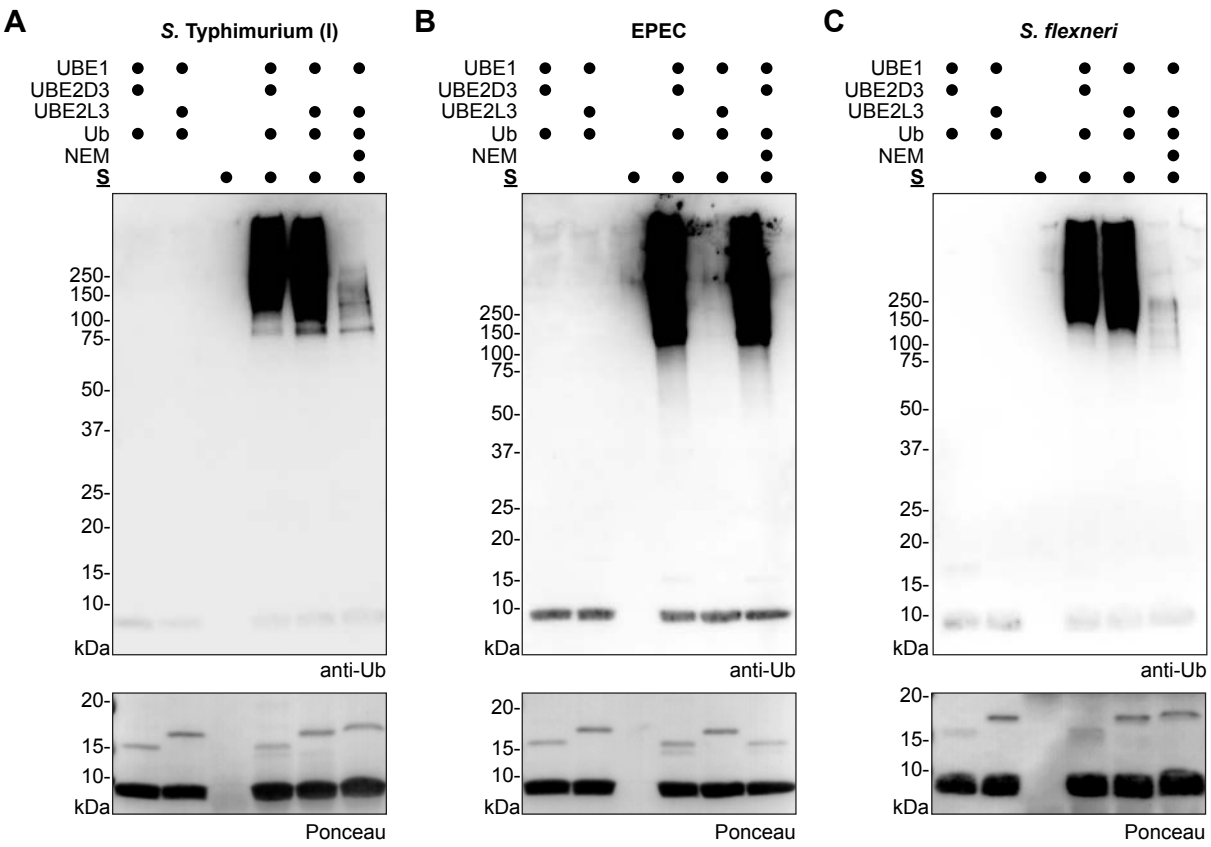
Fig S1. A functional screen for ubiquitin regulation



