

Supplementary Section

ToxR is a c-di-GMP binding protein that modulates surface-associated behaviour in *Pseudomonas aeruginosa*.

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35 **SUPPLEMENTARY TABLES**

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37 **Supplementary Table 1.** Single nucleotide polymorphisms (SNPs) and small insertions/deletions (INDELs) present in the
 38 genomic sequences of PAO1-L and PAO1-N sublines when compared to the reference genome of PAO1-UW¹.

Locus ID	Gene name	SNPs/INDELs	Description	Subline
PA0373	<i>ftsY</i>	GCCGAGC	6-bp insertion, in-frame insertion of glutamic acid-poline (EP) to a stretch of 8 EP repeats	PAO1-N
PA0683	<i>hxcY</i>	GC	1-bp insertion, frameshift at codon 73 (out of 382)	PAO1-L
PA1843	<i>metH</i>	A	GTc to GTt, silent V547V	PAO1-N
PA2492	<i>mexT</i>	-	1-bp deletion, frameshift at codon 83 (out of 348)	PAO1-N
PA2494	<i>mexF</i>	G	TAc to Tag, Y326* premature translation termination (1,062 codons in total)	PAO1-N
PA3578	-	A	Agc to Agt, silent S65S	PAO1-N
-	-	G	Intergenic PA3969- <i>amn</i> region, downstream of both	PAO1-L
-	-	C	Intergenic PA3969- <i>amn</i> region, downstream of both	PAO1-L
PA4367	<i>bifA</i>	G	tAC to gAC, Y442D substitution (687 codons in total)	PAO1-N
PA4498	<i>mdpA</i>	CGGTGC	5-bp insertion, frameshift at codon 333 (out of 406)	PAO1-N
PA4514	<i>piuA</i>	-	1-bp insertion, frameshift at codon 311 (out of 754)	PAO1-N
PA4525	<i>pilA</i>	C	aGC to gGC, S64G substitution (150 codons in total)	PAO1-N
PA5100	<i>hutU</i>	GC	Acg to Agc, T431S substitution (560 codons in total)	PAO1-L

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40 **Supplementary Table 2.** Genetic elements missing in PAO1-N as part of the ~59-kb
 41 deletion

Locus ID	Name	Description
PA0707	<i>toxR</i>	ToxR/RegA protein regulating exotoxin A production. Deletion starts 1 bp before start codon.
PA0706	<i>cat</i>	chloramphenicol acetyltransferase, antibiotic resistance gene
PA0705	<i>migA</i>	alpha-1,6-rhamnosyltransferase MigA, possibly involved in lipopolysaccharide biosynthesis
PA0704	<i>gatA</i>	putative aspartyl/glutamyl-tRNA amidotransferase subunit A
PA0703	-	probable major facilitator superfamily (MFS) transporter
PA0702	-	hypothetical protein similar to sterol desaturase/fatty acid hydroxylase
PA0701.1	-	probable AraC-like transcriptional regulator (unannotated)
PA0701	-	probable LysR-like transcriptional regulator
PA0700	-	hypothetical protein of unknown function
PA0699	-	probable peptidyl-prolyl isomerase (chaperones & heat shock protein family)
PA0698	-	hypothetical protein of unknown function
PA0697	-	hypothetical protein of unknown function
PA0696	-	hypothetical protein of unknown function
PA0695	-	probable TonB-like membrane-linking protein
PA0694	<i>exbD2</i>	predicted biopolymer transport protein ExbD2
PA0693	<i>exbB2</i>	predicted biopolymer transport protein ExbB2
PA0692	<i>pdtB</i>	phosphate depletion regulated TPS partner B
PA0691	<i>phdA</i>	prevent host death protein A
PA0690	<i>pdtA</i>	phosphate depletion regulated TPS partner A
PA0689	<i>lapB</i>	low-molecular-weight alkaline phosphatase B
PA0688	<i>lapA</i>	low-molecular-weight alkaline phosphatase A
PA0687	<i>hxcS</i>	type II secretion system protein involved in alkaline phosphatase secretion
PA0686	<i>hxcR</i>	type II secretion system protein involved in alkaline phosphatase secretion
PA0685	<i>hxcQ</i>	type II secretion system protein involved in alkaline phosphatase secretion
PA0684	<i>hxcZ</i>	type II secretion system protein involved in alkaline phosphatase secretion

