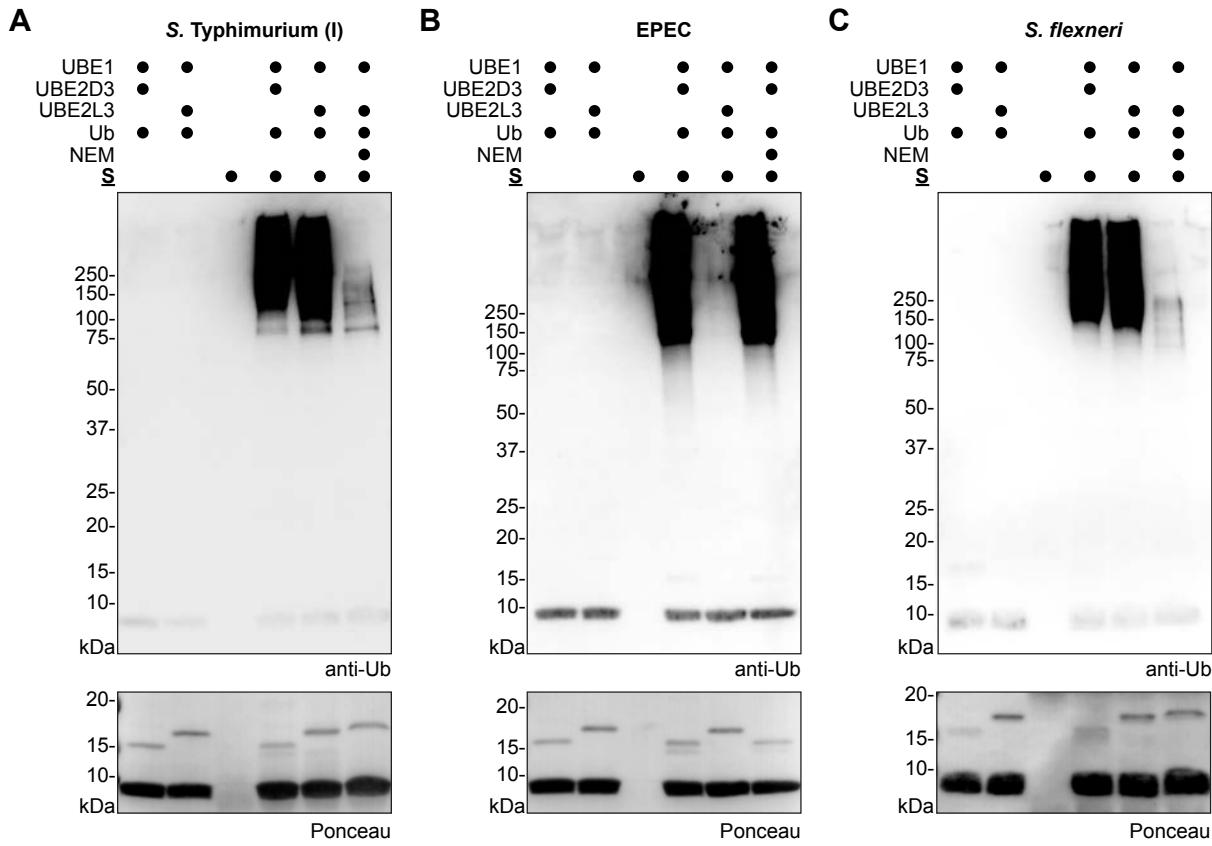


Fig S1. A functional screen for ubiquitin regulation



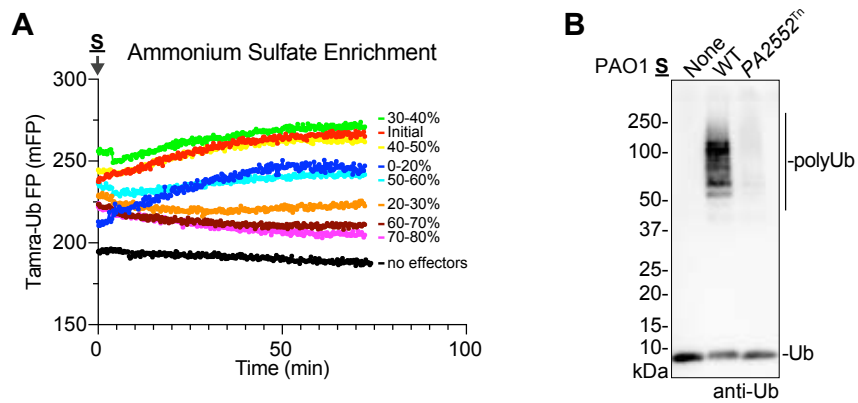
1057 **Supplementary Figure 1: A functional screen for ubiquitin regulation**

