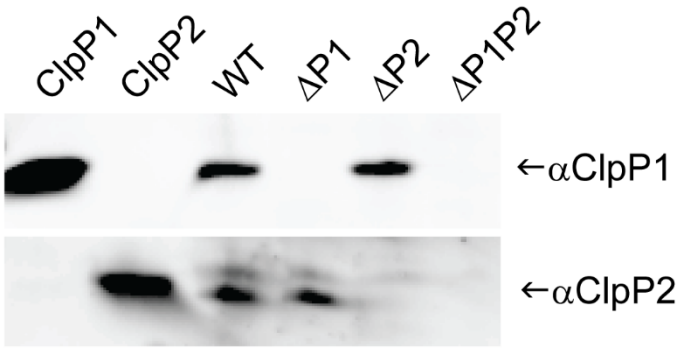


Figure S1. Validation of ClpP1 and ClpP2 specific polyclonal antibodies. Samples of purified ClpP1, purified ClpP2 and cells harvested from overnight cultures of WT, ΔP1, ΔP2 and ΔP1P2 strains of *P. aeruginosa* were subjected to SDS-PAGE and Western blot using primary antibodies specific for either ClpP1 or ClpP2.

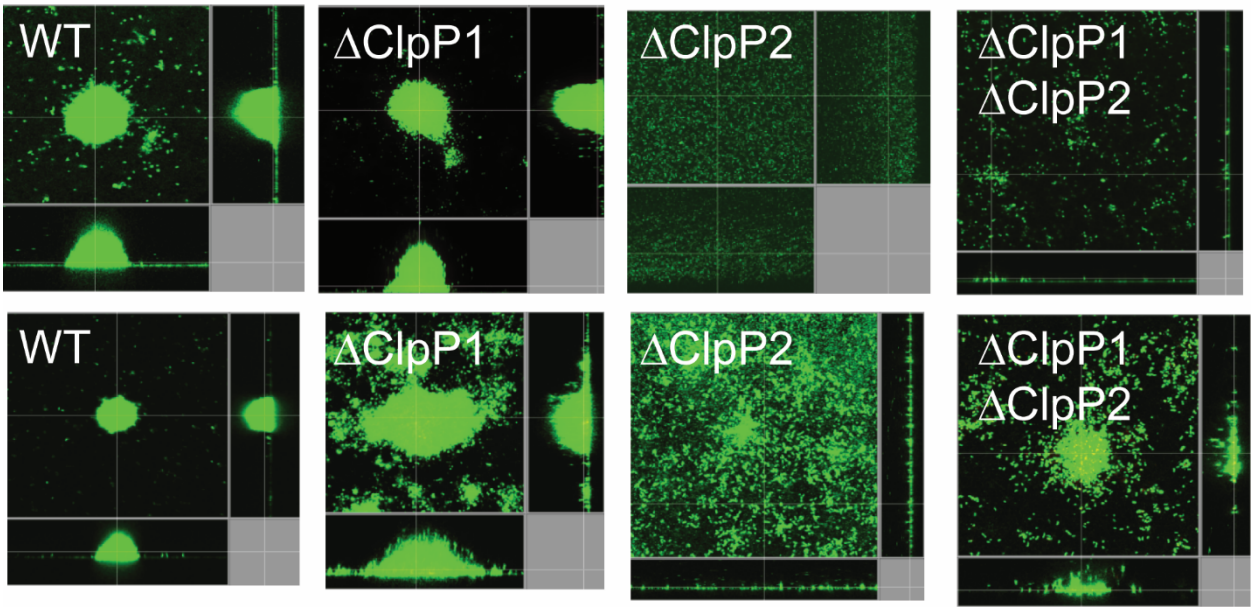
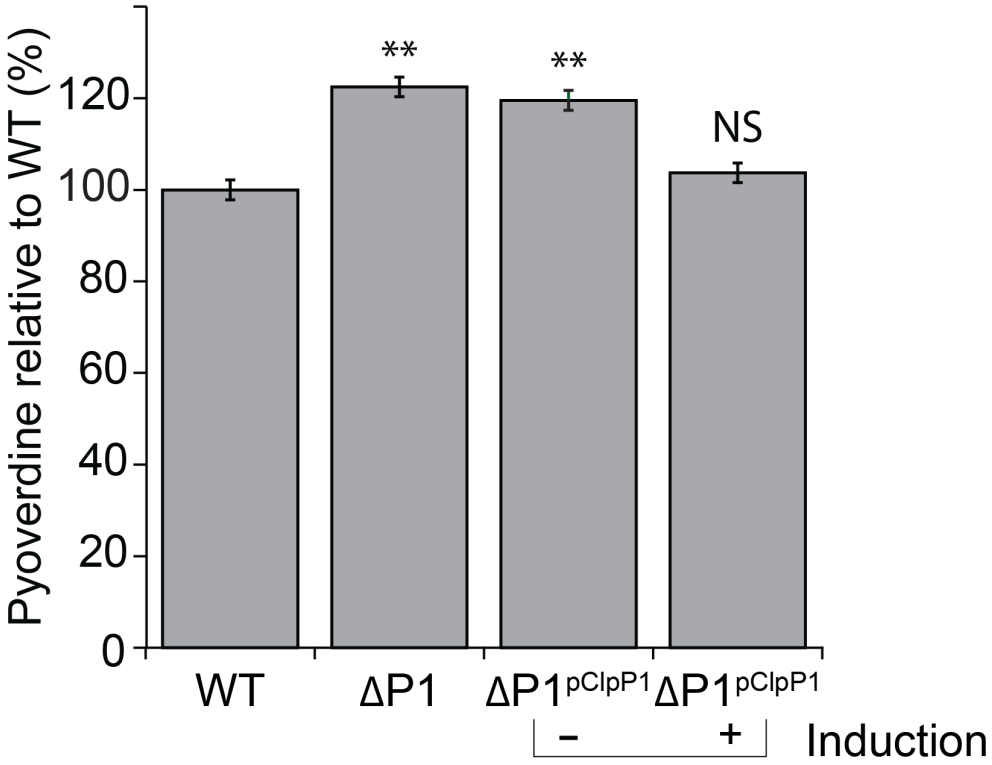