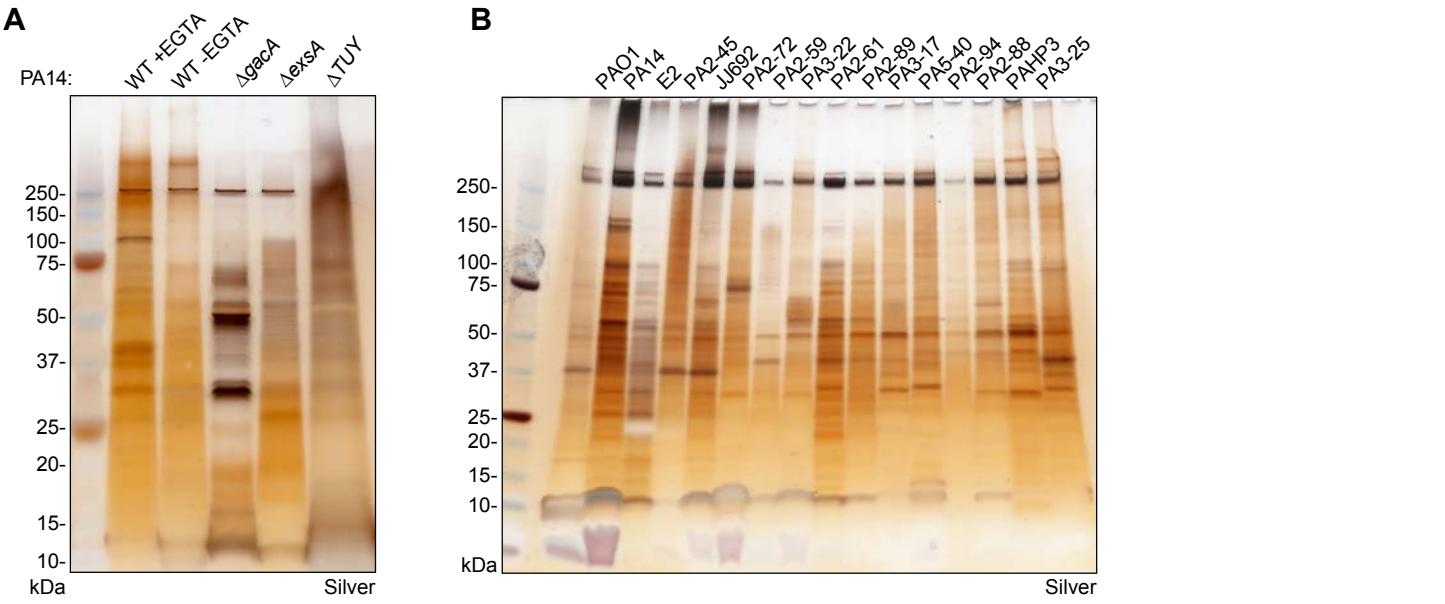
Supplementary Figure 1: A functional screen for ubiquitin regulation

A. E3 ligase assays combining the indicated reaction components with the SPI-I secreted fraction from *S.* Typhimurium, with and without prior treatment with NEM. Reactions were resolved by SDS-PAGE and visualized by anti-Ub western blot.