PA0683	<i>hxcY</i>	type II secretion system protein involved in alkaline phosphatase secretion
PA0682	<i>hxcX</i>	type II secretion system protein involved in alkaline phosphatase secretion
PA0681	<i>hxcT</i>	type II secretion system protein involved in alkaline phosphatase secretion
PA0680	<i>hxcV</i>	type II secretion system protein involved in alkaline phosphatase secretion
PA0679	<i>hxcP</i>	type II secretion system protein involved in alkaline phosphatase secretion
PA0678	<i>hxcU</i>	type II secretion system protein involved in alkaline phosphatase secretion
PA0677	<i>hxcW</i>	type II secretion system protein involved in alkaline phosphatase secretion
PA0676	<i>vreR</i>	sigma factor regulator VreR, PUMA3 cell-surface signaling system
PA0675	<i>vreI</i>	ECF sigma factor Vrel, PUMA3 cell-surface signaling system
PA0674	<i>vreA</i>	TonB-like ECF receptor VreA, PUMA3 cell-surface signaling system
PA0673	-	hypothetical protein of unknown function
PA0672	<i>hemO</i>	predicted heme oxygenase HemO
PA0671	-	probable SOS-response cell division inhibitor
PA0670	-	probable DNA Polymerase Y-family protein
PA0669	-	Probable DNA polymerase alpha chain
PA4280.1	-	5S ribosomal RNA subunit, identical to PA0668.5
PA4280.2	-	23S ribosomal RNA subunit, identical to PA0668.4 but 2 bp. smaller
PA4280.3	-	tRNA-Ala, identical to PA PA0668.3
PA4280.4	-	tRNA-Ile, identical to PA0668.2
PA4280.5	-	16S ribosomal RNA, 1536 bp long, identical to PA0668.1 Deletion starts 308 bp after transcriptional start site

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44 **Supplementary Table 3.** Bacterial strains, plasmids and oligonucleotides used in this
 45 study.

Strain, plasmid or oligonucleotide	Relevant characteristics	Reference or origin
Strain		
<u><i>P. aeruginosa</i></u>		
PAO1-N	Nottingham collection wild type <i>P. aeruginosa</i> strain	Holloway collection, source unknown
PAO1-L	Lausanne collection wild type <i>P. aeruginosa</i> strain	Holloway collection, via D. Haas
PAZH13-N	<i>rsmA</i> in frame deletion mutant, derivative of PAO1-N	²
PAZH13-L	<i>rsmA</i> in frame deletion mutant, derivative of PAO1-L	This study
PASK10-L	<i>rsmA::Sm/Spc-lacIQ-Ptac-rsmA</i> ; IPTG-inducible, conditional <i>rsmA</i> mutant of PAO1-L	³
PAAMP2	<i>toxR::Sm/Spc-lacIQ-Ptac-toxR</i> ; IPTG-inducible, conditional <i>toxR</i> mutant, obtained by allelic exchange using pAMP5 on PAO1-L	This study
PAJD55	<i>toxR</i> in frame deletion mutant, obtained by allelic exchange using pJD22 on PAO1-L	This study
PAJD56	<i>pvdS</i> in frame deletion mutant, obtained by allelic exchange using pJD93 on PAO1-L	This study
PAJD58	<i>toxR</i> in frame deletion mutant, obtained by allelic exchange using pJD22 on PAZH13-L	This study
PAJD173	<i>pvdS</i> in frame deletion mutant, obtained by allelic exchange using pJD93 on PASK10-L	This study
PAJL176	Site-targeted Y442D mutation in <i>bifA</i> , obtained by allelic exchange using pJL108 on PAJD58	This study
PAJL186	Site-targeted D442Y mutation in <i>bifA</i> , obtained by allelic exchange using pJL103 on PAZH13-N	This study
PAJL196	Site-targeted Y442D mutation in <i>bifA</i> , obtained by allelic exchange using pJL108 on PAO1-L	This study
<u><i>E. coli</i></u>		
DH5 α	F ⁻ <i>endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20 ϕ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(<i>r_K⁻m_K⁺)</i>, λ⁻</i>	⁴
S17.1 λ pir	<i>pro</i> , <i>res</i> ⁻ <i>hsdR17</i> (<i>r_K⁻m_K⁺)</i> <i>recA</i> ⁻ with an integrated <i>RP4-2-Tc::Mu-Km::Tn7</i> , Tpr λ pir	⁵
BL21 (DE3)	F ⁻ <i>ompT gal dcm lon hsdS_B(r_B⁻m_B⁻)</i> λ (DE3 [<i>lacI lacUV5-T7p07 ind1 sam7 nin5</i>]) [<i>malB</i> ⁺] _{K-12} (λ ^S)	⁶