Figure S2. Microcolony assay duplicate experiments. Microcolony formation is shown for wild type *P. aeruginosa* (WT), the ClpP1 deficient strain (ΔClpP1), the ClpP2 deficient strain (ΔClpP2) and the strain deficient for both ClpP1 and ClpP2 (ΔClpP1ΔClpP2). Experiments were carried out as described in Materials and Methods.

A.


Pyoverdine relative to WT (%)
0
20
40
60
80
100
120
**
**
NS
WT
ΔP1
ΔP1pClpP1
ΔP1pClpP1
–
+
Induction

B.

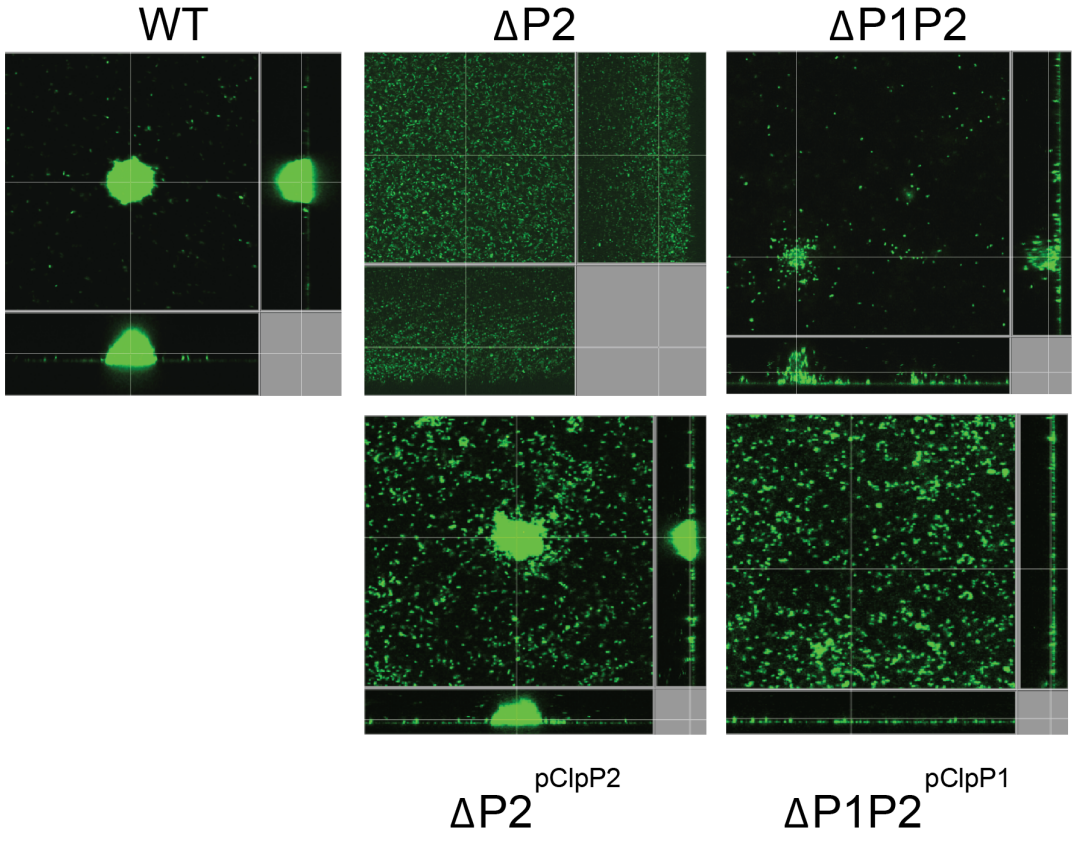

WT
ΔP2
ΔP1P2
ΔP2pClpP2
ΔP1P2pClpP1

Figure S3. Rescue of pyoverdine and microcolony organization by complementation with ClpP1 or ClpP2 expression plasmids. (A) Pyoverdine levels in ΔP1 are restored to wild type by the expression of ClpP1 from a plasmid (pClpP1). Values are normalized averages (n=3) ± 1 SEM. Data were compared to control values (WT) by ANOVA with Dunnet's *post hoc* test (**, P<0.005; NS, not significant). Strains deficient for ClpP1 were transformed as previously described (1) with a pMQ71 based shuttle vector for arabinose-dependent expression of the ClpP1 gene (pClpP1). Growth conditions for cultures are the same as described for pyoverdine assays in Materials and Methods except that media was supplemented with 30 μg/ul gentamycin for pMQ71-containing strains. 2% arabinose was used for induction conditions (+) or was omitted for controls (-). Fluorescence excitation/emission values were measured at 405/465 nm for pyoverdine quantification. (B) Microcolony formation flow cell assay by strains transformed with plasmids for the expression of ClpP1 or ClpP2. Assays carried out with strains without plasmid (WT, ΔP1 and ΔP1P2) are shown in the top row. The ClpP2 deficient strain (ΔP2) was transformed as described in (A) and the strain deficient for both ClpP1 and ClpP2 (ΔP1P2) was transformed similarly with pClpP1. Flow cell biofilm assays were carried out as described in Materials and Methods except that initial overnight cultures were grown in the presence of 30 μg/ml gentamycin and 0.1% arabinose was present throughout the experiment.