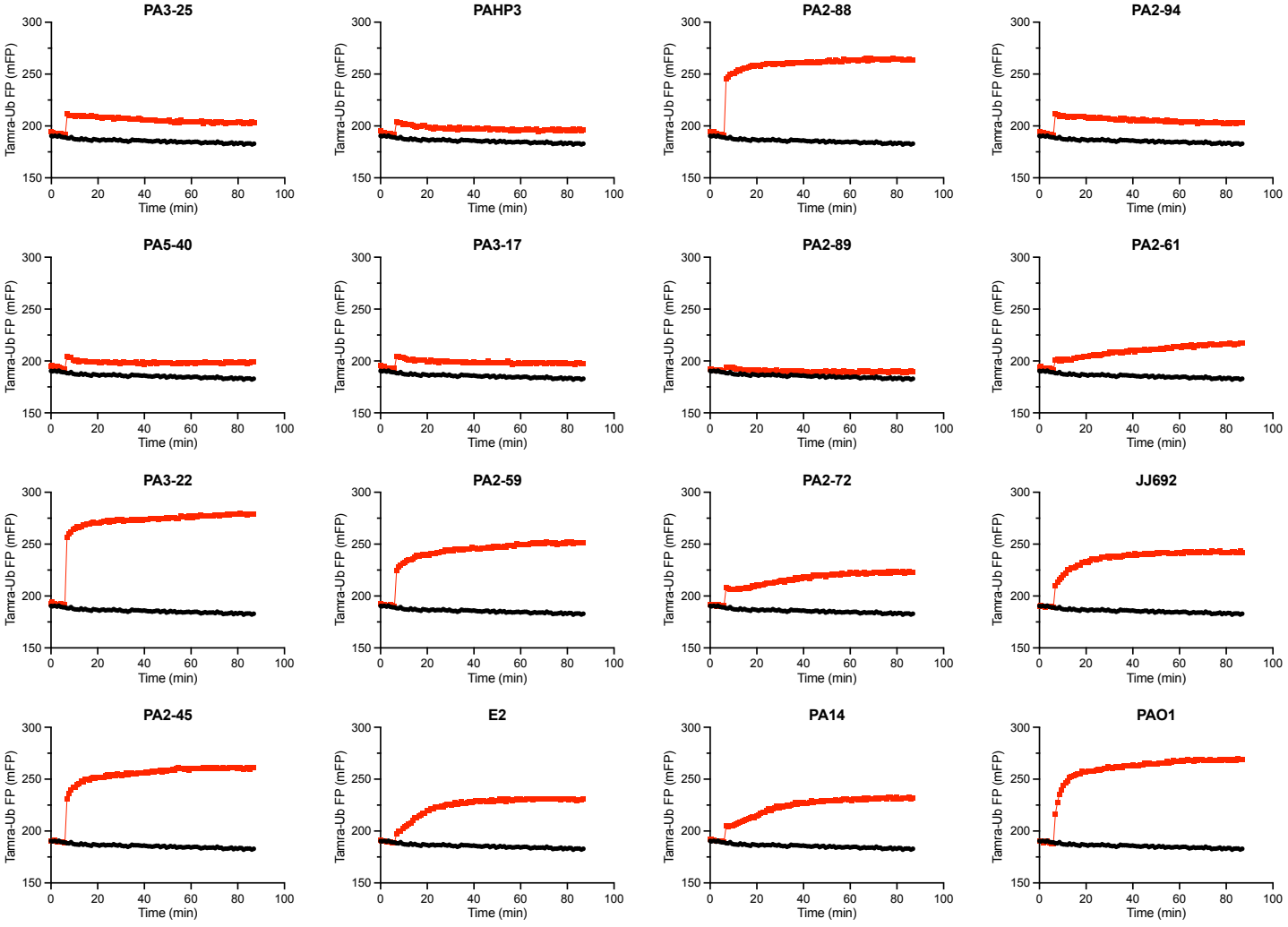
B. As in **A)**, for the secreted fraction from EPEC.

C. As in **A)**, for the secreted fraction from *S. flexneri*.

Fig S2. Detection of E3 ligase activity secreted by *P. aeruginosa*



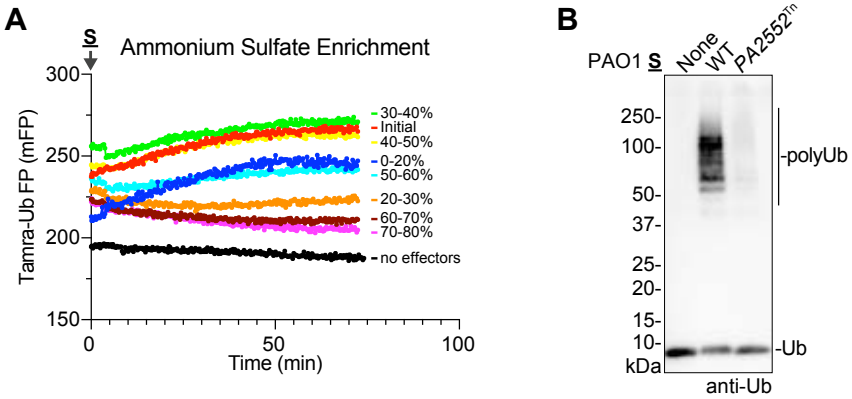
C



Supplementary Figure 2: Detection of E3 ligase activity secreted by *P. aeruginosa*