Plasmids

pBluescript-II KS+	Cloning vector; ColE1 replicon; Amp ^R	Stratagene
mini-CTX <i>lux</i>	Promoter-probe vector containing the <i>luxCDABE</i> operon; Tc ^R	7
pME6000	pBBR1MCS-derived broad host range multicopy vector, Tc ^R	8
pME6032	pVS1-p15A shuttle expression vector; IPTG inducible; Tc ^R	9
pME3087	Suicide vector, ColE1 replicon, Mob; Tc ^R	10
pDM4	Suicide vector; <i>sacBR</i> , oriR6K; Cm ^R	11
pHP45Ω	Source of ΩSm/Spc interposon; Amp ^R	12
pBAD TOPO TA	Topoisomerase I-activated vector for cloning of <i>Taq</i> -amplified PCR products, <i>araBAD</i> promoter for regulated expression, C-terminal polyhistidine (6xHis) tag; Amp ^R	Invitrogen
pUCP18	pUC18 derivative containing a stabilising fragment for maintenance in <i>Pseudomonas</i> ; Amp ^R , <i>E.coli</i> /Cb ^R , <i>P. aeruginosa</i>	13
pCMVDsRed-Express2	pCMV-based vector harbouring <i>dsRed-express2</i> ; Amp ^R	Takara Bio USA, Inc
<i>P_{cdrA} - gfp(ASV)^C</i>	pUCP22Not- <i>P_{cdrA}</i> -RBSII- <i>gfp(ASV)</i> -T0-T1; Amp ^R , Gent ^R	14
pMRE147	pPROBE-based vector harbouring mClover3; Cm ^R , Gent ^R	15
pAMP11	mini-CTX <i>lux</i> based transcriptional fusion containing <i>P_{toxR1}</i> promoter; Tc ^R	This study
pAMP12	mini-CTX <i>lux</i> based transcriptional fusion containing <i>P_{toxR2}</i> promoter; Tc ^R	This study
pAMP13	mini-CTX <i>lux</i> based transcriptional fusion containing <i>P_{toxR1,2}</i> promoter; Tc ^R	This study
pSH30	mini-CTX <i>lux</i> based transcriptional fusion containing <i>P_{rhlA}</i> promoter; Tc ^R	This study
pSH32	mini-CTX <i>lux</i> based transcriptional fusion containing <i>P_{rhlI}</i> promoter; Tc ^R	This study
pSC581	pBluescript II KS ⁺ derivative containing <i>dsRed-express2</i> ; Amp ^R .	This study
pSC855	pUCP18 derivative containing <i>P_{pel} - dsRed</i> transcriptional fusion; Amp ^R	This study
pSC856	pUCP18 derivative containing <i>P_{psl} - dsRed</i> transcriptional fusion; Amp ^R	This study
pMMB-1	pME6000 derivative harbouring a genomic fragment of 2.2 kb; Tc ^R	This study
pMMB-2	pME6000 derivative harbouring a genomic fragment of 2.7 kb; Tc ^R	This study