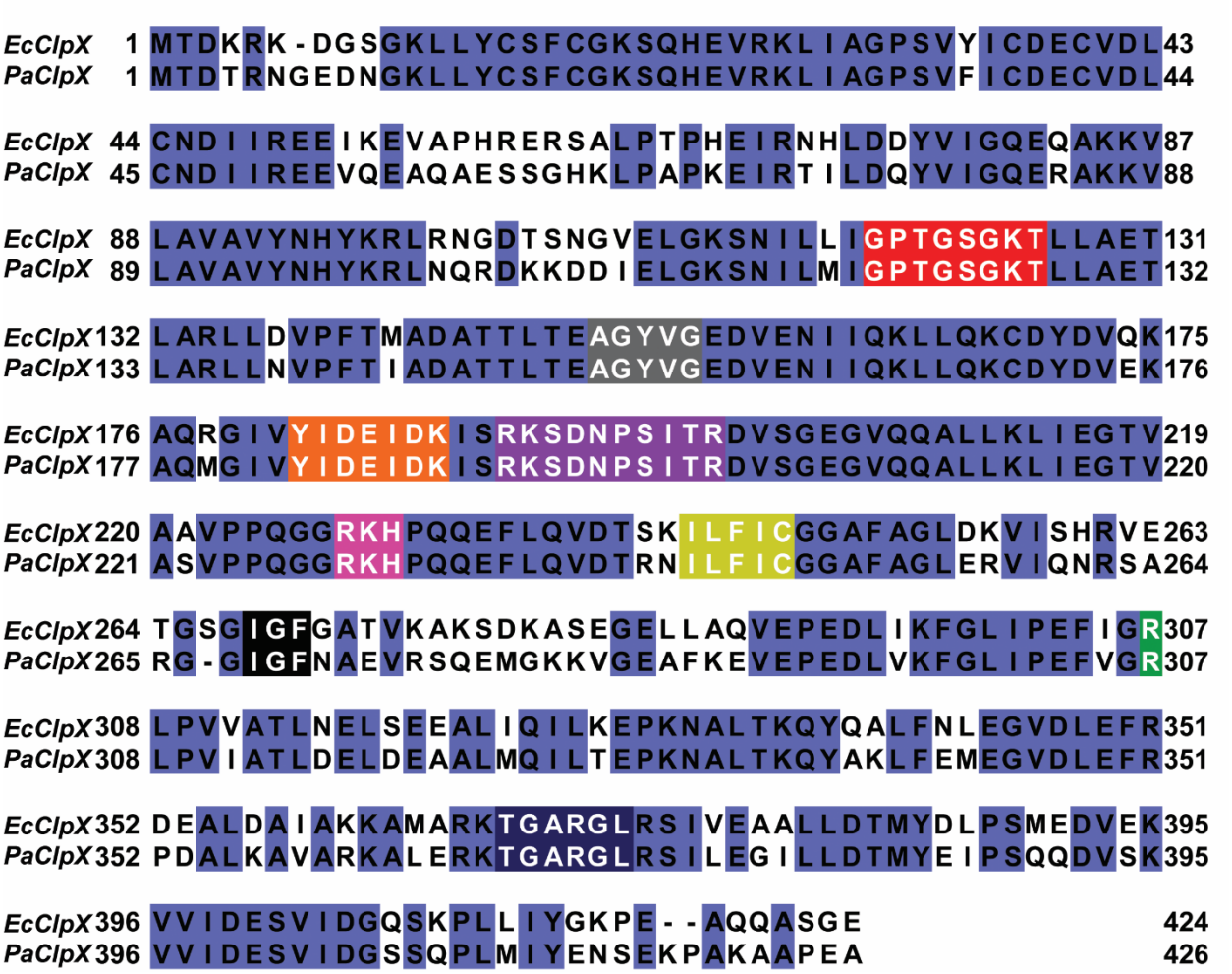


Figure S4. Sequence alignment of ClpX from *E.coli* (*Ec*ClpX) and *P. aeruginosa* (*Pa*ClpX). Identical residues are shown in light blue except for the Walker A motif (red), Walker B motif (orange), pore 1 loops (gray), pore 2 loops (purple), sensor 1 motif (yellow), sensor 2 motif (dark blue), arginine finger residue (green), IGF docking loops (black) and RKH motif (pink). The two proteins have 76% sequence identity. The alignment was carried out with MUSCLE (2) using default values and presented with Jalview (3).

