Supporting Information

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SI Text

Construction of pLapD and Variants. The *lapD* gene, including the preceding 24 bp, was cloned into pMQ72 downstream of the P_{BAD} promoter. A histidine-tagged version was cloned in the same manner, with the addition of 6 codons (CAT) before the stop codon. All site-directed mutations to *lapD* were cloned in yeast using the same vector (pMQ72) and technique: *lapD* was amplified in 2 pieces, the 5′ fragment ending just upstream of the codon to be changed and the 3′ fragment beginning just downstream. The new codon and 21 complementary bases were added to each internal primer so that recombination in yeast would yield a single amino acid change in the translated *lapD* ORF. Deletions of *lapD* were constructed in a similar way, with fragments flanking the sequence to be deleted and primers containing 25–30 bases complementary to the adjacent fragment in the completed construct.

Diguanylate Cyclase and Phosphodiesterase Activity Assays. *E. coli* strains expressing the DGC PleD and the PDE CC3396 were obtained from Urs Jenal, and these proteins were purified as described (1, 2). In vitro activity assays were conducted as described by (3) and modified by (4) with the addition of 1% Triton X-100 to the reaction buffer and an incubation time of 5 h at room temperature. Reactions were stopped with the addition of 10 μl 0.5 M EDTA and an equal volume of running buffer (1:1.5 saturated NH_4SO_4 and 1.5 M KH_2PO_4), and then were resolved by TLC. Results were visualized using a phosphor storage screen (Fig. S1, *C* and *D*), as previously described (4).

Construction of Strains for Testing LapD Responsiveness to DGC Overexpression. DGC overexpression experiments were not performed on in a Δ*lapD* background because of plasmid incompatibility between our DGC construct and pMQ72 and derivatives (the plasmids used in Δ*lapD* complementation assays). Instead, LapD alleles (WT, K446A, and E203K) were reintroduced into the native LapD locus of the Δ*lapD* mutant by single-crossover integration; then the DGC PA1107 was overexpressed from pMQ72 in these genetic backgrounds.

Construction of a Kanamycin (Kan)-marked integration vector: pMQ87 (5) was modified by (*i*) excision of the Gentamycin resistance gene with BsrG1 and SacII, (*ii*) end filling by Klenow DNA polymerase (New England Biolabs), and (*iii*) ligation to a blunt SacI, NheI fragment of pHRB2 containing the Kan resistance gene, generating pDN2.

Construction of *lapD* integration constructs: each *lapD* allele of interest was excised from the corresponding pMQ72-based complementation plasmid with XmnI and KpnI, leaving 48 bp of homologous sequence to pDN2 at the 3′ end. Additionally, a PCR fragment containing the 756 bp immediately upstream of *lapD* was amplified from genomic DNA using primers tagged with 30 bp of sequence homologous to pDN2 on the 5′ end and the *lapD* reading frame on the 3′ end (dubbed "*lapD* upstream"). Each *lapD* restriction fragment was mixed with the *lapD* upstream amplicon and SmaI-digested pDN2 and was used to transform yeast to generate recombinant plasmids by in vivo cloning. The resultant constructs are suicide vectors in *Pseudomonas* that are Kan marked and contain *lapD* with 756 bp of chromosomal sequence upstream to target the *lapD* locus for integration.

Construction of Δ*lapD*::*lapD,* and Δ*lapD*::*K446A* strains: the pDN2-*lapD* construct and derivatives were introduced to the Δ*lapD* mutant by conjugation. Integration of these *lapD* alleles into the native *lapD* locus was confirmed by PCR and phenotypic analysis; Δ*lapD*::*lapD* forms biofilms comparable to those of WT (data not shown). Plasmids containing the DGC PA1107 (and vector controls) were introduced into these strains by electroporation.

Non-Linear Regression Analysis of c-di-GMP–Binding Data. To estimate the K_d of LapD for c-di-GMP, filter-binding assay data were subjected to a non-linear regression analysis (Kaleidograph, Synergy Software). Non-specific binding (the average binding by the ΔPDE protein at a given concentration of c-di-GMP, $n = 3$) was subtracted from each of 3 data sets for LapD binding to yield specific binding data for each data set. The adjusted data were plotted as the amount of c-di-GMP bound verses the $\log_{10}$[c-di-GMP]. The sigmoidal curve of best fit (Fig. S2) and K_d were determined as described by Cushing *et al.* (6). Several additional quantitative binding assays were applied to obtain an independent estimate. However, UV crosslinking of c-di-GMP and protein, or filter-binding assays using purified LapD, reproduced data for our control (PleD) but failed to show binding for LapD comparable to our other assays (data not shown). It is possible that structural support of the Ni-silica resin or the membrane is required for LapD's in vitro activity.