- 1058 A. E3 ligase assays combining the indicated reaction components with the SPI-I secreted
1059 fraction from *S. Typhimurium*, with and without prior treatment with NEM.
1060 Reactions were resolved by SDS-PAGE and visualized by anti-Ub western blot.
- 1061 B. As in **A**), for the secreted fraction from EPEC.
- 1062 C. As in **A**), for the secreted fraction from *S. flexneri*.
- 1063

1064 **Supplementary Figure 2: Detection of E3 ligase activity secreted by *P. aeruginosa***

- 1065 A. Silver-stained SDS-PAGE analysis of secreted fractions generated from the indicated
1066 PA14 mutant strains, or in the absence of EGTA stimulation.
- 1067 B. Silver-stained SDS-PAGE analysis of secreted fractions generated from the indicated
1068 *P. aeruginosa* clinical isolates.
- 1069 C. Representative FP traces monitoring the Tamra-Ub ligase substrate following
1070 addition of secreted fractions from the indicated *P. aeruginosa* clinical isolates.
1071

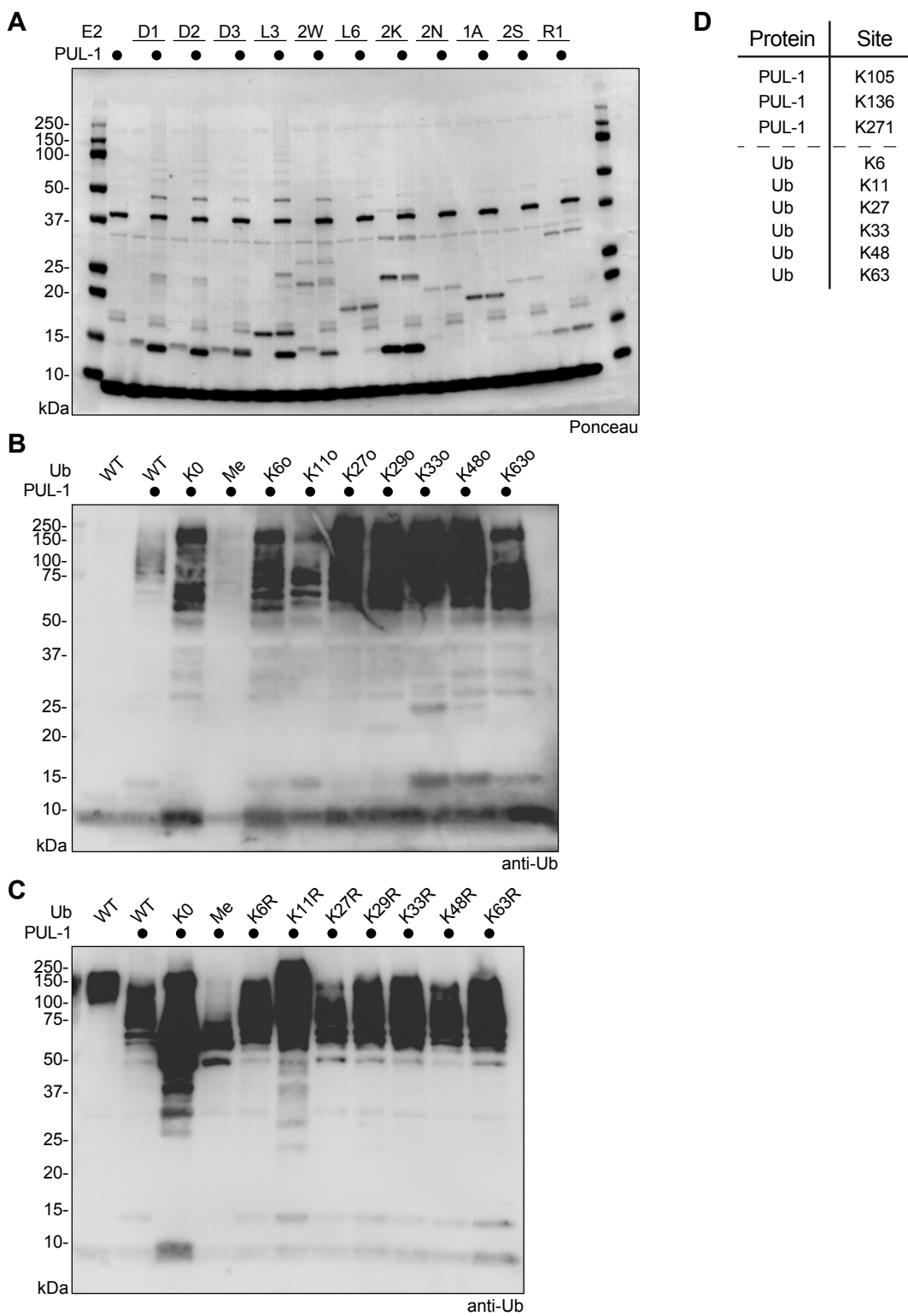
Fig S3. Identification of a *P. aeruginosa* E3 ligase