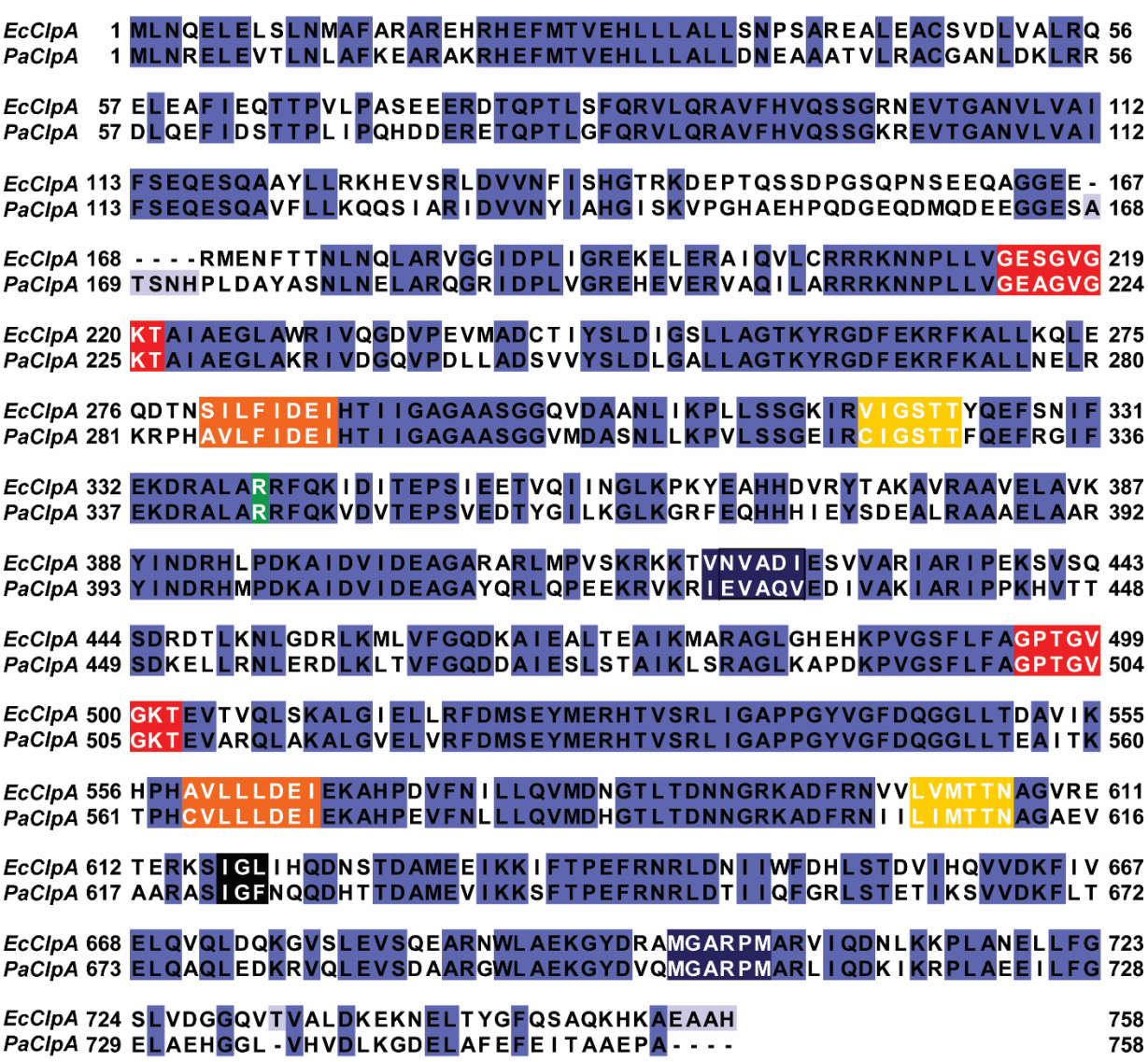


Figure S5. Sequence alignment of ClpA from *E. coli* (*Ec*ClpA) and *P. aeruginosa* (*Pa*ClpA). Identical residues are shown in light blue except for the Walker A motif (red), Walker B motif (orange), pore 1 loops (gray), pore 2 loops (purple), sensor 1 motif (yellow), sensor 2 motif (dark blue), arginine finger residue (green) and IGF docking loops (black). The two proteins have 66% sequence identity. The alignment was carried out with MUSCLE (2) using default values and presented with Jalview (3).