Ponceau S Staining of Quantitative LapA Dot Blots. To control for any differences in the retention of cells on the nitrocellulose membrane during dot blotting, a representative blot membrane was stained for total protein using Ponceau S (Sigma). Samples were spotted onto nitrocellulose, and the membrane was washed as was done for blotting. After washing, the membrane was rinsed and stained in Ponceau S solution according to the manufacturer's instructions. The stained membrane appears in Fig. S3*B*.

Confirmation of the Topology of LapD in the Inner Membrane. After the method of Manoil *et al.* (7), 4 translational fusions were constructed so that *lacZ* or *phoA* was fused to *lapD* after each of the 2 predicted transmembrane helices (amino acid positions 31 and 176, respectively). We assessed the stability and activity of these constructs by enzymatic assays (Fig. S4) and Western blotting (data not shown). β-galactosidase (β-gal) and alkaline phosphatase assays were performed as previously described (8, 9). β-gal activity was assessed through cleavage of the chromogenic substrate o-nitrophenyl-β-galactoside. Phosphatase activity was assessed through cleavage of the fluorogenic substrate methyl-umberriferyl phosphate.

Assessing in Vivo Stability of LapD and Variants. All variants of LapD used in this study were constructed with and without histidine tags and were found to have identical complementation phenotypes with and without the tag (data not shown). To determine the stability of these proteins, the following regimen was applied to the *lapD* mutant carrying each histidine-tagged variant. Strains were grown as if for a LapA localization assay (described in *Materials and Methods*). After 6 h incubation in K10T-1, cells were harvested and lysed in 750 μl of buffer (75 mM Tris pH 8, 150 mM NaCl, and 10 mM MgCl). After lysis, cell debris was pelleted by centrifugation (15 min at 13,000 × *g*). Membranes were purified from the clarified lysate by ultracentrifugation (1 h at 100,000 × *g*, 4 °C) and resuspended in fresh buffer. Western blotting was performed on membrane preparations, and densitometry was used to determine protein stability

relative to LapD6H, as described previously (4). Stability data are listed in Table S1.

Construction of Strains with an Inducible *rapA* Gene. The *rapA* gene (Pfl01_1678) was amplified with primers containing BamH1 and Acc65I restriction sites. This product and pSRK-K_m were digested with the appropriate enzymes, and the resulting fragments were ligated to yield a plasmid in which *rapA* is under the clontrol of LacI (10). A fragment containing *lacI*-Plac-*rapA* was excised from the resulting plasmid with NheI and EcoRI and was subcloned into SpeI-EcoRI–digested pHRB2, within the Tn*7* integration cassette. This plasmid (pHRB2-SRK-*rapA*) was used to introduce the Tn*7* cassette into the chromosome as described (4).

1. Christen B, *et al.* (2006) Allosteric control of cyclic di-GMP signaling. *J Biol Chem* 281(42):32015–32024.
2. Christen M, Christen B, Folcher M, Schauerte A, Jenal U (2005) Identification and characterization of a cyclic di-GMP specific phosphodiesterase and its allosteric control by GTP. *J Biol Chem* 280(35):30829–30837.
3. Paul R, *et al.* (2004) Cell cycle-dependent dynamic localization of a bacterial response regulator with a novel di-guanylate cyclase output domain. *Genes Dev* 18(6):715–727.
4. Monds RD, Newell PD, Gross RH, O'Toole GA (2007) Phosphate-dependent modulation of c-di-GMP levels regulates *Pseudomonas fluorescens* Pf0–1 biofilm formation by controlling secretion of the adhesin LapA. *Mol Microbiol* 63(3):659–679.
5. Shanks RMQ, Caiazza NC, Hinsa SM, Toutain CM, O'Toole GA (2006) A yeast-based molecular tool kit for manipulation of gram-negative bacterial genes. *Appl Environ Microbiol* 72(7):5027–5036.
6. Cushing PR, Fellows A, Villone D, Boisguerin P, Madden DR (2008) The relative binding affinities of PDZ partners for CFTR: A biochemical basis for efficient endocytic recycling. *Biochemistry* 47(38):10084–10098.
7. Manoil C, Beckwith J (1986) A genetic approach to analyzing membrane protein topology. *Science* 233:1403–1408.
8. Miller JH (1992) *A Short Course in Bacterial Genetics* (Cold Spring Harbor Press, Cold Spring Harbor, NY).
9. Monds RD, Newell PD, Schwartzman JA, O'Toole GA (2006) Conservation of the Pho regulon in *Pseudomonas fluorescens* Pf0–1. *Appl Environ Microbiol* 72(3):1910–1924.
10. Khan SR, Gaines J, Roop RM II, Farrand SK (2008) Broad-host-range expression vectors with tightly regulated promoters and their use to examine the influence of TraR and TraM expression on Ti plasmid quorum sensing. *Appl Environ Microbiol* 74(16):5053–5062.