1072 **Supplementary Figure 3: Identification of a *P. aeruginosa* E3 ligase**

- 1073 A. Representative FP traces monitoring the Tamra-Ub ligase substrate following
1074 addition of the indicated ammonium sulfate fractions of PA14 secreted protein.
- 1075 B. E3 ligase assays for secreted fractions generated from PAO1 wild-type or the
1076 *PA2552^{Tn}* mutant strain. Reactions were resolved by SDS-PAGE and visualized by
1077 anti-Ub western blot.
1078

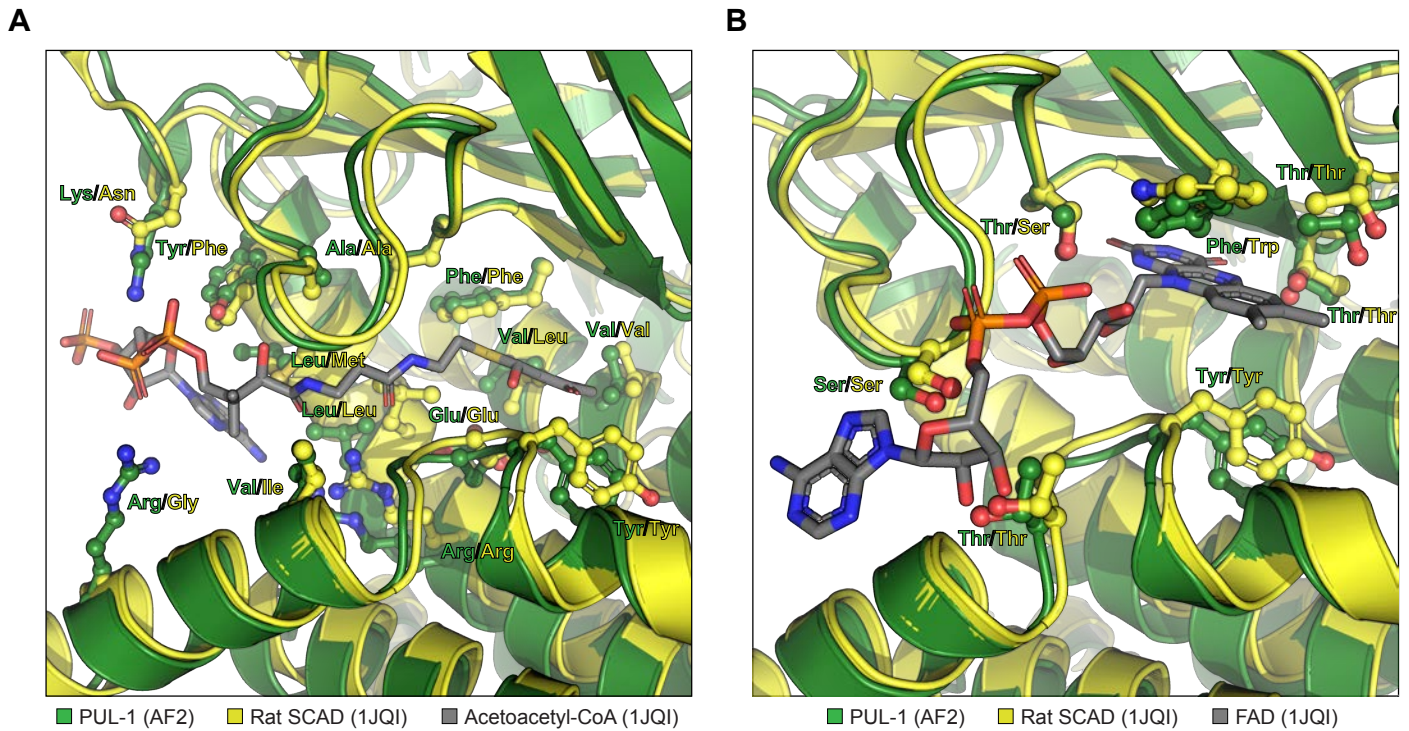
Fig S4. Characterization of PUL-1 E3 ligase activity