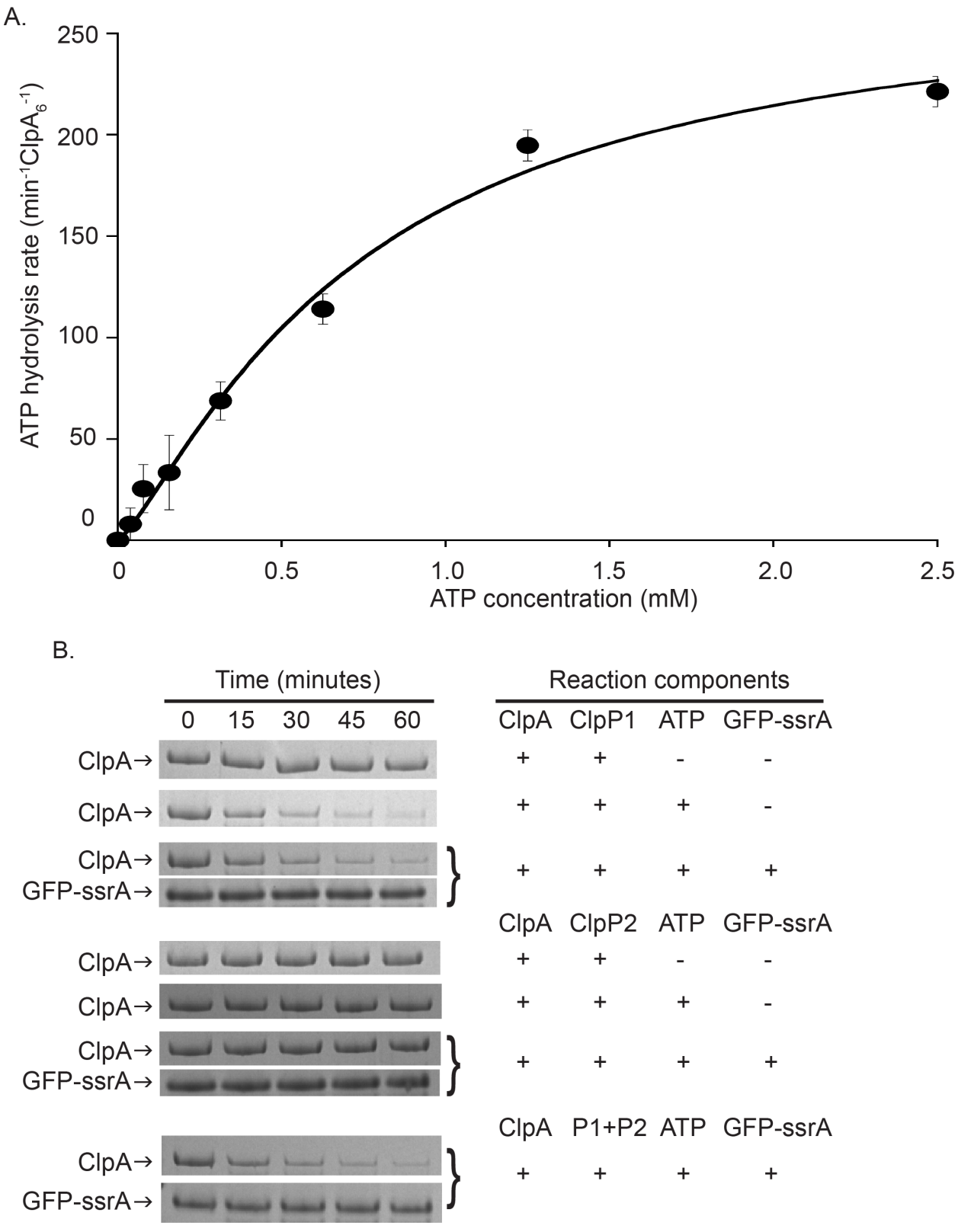

A.
250
200
150
100
50
0
ATP hydrolysis rate (min-1ClpA6-1)
0
0.5
1.0
1.5
2.0
2.5
ATP concentration (mM)
B.
Time (minutes)
0 15 30 45 60
ClpA→
ClpA→
ClpA→
GFP-ssrA→
ClpA→
ClpA→
ClpA→
GFP-ssrA→
ClpA→
GFP-ssrA→
Reaction components
ClpA ClpP1 ATP GFP-ssrA
+ + - -
+ + + -
+ + + +
ClpA ClpP2 ATP GFP-ssrA
+ + - -
+ + + -
+ + + +
ClpA P1+P2 ATP GFP-ssrA
+ + + +

