SI Appendix

SUPPLEMENTAL MATERIAL for:

Evolution of the *Pseudomonas aeruginosa* quorum sensing hierarchy

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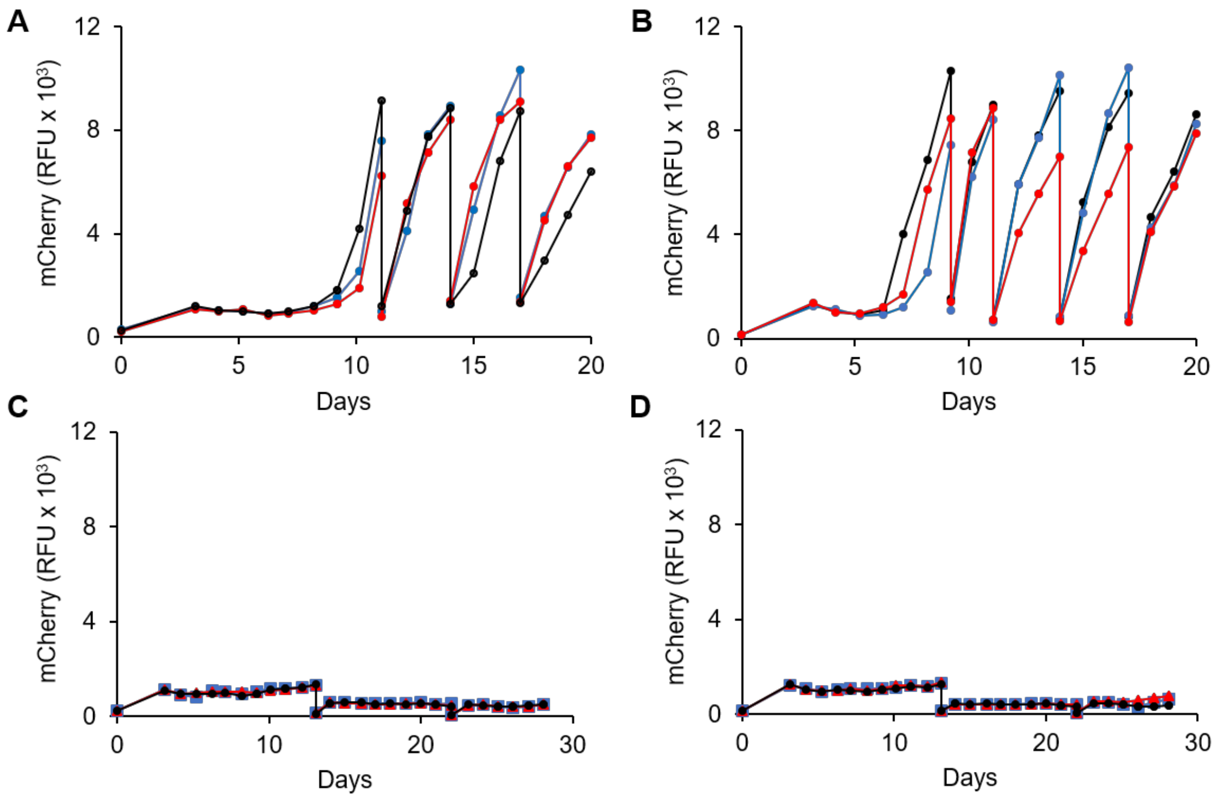

Fig. S1. Emergence of LasR and LasI mutant variants that grow in casein broth with added C4-HSL. The bacteria were marked by inserting *mCherry* in the chromosomal *att* site and growth was monitored as an increase in mCherry fluorescence (RFU). C4-HSL (30 μM) was added at the beginning of experiments and at day-4 in A (the LasR mutant) and B (the LasI mutant). The periodic steep drops in fluorescence correspond to transfers into fresh casein broth. LasR (C) or LasI (D) mutant variants did not emerge without added C4-HSL. In C and D transfers were made at day 13 and day 22. The different colors indicate independent lineages.

