Table S13: Downregulated pathways in sg-lptD knockdown vs sg-CTRL

Significantly downregulated pathways (p-value ≤ 0.001)

| Category | Description | Full set | In subset | Expected in subset | Observed - expected | p-value |
|----------|-------------|----------|-----------|--------------------|---------------------|---------|
| 55085    | transmembrane transport (PMID:24451626 [ISM] InterPro:PS00218) | 177     | 34        | 15                 | 19                  | 4.92E-06 |
| 6810     | transport (PMID:24451626 [ISM] InterPro:TIGR01726) | 321     | 46        | 28                 | 18                  | 2.98E-04 |
| 15891    | siderophore transport (PMID:24451626 [ISM] InterPro:TIGR01783) | 20      | 7         | 2                  | 5                   | 1.02E-03 |
| 15846    | polyamine transport (PMID:24451626 [ISM] InterPro:TIGR01187) | 13      | 5         | 1                  | 4                   | 3.5E-03  |
| 1901053  | sarcosine catabolic process (PMID:24097953 [IDA]) | 5       | 3         | 0                  | 3                   | 5.76E-03 |
| 19439    | aromatic compound catabolic process (PMID:24451626 [ISM] InterPro:PF00848) | 21     | 6         | 2                  | 4                   | 7.37E-03 |
| 6855     | drug transmembrane transport (PMID:24451626 [ISM] InterPro:TIGR00797) | 2       | 2         | 0                  | 2                   | 7.6E-03  |
| 42930    | enterobactin transport (PMID:15899402 [IMP]) | 2       | 2         | 0                  | 2                   | 7.6E-03  |
Legend for Datasets

**Dataset S1: Differentially expressed genes**
Differential log2 Ratios of CRISPRi sg-bamA, sg-ftsH and sg-lptD vs sg-CTRL.

Legend for Movies

**Movie S1: T6-dynamics of control (sg-CTRL)**
Time-lapse imaging of T6SS dynamics in control (sg-CTRL). Images were taken every 10 s. T6SS dynamics were visualized by imaging ClpV-GFP (green). Merged video of phase contrast and GFP channel is depicted. Scale bar: 10 µm.

**Movie S2: T6-dynamics of bamA knockdown (sg-bamA)**
Time-lapse imaging of T6SS dynamics in a bamA knockdown. Images were taken every 10 s. T6SS dynamics were visualized by imaging ClpV-GFP (green). Merged video of phase contrast and GFP channel is depicted. Scale bar: 10 µm.

**Movie S3: T6-dynamics of lptD knockdown (sg-lptD)**
Time-lapse imaging of T6SS dynamics in a lptD knockdown. Images were taken every 10 s. T6SS dynamics were visualized by imaging ClpV-GFP (green). Merged video of phase contrast and GFP channel is depicted. Scale bar: 10 µm.

**Movie S4: T6-dynamics of tolB knockdown (sg-tolB)**
Time-lapse imaging of T6SS dynamics in a tolB knockdown. Images were taken every 10 s. T6SS dynamics were visualized by imaging ClpV-GFP (green). Merged video of phase contrast and GFP channel is depicted. Scale bar: 10 µm.

**Movie S5: P. aeruginosa T6+ sg-bamA vs V. cholerae T6-**
Time-lapse imaging of T6SS + P. aeruginosa bamA knockdown (sg-bamA) in mixture with T6SS- V. cholerae (10:1 ratio). Red: V. cholerae, green: P. aeruginosa. Scale bar: 10 µm.

**Movie S6: P. aeruginosa T6+ sg-bamA vs V. cholerae T6- (crop)**
Time-lapse imaging of T6SS + P. aeruginosa bamA knockdown (sg-bamA) in mixture with T6SS- V. cholerae (10:1 ratio). Red: V. cholerae, green: P. aeruginosa. Scale bar: 10 µm. Cropped version of Movie S5.
**Movie S7: P. aeruginosa T6+ sg-CTRL vs V. cholerae T6-**
Time-lapse imaging of T6SS + P. aeruginosa sg-CTRL (control) in mixture with T6SS- V. cholerae (10:1 ratio). Red: V. cholerae, green: P. aeruginosa. Scale bar: 10 µm.

**Movie S8: P. aeruginosa T6+ sg-CTRL vs V. cholerae T6- (crop)**
Time-lapse imaging of T6SS + P. aeruginosa sg-CTRL (control) in mixture with T6SS- V. cholerae (10:1 ratio). Red: V. cholerae, green: P. aeruginosa. Scale bar: 10 µm. Cropped version of Movie S7.

**Movie S9: P. aeruginosa T6- sg-bamA vs V. cholerae T6-**
Time-lapse imaging of T6SS - P. aeruginosa bamA knockdown in mixture with T6SS- V. cholerae (10:1 ratio). Red: V. cholerae, green: P. aeruginosa. Scale bar: 10 µm.

**Movie S10: P. aeruginosa T6- sg-bamA vs V. cholerae T6- (crop)**
Time-lapse imaging of T6SS - P. aeruginosa bamA knockdown (sg-bamA) in mixture with T6SS- V. cholerae (10:1 ratio). Red: V. cholerae, green: P. aeruginosa. Scale bar: 10 µm. Cropped version of Movie S9.
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