Figure S6. Enzymatic assays with purified ClpA. (A) The rate of ATP hydrolysis by *P. aeruginosa* ClpA increases as a function of ATP concentration. Data were fit to the Hill version of the Michaelis-Menten equation (solid line). Graphic analysis of the data indicated a half-maximal ATP concentration for assembly of ~0.7 mM and a Hill coefficient of 1.3. These data demonstrate cooperative assembly and active hydrolysis of ATP by purified ClpA. (B) ClpA is auto-digested by ClpP1 but not by ClpP2. Reaction mixtures were prepared in HO buffer (50 mM HEPES-KOH pH 7.6, 20 mM $MgCl_2$, 0.3 M NaCl, 10% glycerol, 0.5 mM DTT) using 0.1 μM $ClpA_6$, 0.2 μM $ClpP1_{14}$ or $ClpP2_{14}$, the ATP regeneration system (described in Material and Methods) and 2 μM GFP-ssrA. Mixtures were incubated at 30°C and aliquots were withdrawn for SDS-PAGE analysis at each indicated time point. ClpA was digested in the presence of ClpP1 and ATP regeneration system. GFP-ssrA was not digested under any conditions and did not prevent or delay digestion of ClpA, and reactions with both ClpP1 and ClpP2 behaved the same as those with ClpP1 by itself.