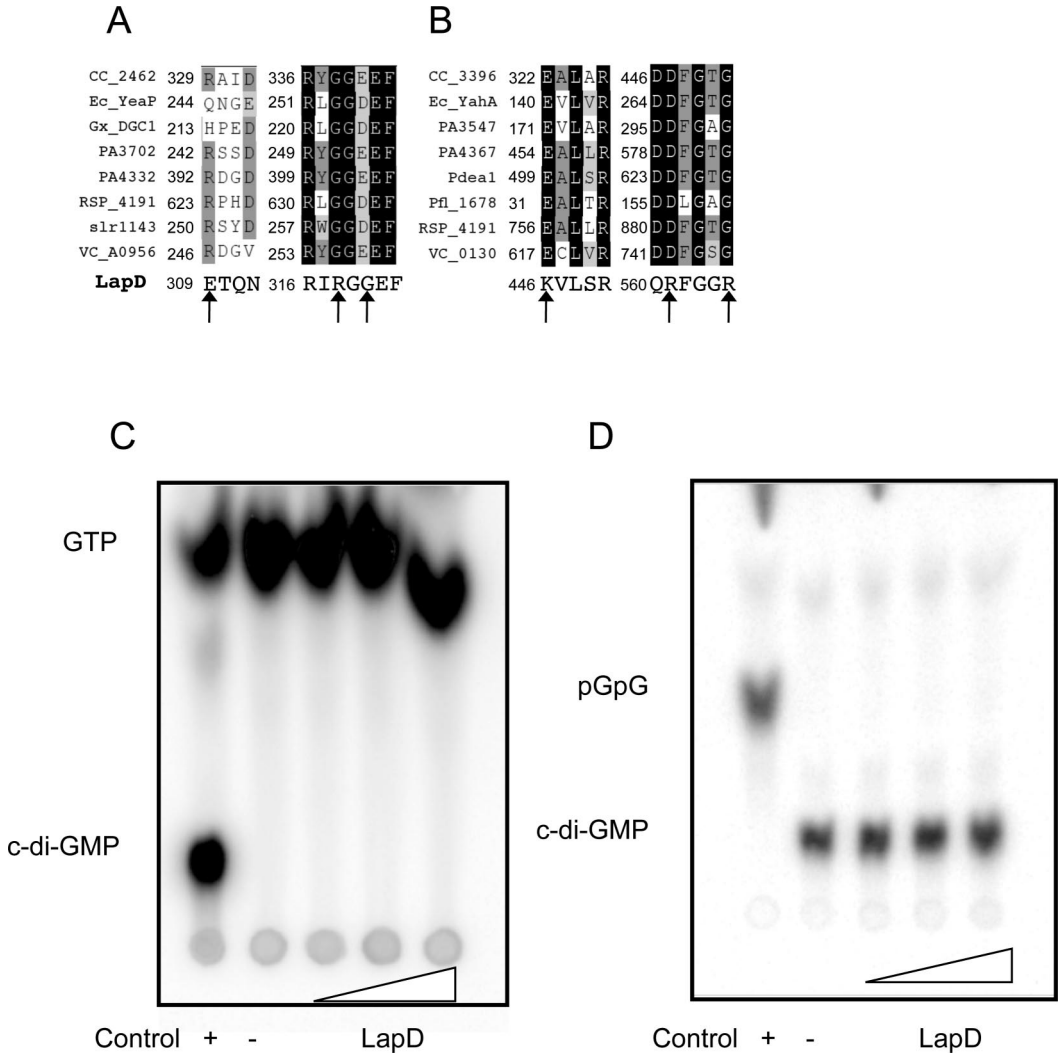

Fig. S1. Testing LapD's DGC and PDE activity in vitro. An alignment of the LapD sequence with sequences of enzymatically active DGCs (*A*) and PDEs (*B*) reveals the absence of key catalytic residues, indicated by shading in the alignment and arrows underneath the LapD sequence. The enzymatic activity of LapD was tested in DGC (*C*) and PDE (*D*) assays, the products of which are shown separated by TLC. As compared with positive controls (PleD and CC3396 of *C. crescentus*), LapD lacks both DGC and PDE activity.