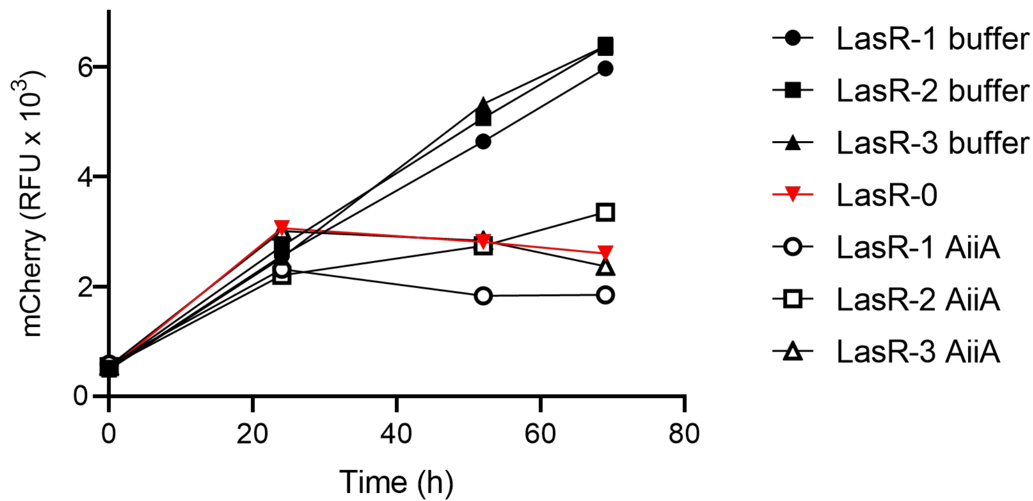

Fig. S2. Quorum sensing-dependent growth of three LasR variants isolated from cultures shown in Fig. S1. The variants (black lines) and the parent LasR mutant (red line) in casein broth (closed symbols) and casein broth plus AiiA lactonase (open symbols); variants LasR-1 (circles), LasR-2 (squares) and LasR-3 (triangles). All variants were marked with *mCherry,* and mCherry fluorescence (RFU) was a proxy for growth. Results are means of duplicate experiments; SEM values are smaller than the symbol size.