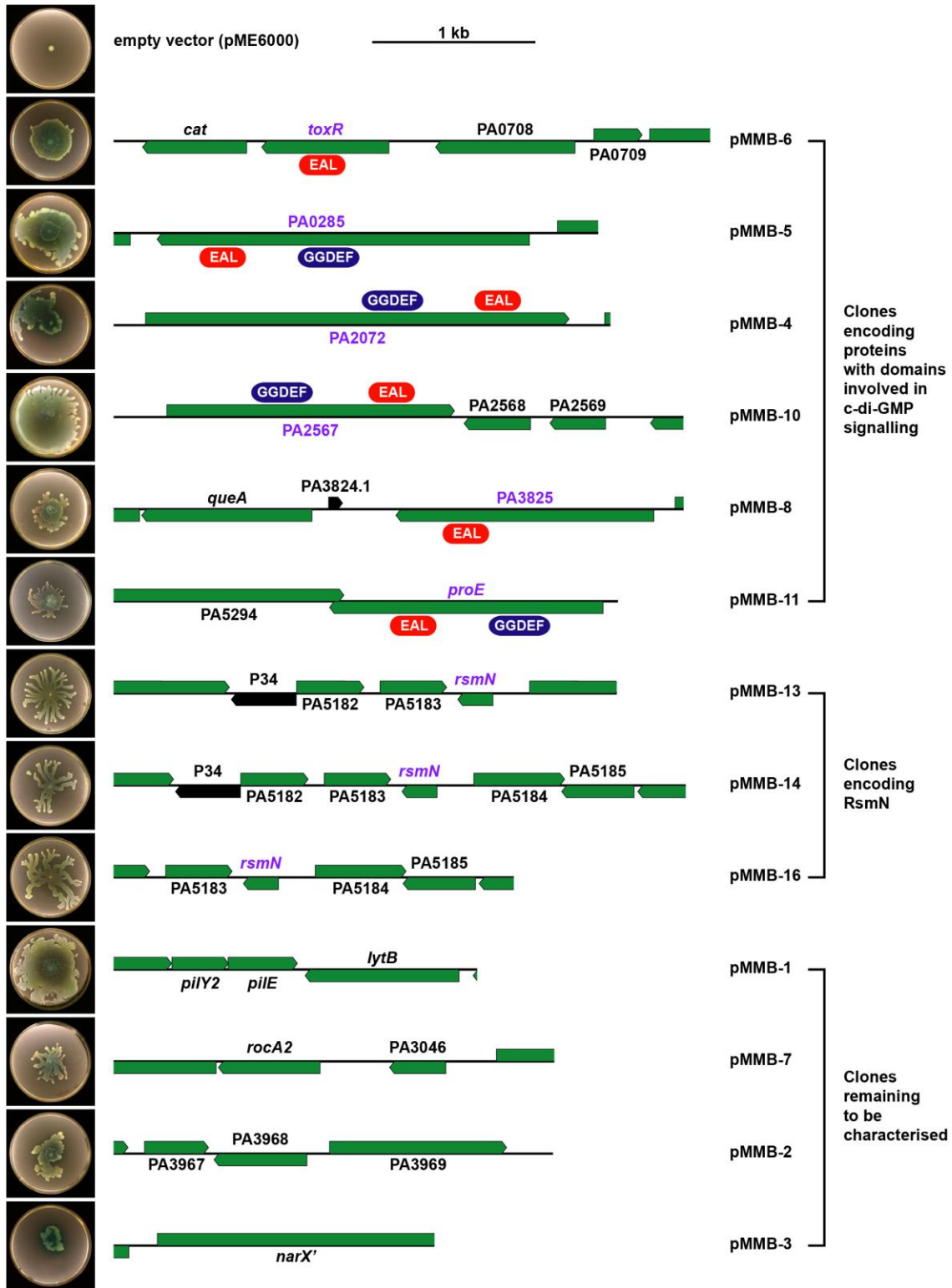
pMMB-3	pME6000 derivative harbouring a genomic fragment of 2 kb; Tc ^R	This study
pMMB-4	pME6000 derivative harbouring a genomic fragment of 3 kb; Tc ^R	This study
pMMB-5	pME6000 derivative harbouring a genomic fragment of 3 kb; Tc ^R	This study
pMMB-6	pME6000 derivative harbouring a genomic fragment of 3.7 kb; Tc ^R	This study
pMMB-7	pME6000 derivative harbouring a genomic fragment of 2.7 kb; Tc ^R	This study
pMMB-8	pME6000 derivative harbouring a genomic fragment of 3.5 kb; Tc ^R	This study
pMMB-10	pME6000 derivative harbouring a genomic fragment of 3.5 kb; Tc ^R	This study
pMMB-11	pME6000 derivative harbouring a genomic fragment of 3.1 kb; Tc ^R	This study
pMMB-13	pME6000 derivative harbouring a genomic fragment of 3.1 kb; Tc ^R	This study
pMMB-14	pME6000 derivative harbouring a genomic fragment of 3.5 kb; Tc ^R	This study
pMMB-16	pME6000 derivative harbouring a genomic fragment of 2.5 kb; Tc ^R	This study
pJD22	pDM4 suicide plasmid with upstream and downstream regions of <i>toxR</i> joined together for the generation of <i>toxR</i> in frame deletion; Cm ^R	This study
pAMP5	pDM4 suicide plasmid for the generation of the <i>toxR</i> -inducible strain; Sm/Spc- <i>lacI</i> Q-Ptac- <i>toxR</i> ; Sm ^R , Spc ^R	This study
pJD93	pME3087 suicide plasmid containing upstream and downstream regions of <i>pvdS</i> joined together for the generation of <i>pvdS</i> in frame deletion; Tc ^R	This study
pJL103	pME3087 suicide plasmid harbouring <i>bifA</i> with a Y442D point mutation from PAO1-N; Tc ^R	This study
pJL108	pME3087-based suicide plasmid harbouring <i>bifA</i> from PAO1-L; Tc ^R	This study
pAMP14	pBAD TOPO-based vector harbouring <i>toxR</i> -6His; Amp ^R	This study
pZH13	pDM4 suicide plasmid with upstream and downstream regions of <i>rsmA</i> joined together for the generation of <i>rsmA</i> in frame deletion; Cm ^R	²
Oligonucleotides	Sequence (5'-3')[#]	Modification
ToxRp1FW	TATA <u>AAGCTT</u> GCGATATCCATTTTCGTCTGG	HindIII
ToxRp1RV	TATGAATTCAGGGTACTTCCGGTGTTCGC	EcoRI
ToxRp2FW	TATA <u>AAGCTT</u> CGGCGGCCGATTGCCGA	HindIII