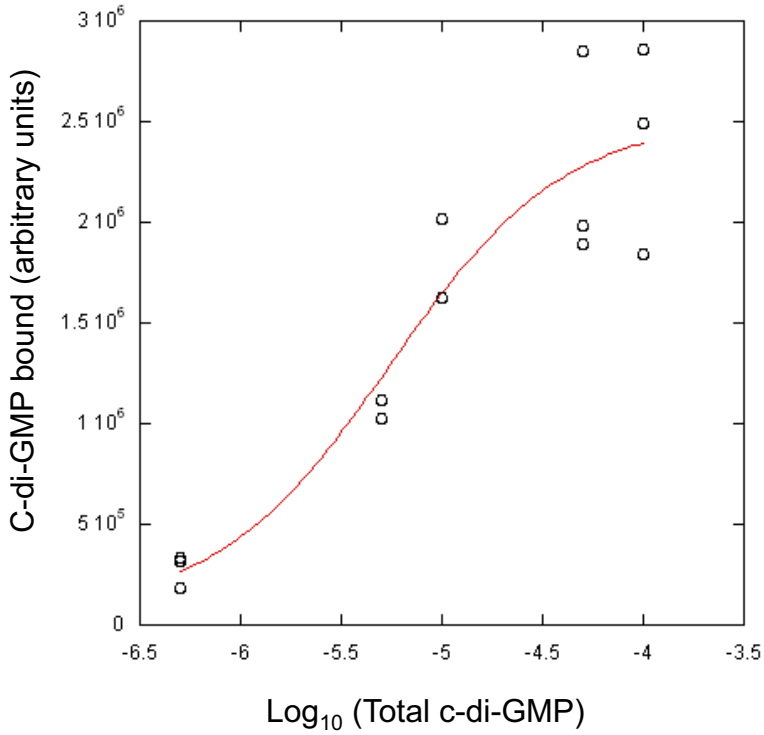

Fig. S2. Non-linear regression of specific binding of c-di-GMP by LapD in filter-binding assays. The amount of radiolabeled c-di-GMP bound by LapD-containing membranes in 3 independent assays is plotted against $\log_{10}$ of the concentration of c-di-GMP.