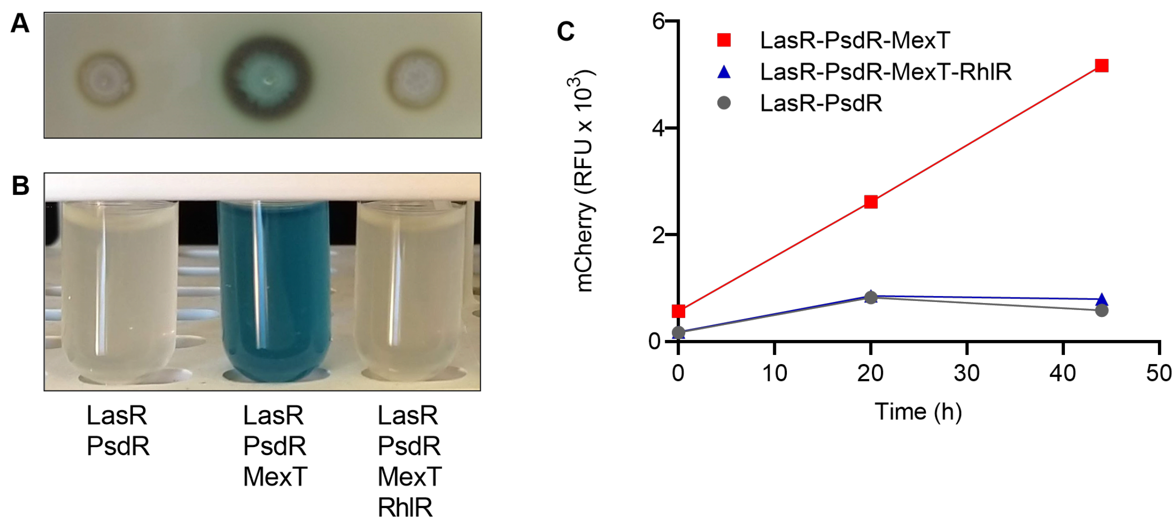

Fig. S3. The involvement of RhlR in protease production, pyocyanin synthesis, and growth on casein in a LasR mutant background. (*A*) A milk agar plate was inoculated by spotting it with 1 µL of overnight LB-MOPS cultures. Image was taken after incubation at 37°C for 22 h. From left to right: LasR-PsdR deletion mutant, LasR-PsdR-MexT mutant, and LasR-PsdR-MexT-RhlR mutant. Only LasR-PsdR-MexT has a large halo of cleared milk, indicating secreted protease activity. (*B*) Image of the mutant cultures grown overnight in *Pseudomonas* P broth, a phosphate-limited medium that enhances the production of pyocyanin (blue-green color). (*C*) The three mutants were marked with *mCherry* and growth in casein broth was followed by monitoring mCherry fluorescence (RFU). Three mL of casein broth in 18 mm tubes was inoculated with 60 µL of overnight LB-MOPS broth cultures. Data are means of duplicate experiments and error bars representing the SEM are smaller than the symbols.

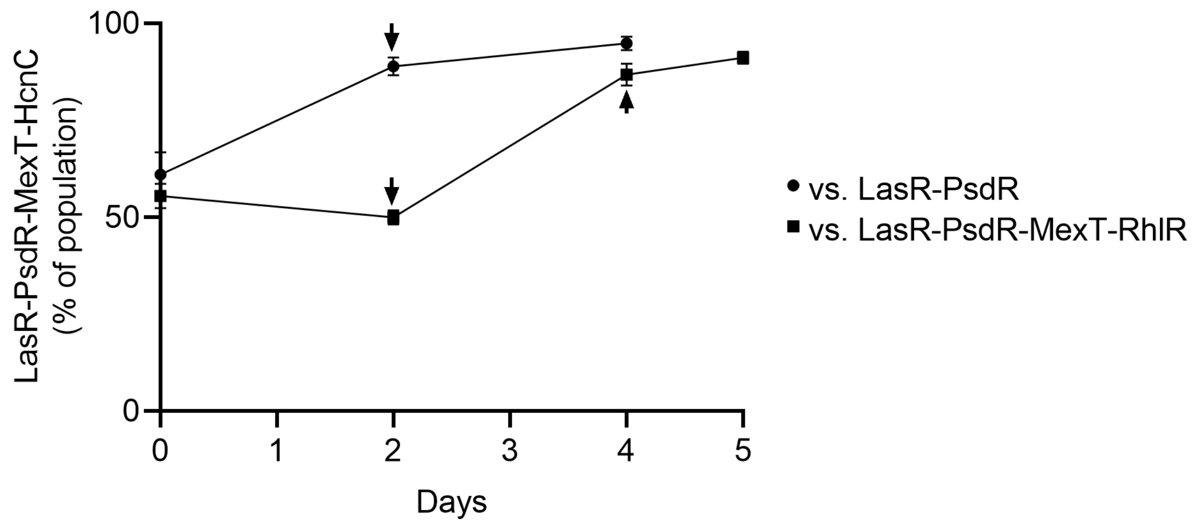

Fig. S4. Competition between the LasR-PsdR-MexT-HcnC mutant and QS mutants that cannot grow in casein broth by themselves. Arrows indicate a transfer to fresh casein broth (inoculum 2% v/v). Data are the means of duplicate experiments and error bars represent the SEM.

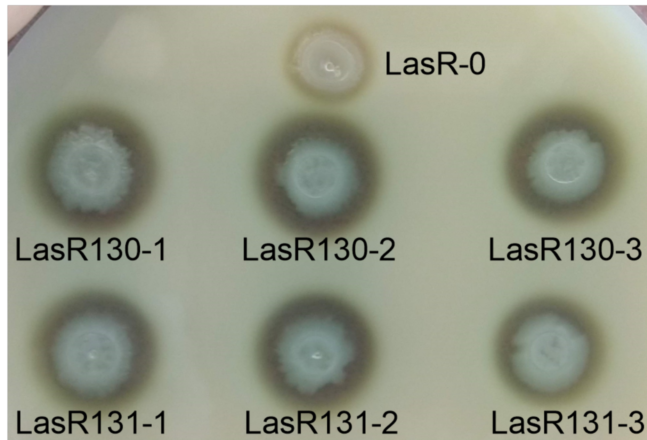

Fig. S5. LasR mutant protease-producing variants isolated from co-cultures of the PAO1 LasR mutant (LasR-0) with the clinical *P. aeruginosa* isolates E131 or E132. A milk agar plate spotted with 1 µL of overnight LB-MOPS cultures. Image was taken after incubation at 37°C for 22 h. The parent non-protease producer is at the top. See Table S1 for details about the variants.