1079 **Supplementary Figure 4: Characterization of PUL-1 E3 ligase activity**

- 1080 A. E3 ligase assays for recombinant PUL-1 and the indicated panel of E2 enzymes.
1081 Ponceau-stained visualization of **Figure 4D**.
- 1082 B. E3 ligase assays for recombinant PUL-1 and Lys-less (K0), methylated, or the
1083 indicated panel of K-only Ub mutants. Reactions were resolved by SDS-PAGE and
1084 visualized by anti-Ub western blot.
- 1085 C. E3 ligase assays for recombinant PUL-1 and Lys-less (K0), methylated, or the
1086 indicated panel of K-to-R Ub mutants. Reactions were resolved by SDS-PAGE and
1087 visualized by anti-Ub western blot.
- 1088 D. Ubiquitination sites identified by mass spectrometry following an *in vitro* PUL-1
1089 ligase reaction.
1090

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



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

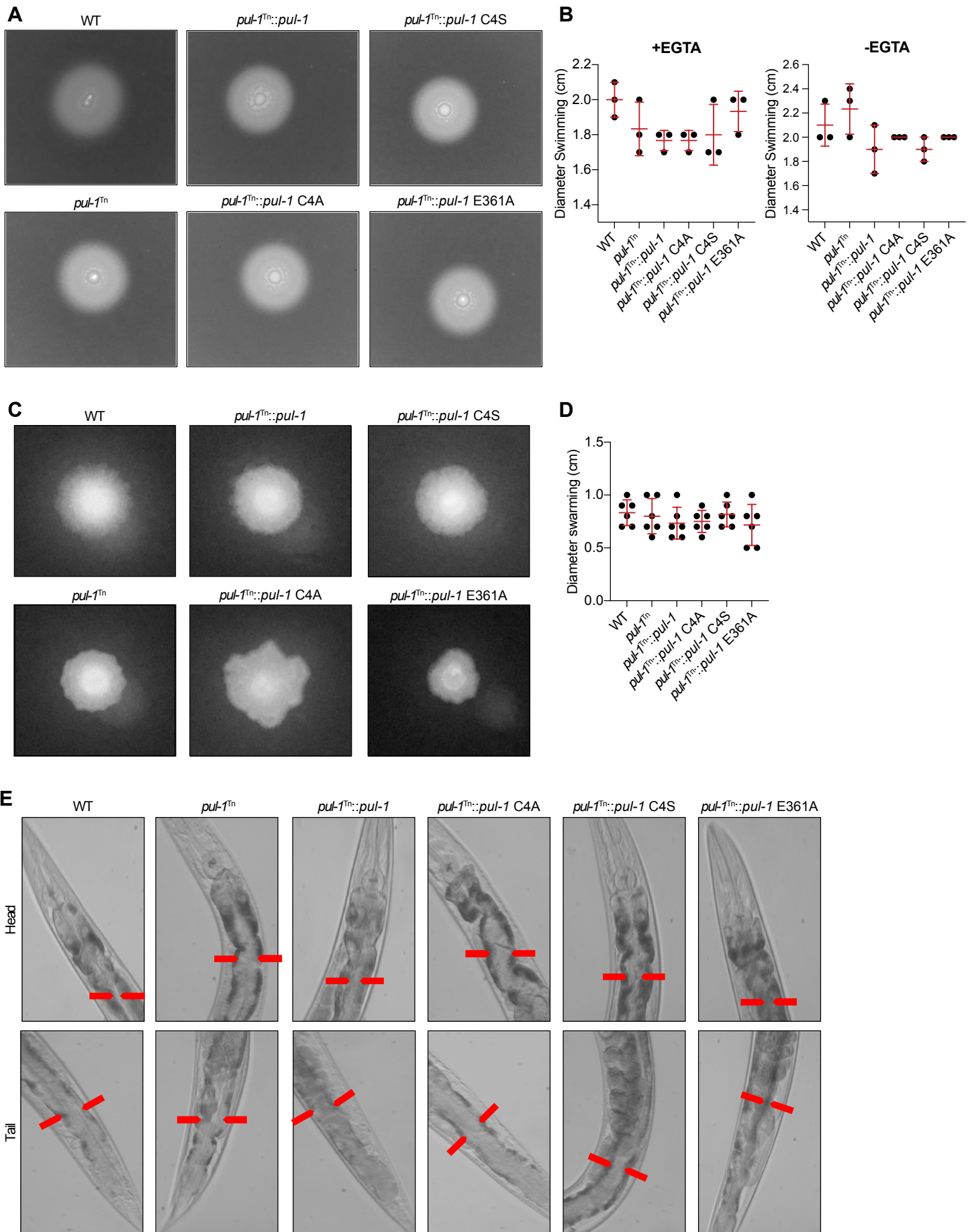
D

	Active site
<i>Pseudomonas aeruginosa</i> PAO1 NP_251242.1	MIP---C ^{EE} EIQI
<i>Pseudomonas aeruginosa</i> UCBPP-PA14 ABJ11732.1	MIP---C ^{EE} EIQI
<i>Acinetobacter baumannii</i> SST13011.1	MIP---C ^{EE} EIQI
<i>Pseudomonas otitidis</i> WP_165675478.1	MIP---S ^{ED} DIQI
<i>Pseudomonas alcaligenes</i> WP_203792207.1	MLP---S ^{EQ} DLLI
<i>Pseudomonas syringae</i> WP_004418511.1	M ^H DLEL ^S EEQVMI
<i>Pseudomonas nitritireducens</i> WP_184593261.1	MIP---S ^{EE} DIQI
<i>Pseudomonas fluorescens</i> WP_039768646.1	MIP---N ^{DD} QQQI
<i>Pseudomonas putida</i> WP_019437599.1	MLV---N ^{DE} QQQI

1091 **Supplementary Figure 5: Structural analysis of the PUL-1 ligase fold**

- 1092 A. Structural overlay of the mitochondrial short-chain specific acyl-CoA dehydrogenase