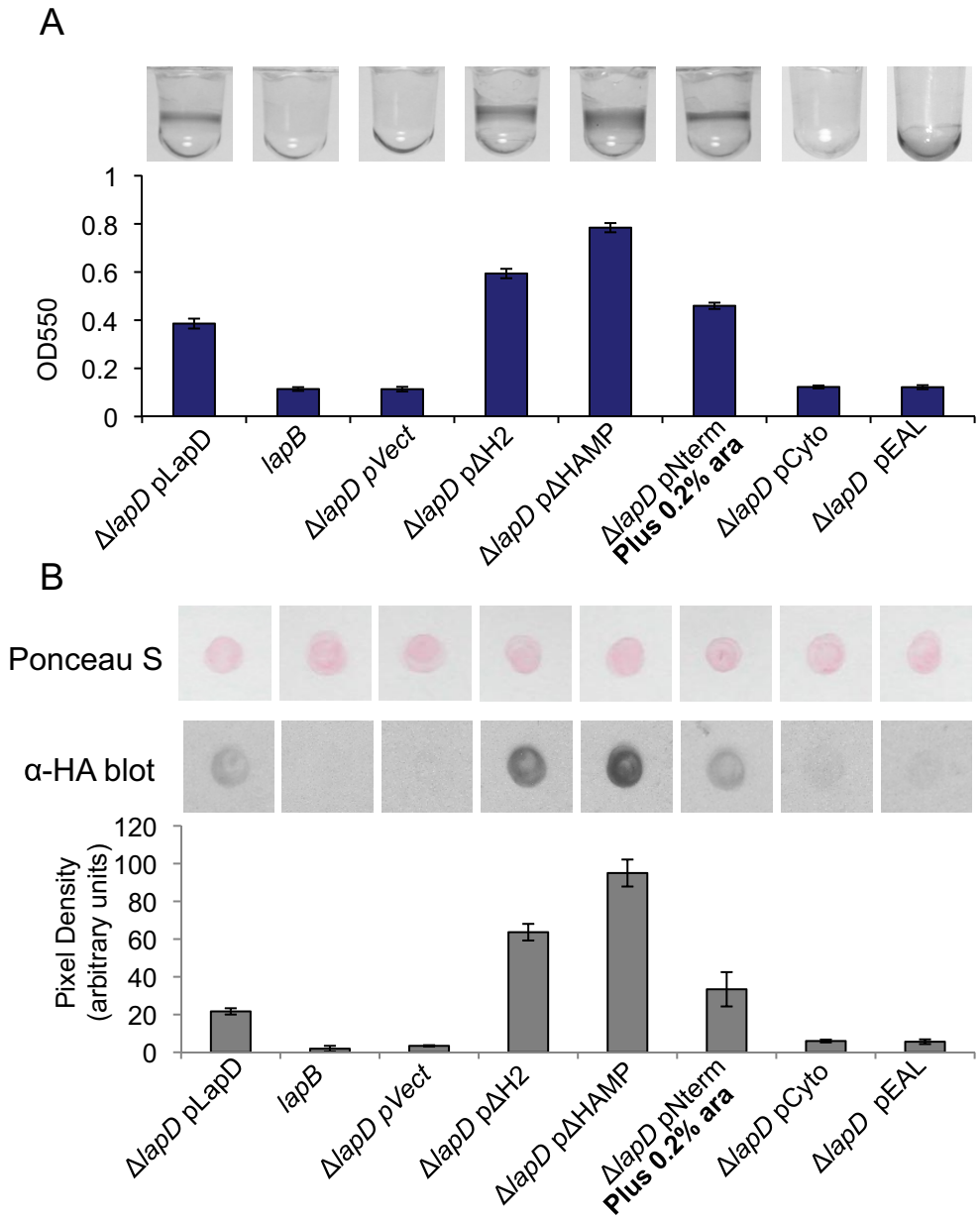

Fig. S3. Biofilm formation phenotypes and surface LapA levels of control strains and additional *lapD* derivatives. (*A*) Biofilm phenotypes of additional strains described in *Results*. The Δ*lapD* pLoop strain was grown in 0.2% arabinose; the overexpression of the first 30 codons of LapD from pLoop restores biofilm formation to Δ*lapD*. The cytoplasmic portion of the protein (pCyto) and the EAL domain alone (pEAL) do not complement Δ*lapD*. (*B*) Cell surface LapA from strains shown in (*A*) was quantified by α-LapA dot blotting ($n = 3 \pm$ SD). A Ponceau S-stained dot blot membrane and representative dot blot are shown, both treated with the same washing regimen before staining/visualization.