ToxRp2RV	TATGAATT <u>CGCG</u> CGTTATCGGATGATTAGG	EcoRI
ToxRp12FW	TATA <u>AAGCTT</u> GCGATATCCATTTTCGTCTGG	HindIII
ToxRp12RV	TATGAATT <u>CGCG</u> GAGACGACCCTACCTC	EcoRI
RhIApFW	TAT <u>CTCGAG</u> GTTTCGACACCGGAAACCG	XhoI
RhIApRV	TAT <u>CTGCAG</u> TACCAACAGACTTTTCGCGCC	PstI
RhIIpFW	TAT <u>CTCGAG</u> CGCTGGGTCTCATCTGAAG	XhoI
RhIIpRV	TAT <u>CTGCAG</u> CGATTTCAGAGAGCAATTCGA	PstI
PpelAdsRedFW	ATAGGTACCACGCAACTGAAGGCGGTGCGA	KpnI
PpelAdsRedRV	ATA <u>AAGCTT</u> CCATGCCAGCCTACGCGGCA	HindIII
PpsIAdsRedFW	ATAGGTACCTCTTCCGCCTTCGACGAGGGC	KpnI
PpsIAdsRedRV	ATA <u>AAGCTT</u> CCATGTTGTTTGCTCTGCCGA	HindIII
dsRedFW	ATAGGATCCATGGaagcttGGATAGCACT*	BamHI
dsRedRV	ATAGAATTCCTACTGGAACAGGTGGTGG	EcoRI
ToxRtopoFW	GAGGAATAATAAATGACTGCGACAGACAGAACG CCCC	N/A
ToxRtopoRV	GCAGGCCGGACTGCTGAACGGCCA	N/A
rsmAD1FW	GCTCTTGATTTCTGCGGATCCGCGCC	BamHI
rsmAD1RV	GGTCTCTCCGACCCG <u>TCTAGAT</u> TAAATCAGCAT TCC	XbaI
rsmAD2FW	CATCCAGAAAGAGAAAGG <u>TACCGAGCCAAACCA</u> TTAA	KpnI
rsmAD2RV	<u>CGGTACCCTGCAGGCCCTTT</u> CGGTATGGCGCA C	KpnI
toxRD1FW	TATGAATT <u>CGCG</u> GATATCCATTTTCGTCTGG	EcoRI
toxRD1RV	ATAGGATCCAGAGCTCGAATCCGTCGTT	BamHI
toxRD2FW	ATAGGATCCCACTATTCGGAGCCTTTCCA	BamHI
toxRD2RV	ATATCTAGAAAGACAGGCTCTTCGTGCAT	XbaI
toxRpFW	TATA <u>AAGCTT</u> GCGATATCCATTTTCGTCTGG	HindIII
toxRpRV	TATGAATTCAAGTGATGGCTCTATGGGC	EcoRI
pvdSD1FW	TATAAAGCTTATAGCGATGTACGCCATGGA	HindIII
pvdSD1RV	TATAGAATTCGTATCGCATCTGCGGGTAGA	EcoRI
pvdSD2FW	TATAGAATTCGGTGCAGTCCGCAAGGTCA	EcoRI
pvdSD2RV	TATATCTAGAACCTGCGCATTTCATCGAGGA	XbaI
BifAFW	TATGAATTCAGCAACTCGACCAGATCCTC	EcoRI
BifARS	TATA <u>AAGCTT</u> GGTCCTGGACGAAACTCTTG	HindIII