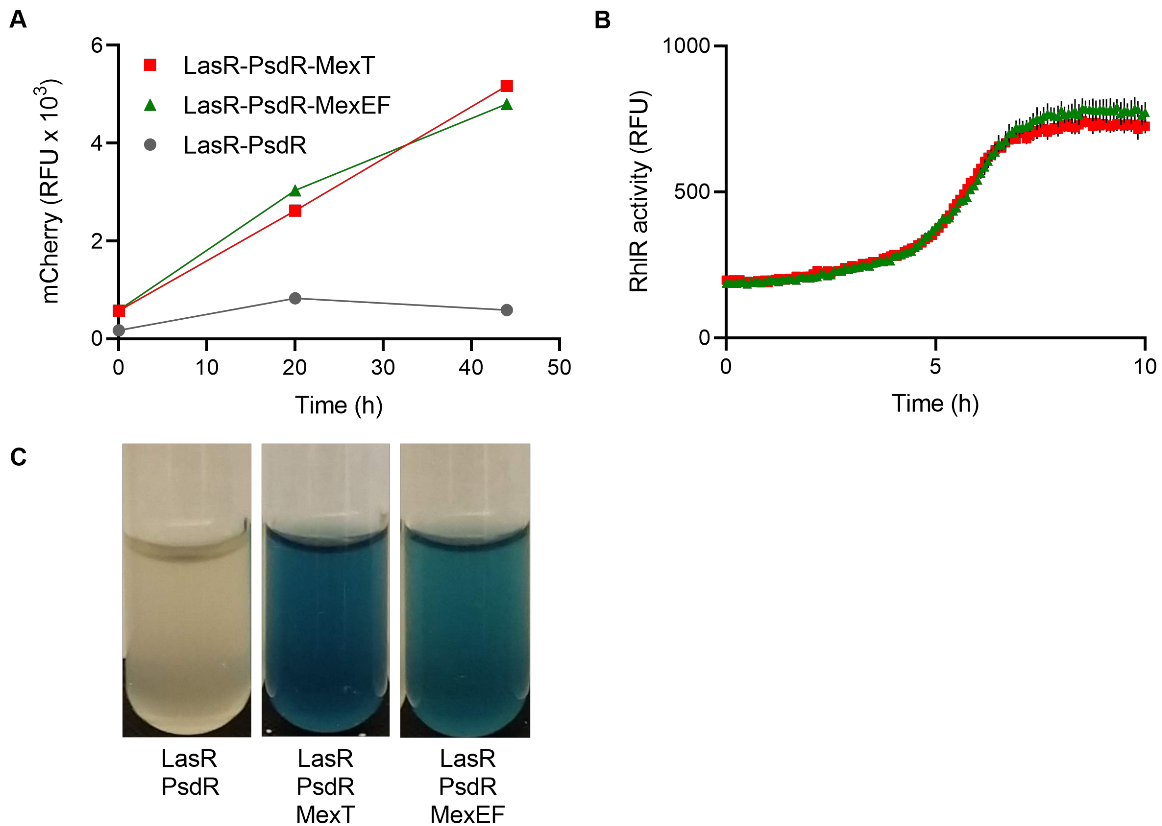

Fig. S6. Deletion of either *mexEF* or *mexT* in a LasR-PsdR mutant results in similar phenotypes. (*A*) Mutants were grown in casein broth and their growth was measured by monitoring mCherry fluorescence (RFU). Three mL of casein broth in 18 mm tubes was inoculated with 60 µL of overnight LB-MOPS broth cultures. (*B*) RhlR activity of both mutants, measured as GFP fluorescence and reported as relative fluorescence units (RFU). Both strains contained the P_{rhlA}-*gfp* fusion plasmid pProbe-GT-P_{rhlA}*gfp*. (*C*) Images of the mutant cultures grown for 20 h in *Pseudomonas* P broth, a phosphate-limited medium that enhances the production of pyocyanin (blue-green color). In panels A and B, data are means of duplicate and triplicate experiments, respectively. Error bars represent the SEM. In panel A, the error bars are smaller than the symbol size.