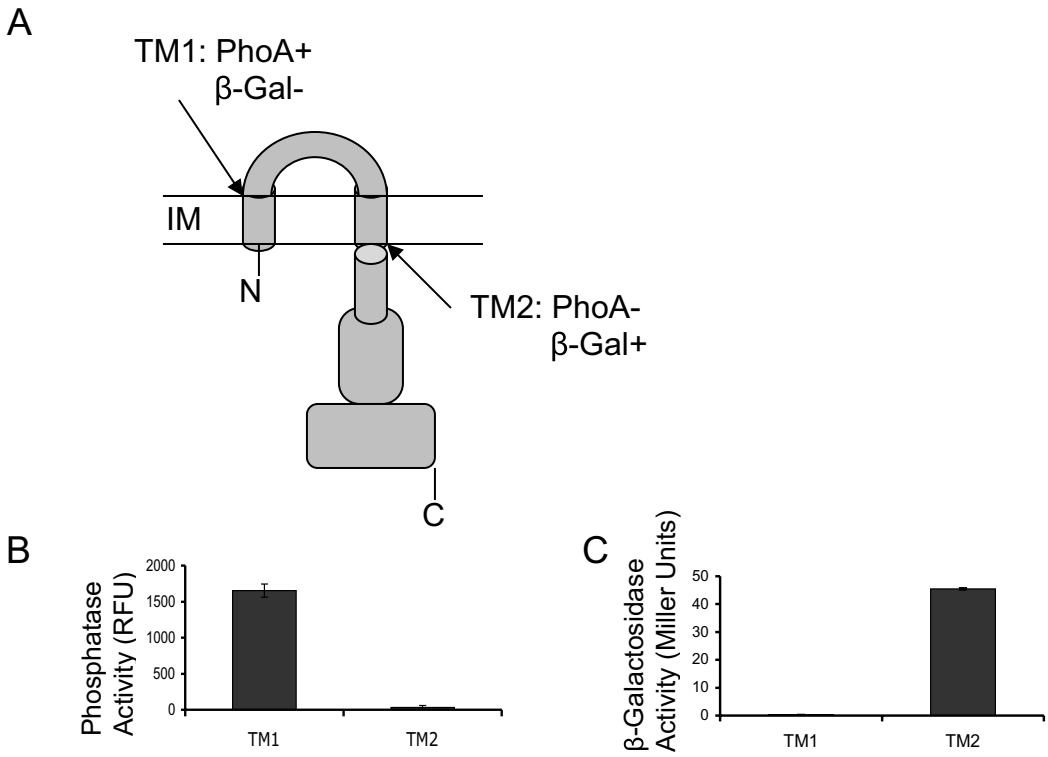

Fig. S4. Testing the predicted topology of LapD in the inner membrane. (*A*) A schematic of LapD shows the location of and activity of fusions (TM1 and TM2, respectively, indicated by arrows). (*B*) Phosphatase activity of PhoA fusions to TM1 and TM2 expressed in relative fluorescence units (RFU). Fusion of PhoA to TM1 results in enzymatic activity, but fusion to TM2 does not, suggesting that residues between TM1 and TM2 are periplasmic and residues after TM2 are cytoplasmic. (*C*) β-Galactosidase activity of fusions to TM1 and TM2, in Miller units. Only fusion of β-galactosidase to TM2 results in enzymatic activity. This finding suggests a cytoplasmic localization of residues after TM2, consistent with the PhoA fusion data.