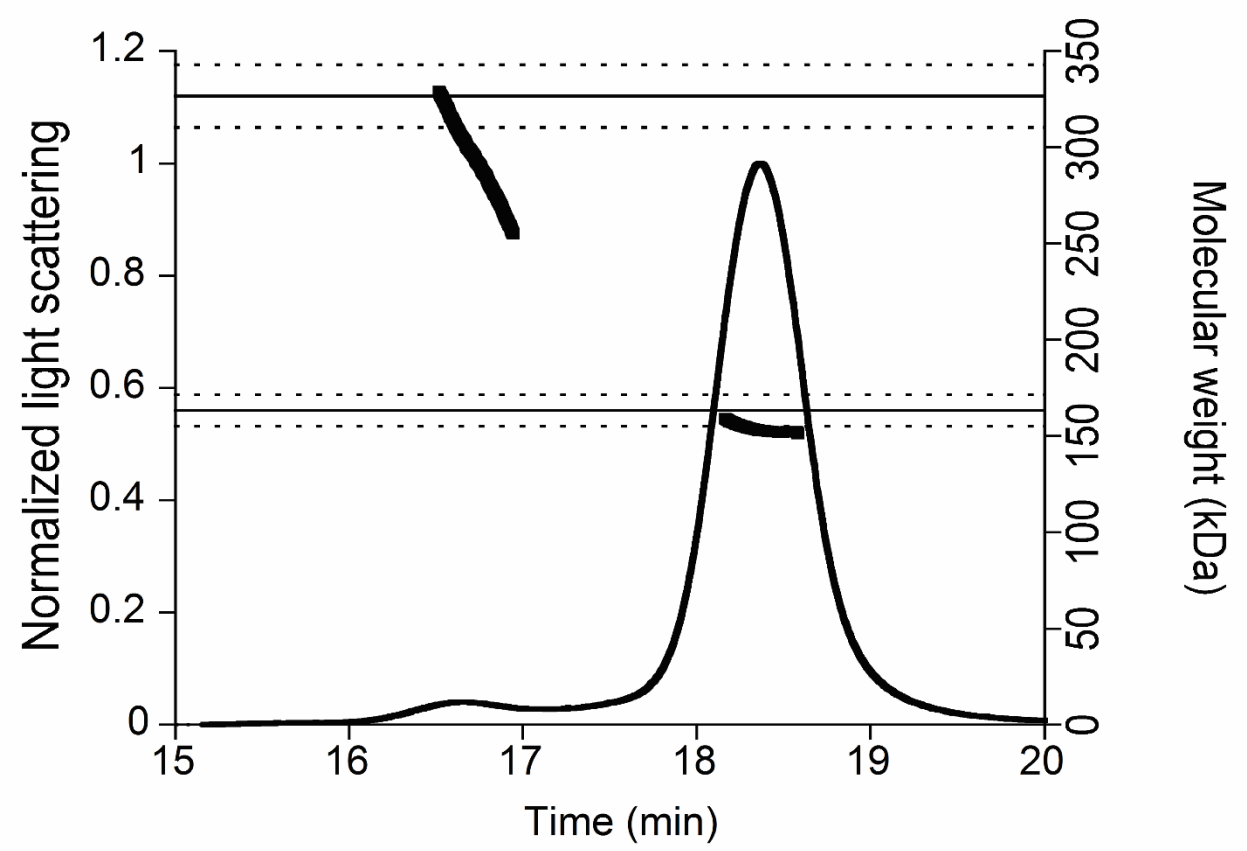


Figure S7. ClpP2 assembles as heptamers at high concentrations. SEC-MALS experiments were used to demonstrate that ClpP2 (93 μM or 30 mg/ml loading concentration) formed heptamers (expected M_R ~163 kDa) and potentially a very small population of tetradecamers (expected M_R ~327 kDa). Dashed lines represent an error of 5% in measurement of molecular weight. Calculated molecular weights for the heptamer and tetradecamer peaks were 155 ± 0.9 kDa and 272 ± 0.2 kDa, respectively. Size exclusion chromatography was performed on a Wyatt WTC-030S5 size exclusion column using an Agilent HPLC. MALS was measured in line using a Wyatt Dawn-HELEOS instrument and concentrations were determined using a Wyatt OptilabrEX instrument. Standard Zimm-plot analysis was carried out with ASTRA software 5.3.4

(Wyatt Technology). ClpP2 samples were run in triplicate with 25 μl load volume of 30 mg/ml protein in 0.1 M Tris pH 8, 0.15 M NaCl at room temperature.

Table S1. Primers used in this study.

Primer name	Sequence
ΔP1-pMQ30-1	GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGC TTGATGTCGAC
ΔP1-	GGAAAACCCTGGCGTTACCCGTCTTGCGATCACTCCCTAA
ΔP1-pMQ30-3	GGGTAACGCCAGGGTTTTCCGGCTCCGCAATACTGGCGGT
ΔP1-pMQ30-4	CCAGGCAAATTCTGTTTTATCAGACCGCTTCTGCGTTCTGA TCGAACTTGACGAGATCTTCC
ΔP2-pMQ30-1	GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCT CCAGGAGGGAAGCCGTCGGC