Table S1. Bacterial strains, and plasmids

Bacterial strain or plasmid	Description	Reference or source
P. aeruginosa		
PAO1	Wildtype	(1)
E92	Cystic fibrosis lung clinical isolate	(2)
E130	Cystic fibrosis lung clinical isolate	(2)
E131	Cystic fibrosis lung clinical isolate	(2)
LasR	PAO1 containing an unmarked, in-frame *lasR* deletion (3), marked with *mCherry* at the *att* site	This study
LasI	PAO1 containing an unmarked, in-frame *lasI* deletion (4) and marked with *mCherry* at the *att* site	This study
LasR-PsdR	Derived from LasR; contains an in-frame *psdR* deletion	This study
LasR-PsdR-MexT	Derived from LasR-PsdR; contains an in-frame *mexT* deletion	This study
LasR-PsdR-MexEF	Derived from LasR-PsdR; contains a deletion, Δ(2808249-2810363), which spans all *mexE* and part of *mexF*	This study
LasR-PsdR-MexT-RhlR	Derived from LasR-PsdR-MexT; contains an in-frame deletion in *rhlR*	This study
LasR-PsdR-MexT-PqsA	Derived from LasR-PsdR-MexT; contains a deletion in *pqsA* Δ(1078495-1079995)	This study
LasR-PsdR-MexT-PqsE	Derived from LasR-PsdR-MexT; contains an in-frame deletion in *pqsE*	This study
LasR-PsdR-MexT-PqsA/E	Derived from LasR-PsdR-MexT-PqsA; contains an in-frame deletion of *pqsE*	This study
LasR-PsdR-MexT-HcnC	Derived from LasR-PsdR-MexT; contains a deletion in *hcnC*	This study
LasR mutant variants		
LasR-1	Isolated after 7 transfers in 1% casein	This study
LasR-2	Isolated after 10 transfers in 1% casein	This study
LasR-3	Isolated after 8 transfers in 1% casein	This study
LasR130-1	Isolated after 2 transfers in 1% casein	This study
LasR130-2	Isolated after 2 transfers in 1% casein	This study
LasR130-3	Isolated after 2 transfers in 1% casein	This study
LasR131-1	Isolated after 2 transfers in 1% casein	This study
LasR131-2	Isolated after 2 transfers in 1% casein	This study
LasR131-3	Isolated after 2 transfers in 1% casein	This study
LasI mutant variants		
LasI-1	Isolated after 8 transfers in 1% casein	This study
LasI-2	Isolated after 8 transfers in 1% casein	This study
LasI-3	Isolated after 8 transfers in 1% casein	This study
E. coli		
NEB5alpha	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	New England Biolabs
S17-1	*recA* pro *hsdR* RP4-2Tc::Mu-Km::Tn7	(5)
Plasmids		
pProbe-GT-P_{rhlA}*gfp*	pVS1/p15a vector with *gfp* under the control of P_{rhlA}	(2, 6)
pEXG2	Allelic exchange vector with pBR origin, gentamicin resistance, *sacB*	(7)
pEXG2-psdR-KO	pEXG2 containing sequences for *psdR* knockout	(8)
pEXG2-mexT-KO	pEXG2 containing sequences for *mexT* knockout	This study
pEXG2-mexEF-KO	pEXG2 containing sequences for *mexEF* knockout	This study
pEXG2-rhlR-KO	pEXG2 containing sequences for *rhlR* knockout	This study
pEXG2-pqsA-KO	pEXG2 containing sequences for *pqsA* knockout	This study
pEXG2-pqsE-KO	pEXG2 containing sequences for *pqsE* knockout	This study
pEXG2-hcnC-KO	pEXG2 containing sequences for *hcnC* knockout	(3)
pUC18-mini-Tn7T-Gm-*mCherry*	Derived from pUC18-mini-Tn7T-Gm (9); used for the integration of *mCherry* at the *att* site	(10)
pFLP2	Used to express the *flp* recombinase gene for excising a resistance marker from the *mCherry* cassette delivered in the pUC18-mini-Tn7T-Gm-*mCherry*	(11)
pECP61_5_mCherry-PrhlA	Contains *mCherry* under the control of promoter from *rhlA;* used in the C4-HSL bioassay	(12)