Table S1. LapD stability in vivo

Allele	Phenotype[a]	Stability[b]
LapD	WT biofilm	100
K446A	no biofilm	92.9
R450A	no biofilm	93.8
K581A	no biofilm	98.7
E617A	no biofilm	105
E203K	biofilm reduced	90.9
E203D	WT biofilm	103
ΔH1	hyperbiofilm	156
ΔH2	hyperbiofilm	52.3
ΔHAMP	hyperbiofilm	23.8
ΔH1, R450A	hyperbiofilm	92.9
pNterm	WT biofilm upon overexpression	undetectable
pNterm-βgal	hyperbiofilm	120
pCytoplasmic	no biofilm	73.2
pEAL	no biofilm	95.8
L152P	biofilm reduced	84.1
L152P, ΔH1	biofilm reduced	97.7

[a]Phenotype indicates the ability of the allele to complement the Δ *lapD* mutant for biofilm formation, relative to the WT allele.
[b]Stability is given as percent abundance relative to WT LapD, calculated by densitometry using scanned images of representative western blots.

Table S2. Bacterial strains and plasmids

Strain or Plasmid	Genotype or Description	Reference
	Escherichia coli	
Top-10	Relevant characteristics: *recA1 araD*139 Δ (ara-leu)7697	Invitrogen
S17–1(λpir)	*thi pro hsdR- hsdM+* Δ *recA* RP4–2::TcMu-Km::Tn7	(Simon *et al.*, 1983)
	Pseudomonas fluorescens	
Pf0–1	Wild type	(Compeau *et al.*, 1988)
Δ *lapD*	Pf0–1 with unmarked deletion of *lapD*	This study
PF-013	Pf0–1 expressing *lapA*-HA	(Monds *et al.*, 2007)
PFN-001	Δ *lapD* expressing *lapA*-HA	This study
Δ *pst*	Pf0–1 with deletion of *pstSCAB-phoU*; Gm(r)	(Monds *et al.*, 2006)
Δ *pst*Δ *rapA*	Δ *pst* with unmarked deletion of *rapA*; Gm(r)	(Monds *et al.*, 2007)
Δ *pst*Δ *rapA, lapD*	Δ *pst*Δ *rapA*::pUC-*lapD*; Km(r)	This study
pst	Pf0–1::pKO-*pstC*; Tc(r)	This study
Δ *lapD, pst*	Δ *lapD*::pKO-*pstC*; Tc(r)	This study
Δ *rapA lapD* pLapD	Δ *rapA*::pUC-*lapD*; pLapD Km(r) Gm(r)	This study
Δ *lapD* Tn*7* Plac-*rapA*	Δ *lapD* with with *lacI*-Plac-*rapA* integrated at the Tn*7* site	This study
Δ *lapD* Tn*7* vector	Δ *lapD* with the HRB2 vector at the Tn*7* site	This study
Δ *lapD*::*lapD*	Δ *lapD* with the *lapD* gene integrated into the native locus via pDN2	This study
Δ *lapD*::*K446A*	Δ *lapD*::*lapD* with the K446A allele of *lapD* at the native locus	This study
Δ *lapD*::*E203K*	Δ *lapD*::*lapD* with the E203K allele of *lapD* at the native locus	This study
lapB	Pf0–1::pMQ89-*lapB.* Cannot secrete LapA due to loss of transporter	(Monds *et al.*, 2007)
	Plasmids	
p72	pMQ72	(Shanks *et al.*, 2006)
pLapD	pMQ72 expressing *lapD*	This study
pLapD6H	pLapD with the addition of six histidine codons to *lapD*	This study
pΔ GGDEF	pLapD6H with deletion of codons for R247-A387 of LapD	This study
pΔ EAL	pLapD6H with deletion of codons for H412-G649 of LapD	This study
pEAL	pLapD6H with deletion of codons for S2-G391 of LapD	This study
pK446A	pLapD6H with point mutation K446A in LapD	This study
pR450A	pLapD6H with point mutation R450A in LapD	This study
pK581A	pLapD6H with point mutation K581A in LapD	This study
pE617A	pLapD6H with point mutation E617A in LapD	This study
pΔ H1	pLapD6H with deletion of codons for M180-A186 of LapD	This study
pΔ H2	pLapD6H with deletion of codons for V207-Q213 of LapD	This study
pΔ HAMP	pLapD6H with deletion of codons for P176-Q223 of LapD	This study
pΔ H1-R450A	pΔ H1 with point mutation R450A in LapD	This study
pE203K	pLapD6H with point mutation E203K in LapD	This study
pE203D	pLapD6H with point mutation E203D in LapD	This study
pL152P	pLapD6H with point mutation L152P in LapD	This study
pΔ H1-L152P	pΔ H1 with point mutation R450A in LapD	This study
pNterm	pMQ72 expressing the first 153 codons of LapD	This study
pNterm+βgal	pLapD6H with replacement of codons P176-G649 with *lacZ*	This study
pCytoplasmic	pMQ72 expressing P176-H655 of lapD6H plus an N-term M codon	This study
pMQ83	*sacB tetR ColE1 lacZalpha oriT CEN4 ARSH6 URA3*	(Shanks *et al.*, 2006)
pLapDKO	pMQ83 based allelic exchange construct for deletion of *lapD*	This study
pKO3	Pseudomonas integration vector; MCS, *oriT, lacZ'*, Tc(r)	(Monds *et al.*, 2006)
pKO-*pstC*	Integration construct for insertional inactivation of *pstC*	This study
pLapD-TM1-Bgal	pLapD6H with replacement of codons R31-G649 with *lacZ*	This study
pLapD-TM1-PhoA	pLapD6H with replacement of codons R31-G649 with *phoA*	This study
pLapD-TM2-PhoA	pLapD6H with replacement of codons P176-G649 with *phoA*	This study
pHRB2	Vector containing Km marked Tn*7* construct	(Monds *et al.*, 2007)
pSRKKm	pBBR based vector with tightly controlled *lacI*-Plac	(Khan *et al.*, 2008)
pSRK-*rapA*	pSRK with *rapA* cloned downstream of Plac (via HindIII and XhoI)	This study
pHRB2-SRK-*rapA*	pHRB2 with *lacI*-Plac-*rapA* inside Tn*7*	This study
pPA1107	pMQ72 expressing PA1107 of *Pseudomonas aeruginosa* PA14	This study
pDN2	pMQ87 based vector with a Kan(r) in place of Gm (r)	This study
pDN2-*lapD*	pDN2 with *lapD* and 756bp of upstream genomic sequence	This study
pDN2-K446A	pDN2-*lapD* with the K446A mutation	This study
pDN2-E203K	pDN2-*lapD* with the E203K mutation	This study