A. Silver-stained SDS-PAGE analysis of secreted fractions generated from the indicated PA14 mutant strains, or in the absence of EGTA stimulation.
B. Silver-stained SDS-PAGE analysis of secreted fractions generated from the indicated *P. aeruginosa* clinical isolates.
C. Representative FP traces monitoring the Tamra-Ub ligase substrate following addition of secreted fractions from the indicated *P. aeruginosa* clinical isolates.

Fig S3. Identification of a *P. aeruginosa* E3 ligase

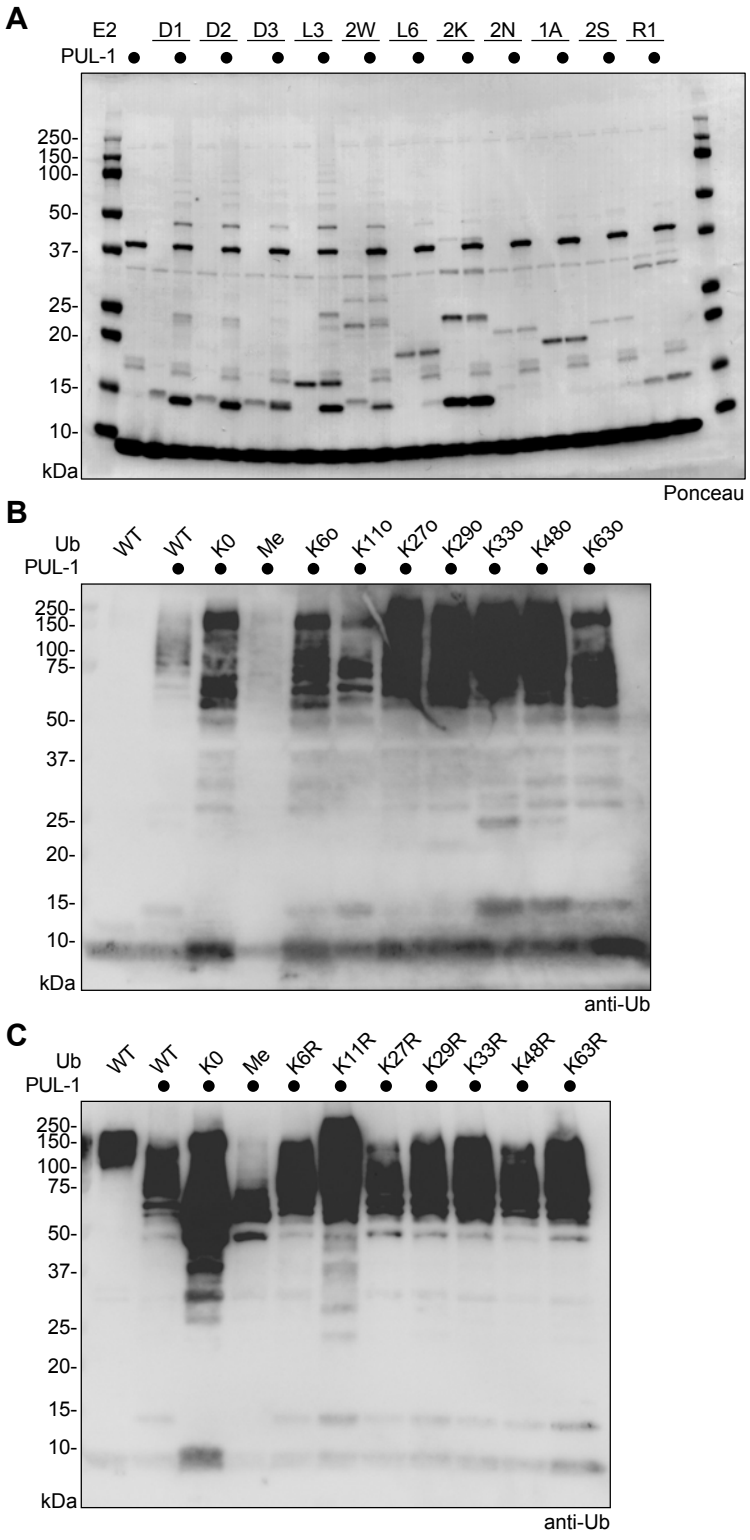


Supplementary Figure 3: Identification of a *P. aeruginosa* E3 ligase

A. Representative FP traces monitoring the Tamra-Ub ligase substrate following addition of the indicated ammonium sulfate fractions of PA14 secreted protein.

B. E3 ligase assays for secreted fractions generated from PAO1 wild-type or the *PA2552*^{Tn} mutant strain. Reactions were resolved by SDS-PAGE and visualized by anti-Ub western blot.