Table S2. Primers used for knockout plasmid construction[a]

Construct	Fragment	Direction	Primer sequence
pEXG2-mexT-KO	vector	fwd	GTGGCCTTCCATGAAGGCGCACTGGTGAAGAAAGGCGACCTCGAGCTCGAATTC GGTACC
		rev	CTACTACACCGGCCTGCTGGTGGCCTACCCGGGGATCCTCTAGAGTCGAC
	upstream sequence	fwd	TGCAGGTCGACTCTAGAGGATCCCCGGGTAGGCCACCAGCAGGCCGGTG
		rev	GACGCCTCGTGCGGGTAGCTGCCGGAGGGCTCACATAGCCATTATCAATAACGA CGGGTG
	downstream sequence	fwd	TGCCTGACAAAACCACCCGTCGTTATTGATAATGGCTATGTGAGCCCTCCGGCAG CTACC
		rev	GGTACCGAATTCGAGCTCGAGGTCGCCTTTCTTCACCAGTGC
pexG2-mexEF-KO	vector	fwd	AACCAGTACGCCCTGCGTTCGCTGCTGAACTCGAGCTCGAATTCGGTACC
		rev	CACCGCCAGGACCACCTTGCGCTTGCGCCCGGGGATCCTCTAGAGTCGAC
	upstream sequence	fwd	TGCAGGTCGACTCTAGAGGATCCCCGGGCGCAAGCGCAAGGTGGTCCTG
		rev	AGCCGGGTCACTTCTTCCGGGAGCTTTTCCATGCTTGACTCCGCCAGT
	downstream sequence	fwd	AACCGACTGGCGGAGTCAAGCATGGAAAAGCTCCCGGAAGAAGTGACC
		rev	GGTACCGAATTCGAGCTCGAGTTCAGCAGCGAACGCAGGGCGTAC
pEXG2-pqsE-KO	vector	fwd	GACGCCTGCATGGCCTGTCCAGCCTCTCGAGCTCGAATTCGGTACC
		rev	CGTCTTCGAGACTCTCGCCGGCGCTCCCGGGGATCCTCTAGAGTCGAC
	upstream sequence	fwd	AGGTCGACTCTAGAGGATCCCCGGGAGCGCCGGCGAGAGTCTC
		rev	CCGCAATGGATGTCCCGTCTCACCTCAACATGGCCGGTTCACCTCCTC
	downstream sequence	fwd	AACCTGAGGAGGTGAACCGGCCATGTTGAGGTGAGACGGGACATCCATTGC
		rev	TTAAGGTACCGAATTCGAGCTCGAGAGGCTGGACAGGCCATGCAG
pEXG2-rhlR-KO	upstream sequence	fwd	TTTTTGAGCTCACGAACTGCAACGCTTTCT
		rev	TCATGGCAACCCTATCTGTTAT
	downstream sequence	fwd	CTCATGTGTGTGCTGGTATGT
		rev	TTTTTTAAGCTTGTCGAACTGGTCGAATTCCT
pEXG2-pqsA-KO	upstream sequence	fwd	gaccggaagcttTCGAGCAAGGGTTGTAACGGTT
		rev	TCAACATGCCCGTTCCTCCGGAACAGAACCTCGGTCAG
	downstream sequence	fwd	CTGACCGAGGTTCTGTTCCGGAGGAACGGGCATGTTGA
		rev	gccacggaattcCGGTTCTCGATCAGATGGTC

[a] pEXG2-mexT-KO, pEXG2-mexEF-KO, and pEXG2-pqsE-KO constructs were made by using *E. coli*-mediated DNA assembly, as described previously (13); pEXG-rhlR-KO and pEXG-pqsA-KO constructs were made using restriction enzyme-based methods, following a protocol described in ref. 14.

SI References

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