Table S3. Oligonucleotides used in this study

No.	Sequence	Purpose
1	5′-CCCGTTTTTTTGGGCTAGCGA ATTCGAGCTCGGTACCCGACTAACTAGGAGCACGCGCTC	Cloning *lapD* and all derivatives
2	5′-AGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGAGCGTGGGAAAGATCAGTCAGC	Cloning untagged LapD
3	5′-TCTAGAGGATCCCCTTAATGATGATGATGATGATGTCCCCAGGGTTTTGGCTCACCGAAC	Cloning 6His-tagged LapD and all derivatives
4	5′-CAGCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCTTAATGATGATGATGA	Linker oligo for yeast cloning with primer 03
5	5′-CTC GCC CTG CCC TTC GTT GGC CAA CCC TGT GAG GTT GTC	Reverse primer for deletion of DGC domain
6	5′-CAC AGG GTT GGC CAA CGA AGG GCA GGG CGA GCA GAA C	Forward primer for deletion of DGC domain
7	5′-AGGTCGACTCTAGAGGATCCCCTTAGTGGTGGTGGTGGTGGTGATCGTCACCGACGTCTG	Reverse primer for deletion of PDE domain
8	5′-CCAAAACAGCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCTTAGTGGTG	Linker oligo for yeast cloning with primer 07
9	5′-CTCGGTACCCGACTAACTAGGAGCACGCGCTCAGATGGGCGAGCAGAACTGGGCGTG	Cloning the PDE domain of LapD
10	5′-CTC ACG ACG AGC GAT GTA ATC GAG CGG CTT CAA TTG CC	Reverse primer for making H1 deletion
11	5′-AAG CCG CTC GAT TAC ATC GCT CGT CGT GAG TTC CTG	Forward primer for making H1 deletion
12	5′-AAG CGC GAC AGC ACT GCG TAA TGC AGC ACC AGT TGC GTG	Reverse primer for point mutant K446A
13	5′-AAC TGG TGC TGC ATT ACG CAG TGC TGT CGC GCT TGC TCG	Forward primer for point mutant K446A
14	5′-GCA ATC GAT GGC AGC TAC ATC CGG G	Reverse primer for point mutant K581A
15	5′-TAG CTG CCA TCG ATT GCC AGG TAC GCC AGC CCC AGC	Forward primer for point mutant K581A
16	5′-GCT GGG GCT GGC GTA CCT GGC AAT CGA TGG CAG CTA CAT CCG GG	Linker oligo for yeast cloning with primer 14
17	5′-GTC TCG ACC CG TGC GGC AAT CAA CGG CAG ATC GAT GC	Reverse primer for point mutant E617A
18	5′-ATC TGC CGT TGA TTG CC GCA CG GGT CGA GAC GGA AGG G	Forward primer for point mutant E617A
19	5′-CTG CTC GTC GAG CAA AGC CGA CAG CAC CTT GTA ATG CAG CAC	Reverse primer for point mutant R450A
20	5′-TAC AAG GTG CTG TCG GCT TTG CTC GAC GAG CAG GGT CAG	Forward primer for point mutant R450A
21	5′-TTG CGC CGC GTA GTG CTG G	Forward primer for point mutants of E203
22	5′-AGG CGT GCG CGG CAG GTC CG	Reverse primer for point mutants of E203
23	5′-GTCTGCCGGACCTGCCGCGCACGCCTAAATTGCGCCGCGTAGTGCTGGCGATGAACCA	Linker oligo for yeast cloning with primer 19
24	5′-GTCTGCCGGACCTGCCGCGCACGCCTGATTTGCGCCGCGTAGTGCTGGCGATGAACCA	Linker oligo for yeast cloning with primer 21
25	5′-AGG ATC CCC TTA ATG ATG ATG ATG ATG ATG GCT TCA ATT GCC GAC GCA ACA AC	Cloning the periplasmic domain of LapD
26	5′-TTCACCGGCAGCTTCATGGTCAGTCTGGAGAGCTCGCCTGTTCTGGAAAACCGGGCTGC	Cloning *phoA* as a fusion to TM1 of LapD
27	5′-TAGAGGATCCCCTTAATGATGATGATGATGATGTCCTTTCAGCCCCAGAGCGGCTTTC	Cloning *phoA* as a fusion to TM1 of LapD
28	5′-TCACCGGCAGCTTCATGGTCAGTCTGGAGAGCTCGACCATGATTACGGATTCACTGGCCG	Cloning *lacZ* as a fusion to TM1 of LapD
29	5′-GGATCCCCTTAATGATGATGATGATGATGTCCTTGACACCAGACCAACTGGTAATGGTAG	Cloning *lacZ* as a fusion to TM1 of LapD
30	5′-TTG TTG CGT CGG CAA TTG AAG CCT GTT CTG GAA AAC CGG GCT GC	Cloning *phoA* as a fusion to TM2 of LapD
31	5′-TTG TTG CGT CGG CAA TTG AAG ACC ATG ATT ACG GAT TCA CTG GCC G	Cloning *lacZ* as a fusion to TM2 of LapD
32	5′-GGC CAG TGA ATC CGT AAT CAT GGT CTT CAA TTG CCG ACG CAA CAA CAA C	Cloning *lapD* portion of TM2-*phoA*
33	5′-AGC CCG GTT TTC CAG AAC AGG CTT CAA TTG CCG ACG CAA CAA CAA C	Cloning *lapD* portion of TM2-*lacZ*
34	5′-CAGCTATGACCATGATTACGAATTCGAGCTCGGTACCCCGACTTTGATGCTGTGGAGTG	Upstream homologous sequence for pLapDKO
35	5′-TCTTTCCCACGCGGAGCGTGGGAAAGATCAGTCAGGTGCTCCTAGTTAGTCGTCTCG	Upstream homologous sequence for pLapDKO
36	5′-CGAAGGATTTCCGGTCGAGACGACTAACTAGGAGCACCTGACTGATCTTTCCCACGCTC	Downstream sequence for pLapDKO
37	5′-CAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCGGCAACGACACGCTGATCG	Downstream sequence for pLapDKO
38	5′-TTA CAA GGA TCC TAA AGA GGA GAA ATT AAC TAT GCA TC	Cloning *rapA* into pSRKKm, BamH1 site
39	5′-TCA TTA GGT ACC CAT TTG AAG AGG TCA GAA CTG CTC	Cloning *rapA* into pSRKKm, Acc651 site
40	5′-CAGCTATGACCATGATTACGAATTCGAGCTCGGTACCCCGACTTTGATGCTGTGGAGTGG	Cloning upstream sequence into pDN2
41	5′-GAC AGA TAG CGA TCA ACA GCT G	Cloning upstream sequence into pDN2