Fig S4. Characterization of PUL-1 E3 ligase activity



D

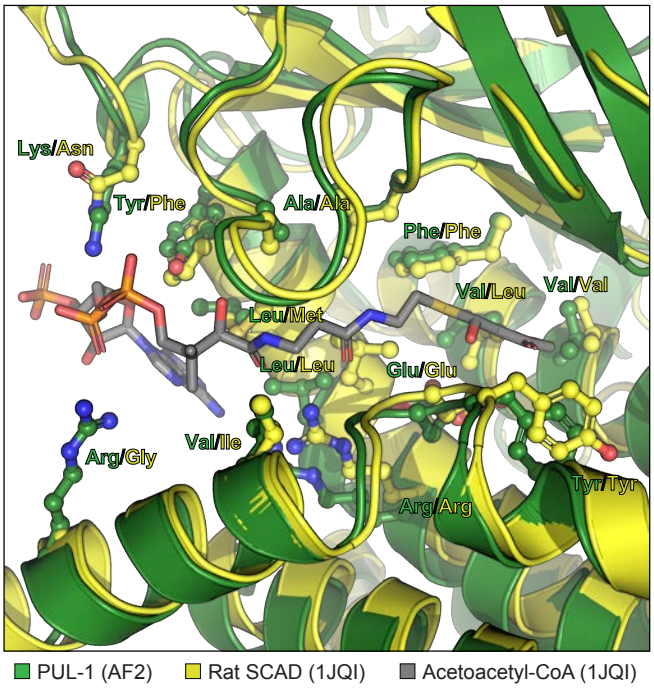
Protein	Site
PUL-1	K105
PUL-1	K136
PUL-1	K271
Ub	K6
Ub	K11
Ub	K27
Ub	K33
Ub	K48
Ub	K63

Supplementary Figure 4: Characterization of PUL-1 E3 ligase activity

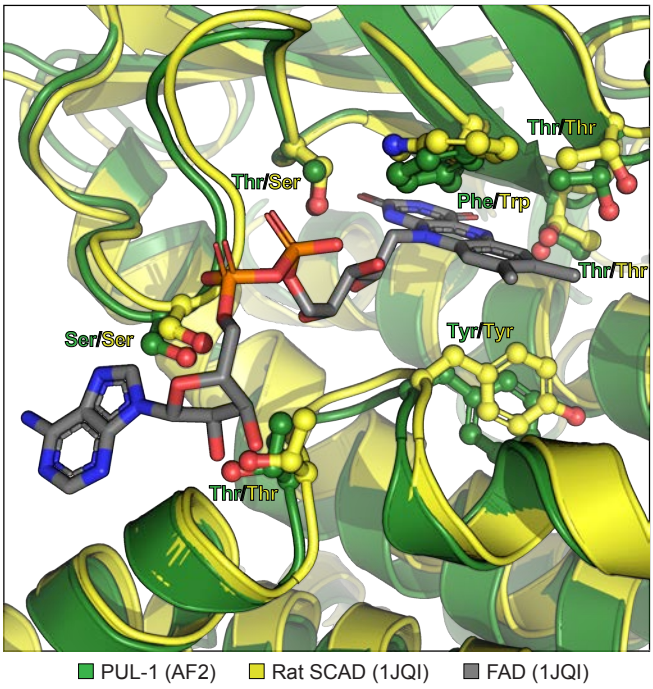
A. E3 ligase assays for recombinant PUL-1 and the indicated panel of E2 enzymes. Ponceau-stained visualization of **Figure 4D**.
B. E3 ligase assays for recombinant PUL-1 and Lys-less (K0), methylated, or the indicated panel of K-only Ub mutants. Reactions were resolved by SDS-PAGE and visualized by anti-Ub western blot.
C. E3 ligase assays for recombinant PUL-1 and Lys-less (K0), methylated, or the indicated panel of K-to-R Ub mutants. Reactions were resolved by SDS-PAGE and visualized by anti-Ub western blot.
D. Ubiquitination sites identified by mass spectrometry following an *in vitro* PUL-1 ligase reaction.