1093 (SCAD) from rat (yellow, PDB: 1JQI), with acetoacetyl-CoA bound (grey sticks),
1094 and the PUL-1 AlphaFold2 model (green). Residues within the acyl-CoA-binding
1095 pocket are shown in ball-and-stick for both enzymes.
- 1096 B. Structural overlay of the mitochondrial short-chain specific acyl-CoA dehydrogenase
1097 (SCAD) from rat (yellow, PDB: 1JQI), with FAD bound (grey sticks), and the PUL-1
1098 AlphaFold2 model (green). Residues within the FAD-binding pocket are shown in
1099 ball-and-stick for both enzymes.
- 1100 C. Conservation of PUL-1 orthologues among all *P. aeruginosa* clinical isolates
1101 presented in **Figure 2F**. Amino acid substitutions relative to PAO1 are listed.
- 1102 D. Sequence alignment of PUL-1 orthologues, focused on the region surrounding Cys4
1103 of PAO1.
1104

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



1105 **Supplementary Figure 6: PUL-1 ligase activity modulates *P. aeruginosa* virulence**

1106 A. Representative images of *P. aeruginosa* swimming for WT PAO1 and the indicated
1107 *pul-I*^{Tn} mutant strains.

1108 B. Quantification of **A**), under conditions with and without EGTA. Mean values and
1109 standard deviation are indicated in red.

1110 C. Representative images of *P. aeruginosa* swarming for WT PAO1 and the indicated
1111 *pul-I*^{Tn} mutant strains.

1112 D. Quantification of **C**). Mean values and standard deviation are indicated in red.

1113 E. Representative images of *C. elegans* intestinal bloating near the head and tail,
1114 following infection with WT PAO1 or the indicated *pul-I*^{Tn} mutant strains. The
1115 intestinal lumen diameter is indicated by black arrows.

1116