ΔP2-pMQ30-2	GGAAAACCCTGGCGTTACCCGCAACCTCCTGGAACTGTTG
ΔP2-pMQ30-3	GGGTAACGCCAGGGTTTTCCGTTTCCGCAGGCCGGATAAA
ΔP2-pMQ30-4	CCAGGCAAATTCTGTTTTATCAGACCGCTTCTGCGTTCTGATTGCATTCGC GGGCCACGAC
ClpP	GAGATGAATTCGTGATTATGTCTCGCAACTCTTTTATTCCGCAC

pET2	
ClpP1 XhoI pET23b	CACGCTCGAGTCAGTGATGGTGATGGTGATGGCCGGACCCGCTGCCGGAGACGGCCAGGTCGCGCTG
ClpP1 NheI pMQ71	GGGCTAGCATGTCTCGCAACTC
ClpP1 KpnI pMQ71	GGTACCTTAGACGGCCAGGTCGCG
ClpP2 NdeI pET23b	CGTGCATCATATGAAAACCGATGACAAGGAC
ClpP2 XhoI pET23b	ATGCACGCTCGAGTTATTTTTCGAACTGCGGGTGGCTCCACTGGCCAGGCAGCGTGATCTC
ClpP2 EcoRI pMQ71	GAATTCATGAAAACCGATGACAAGGACCG
ClpP2 SphI pMQ71	GCATGCTCACTGGCCAGGCAGCGT
ClpX NdeI pET23b	CGTGCATGCCATATGATGACTGATACCCGCAACGGCGAG

ClpX XhoI pET23b	GTAGCATGCTAAATGAAAACCGATGACAAGGACCGCGAAGG
ClpA AgeI pET23b	AGAGAACAGACCGGTGGTATGTTGAATCGAGAGCTCGAAGTCACC
ClpA HindIII pET23b	TCGAGTGCGGCAAGCTTTCAGGCGGGCTCCGCCGCC

Supplemental references

1. **Choi KH, Kumar A, Schweizer HP.** 2006. A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: application for DNA fragment transfer between chromosomes and plasmid transformation. J Microbiol Methods **64:**391-397.
2. **Edgar RC.** 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res **32:**1792-1797.
3. **Waterhouse AM, Procter JB, Martin DM, Clamp M, Barton GJ.** 2009. Jalview Version 2--a multiple sequence alignment editor and analysis workbench. Bioinformatics **25:**1189-1191.