Fig S5. Structural analysis of the PUL-1 ligase fold

A



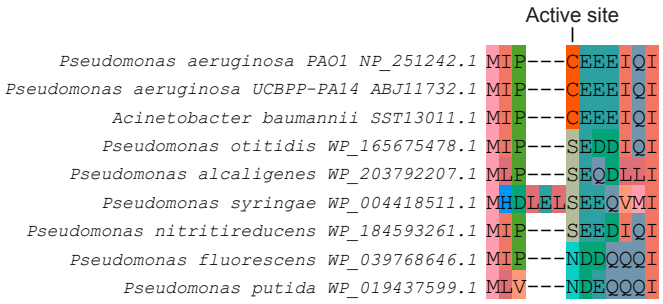
B



C

Strain	PUL-1 sequence substitutions (relative to PAO1)
PAO1	-
PA14	A372V
PA2-45	None
PA2-59	None
PA2-61	E7D, T220A
PA2-72	None
PA2-88	None
PA2-89	None
PA2-94	None
PA3-17	None
PA3-22	None
PA3-25	None
PA5-40	None
PAHP3	None
E2	R260C
JJ692	None

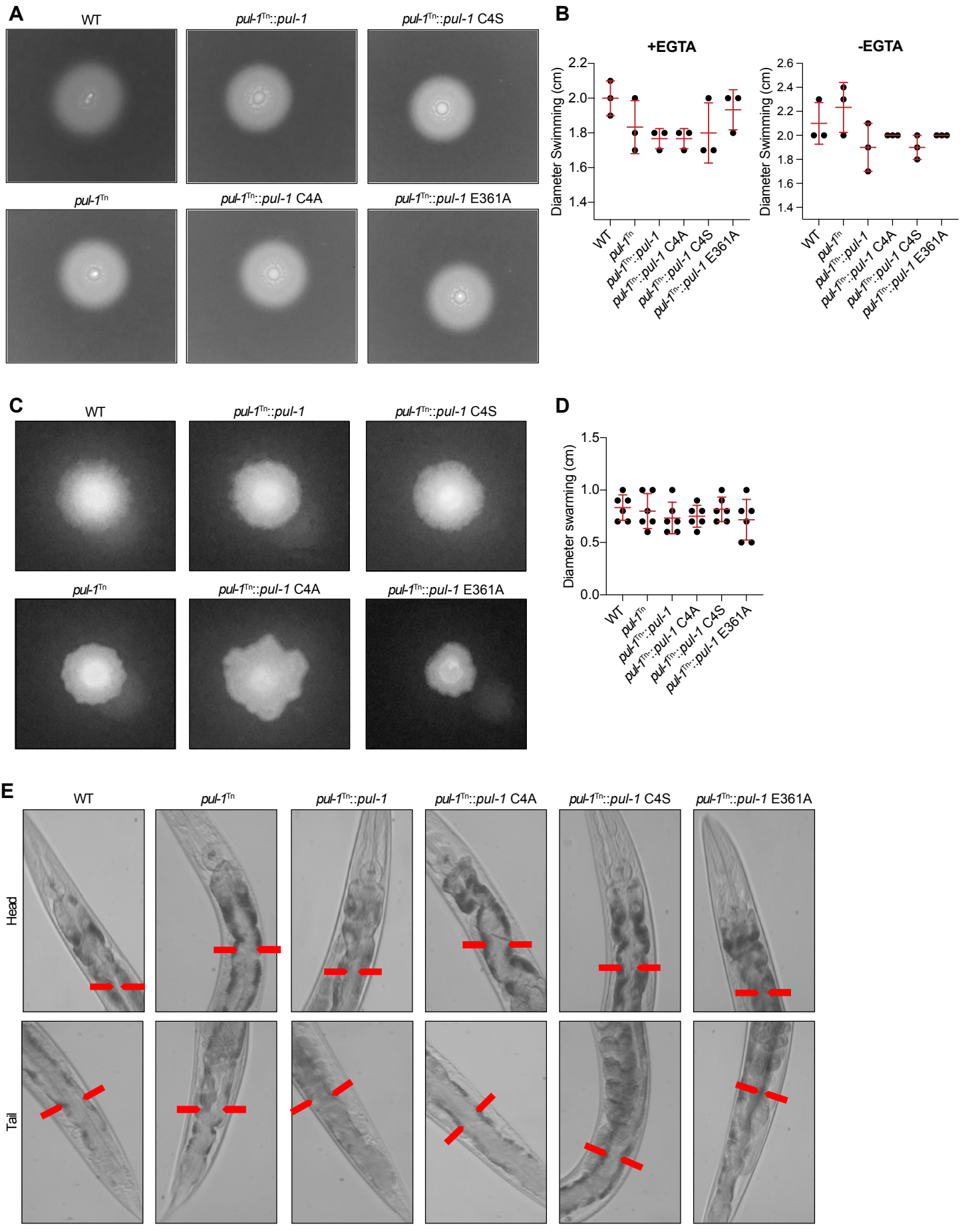
D



Supplementary Figure 5: Structural analysis of the PUL-1 ligase fold

A. Structural overlay of the mitochondrial short-chain specific acyl-CoA dehydrogenase (SCAD) from rat (yellow, PDB: 1JQI), with acetoacetyl-CoA bound (grey sticks), and the PUL-1 AlphaFold2 model (green). Residues within the acyl-CoA-binding pocket are shown in ball-and-stick for both enzymes.
B. Structural overlay of the mitochondrial short-chain specific acyl-CoA dehydrogenase (SCAD) from rat (yellow, PDB: 1JQI), with FAD bound (grey sticks), and the PUL-1 AlphaFold2 model (green). Residues within the FAD-binding pocket are shown in ball-and-stick for both enzymes.
C. Conservation of PUL-1 orthologues among all *P. aeruginosa* clinical isolates presented in **Figure 2F**. Amino acid substitutions relative to PAO1 are listed.
D. Sequence alignment of PUL-1 orthologues, focused on the region surrounding Cys4 of PAO1.

Fig S6. PUL-1 ligase activity modulates *P. aeruginosa* virulence



Supplementary Figure 6: PUL-1 ligase activity modulates *P. aeruginosa* virulence

A. Representative images of *P. aeruginosa* swimming for WT PAO1 and the indicated *pul-1*^{Tn} mutant strains.
B. Quantification of **A)**, under conditions with and without EGTA. Mean values and standard deviation are indicated in red.
C. Representative images of *P. aeruginosa* swarming for WT PAO1 and the indicated *pul-1*^{Tn} mutant strains.
D. Quantification of **C)**. Mean values and standard deviation are indicated in red.
E. Representative images of *C. elegans* intestinal bloating near the head and tail, following infection with WT PAO1 or the indicated *pul-1*^{Tn} mutant strains. The intestinal lumen diameter is indicated by black arrows.