46 * Lowercase bases indicate an internal HindIII restriction site located 1bp from the ATG translational start
47 site in dsRed coding nucleotide sequence.
48 #nucleotide sequences of restriction sites are underlined.
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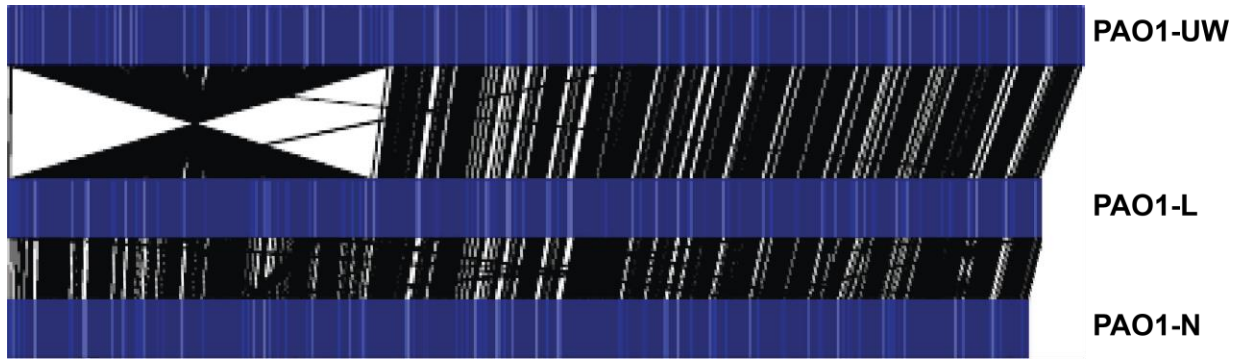
50 SUPPLEMENTARY FIGURES

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52

53 **Supplementary Figure 1. Clones complementing swarming motility in PAO1-N.**
54 DNA fragments from PAO1-L WT restoring swarming motility (insert images) in the
55 PAO1-N $\Delta rsmA$ mutant when overexpressed from the pME6000 vector. Typical EAL
56 (red framed) and GGDEF (blue framed) domains of proteins involved in c-di-GMP
57 metabolism were identified in the predicted proteins. Green arrows represent ORFs and
58 black arrows non-coding RNAs. Genes labelled purple were identified as
59 complementing swarming in PAO1-N $\Delta rsmA$ mutant when individually sub-cloned.
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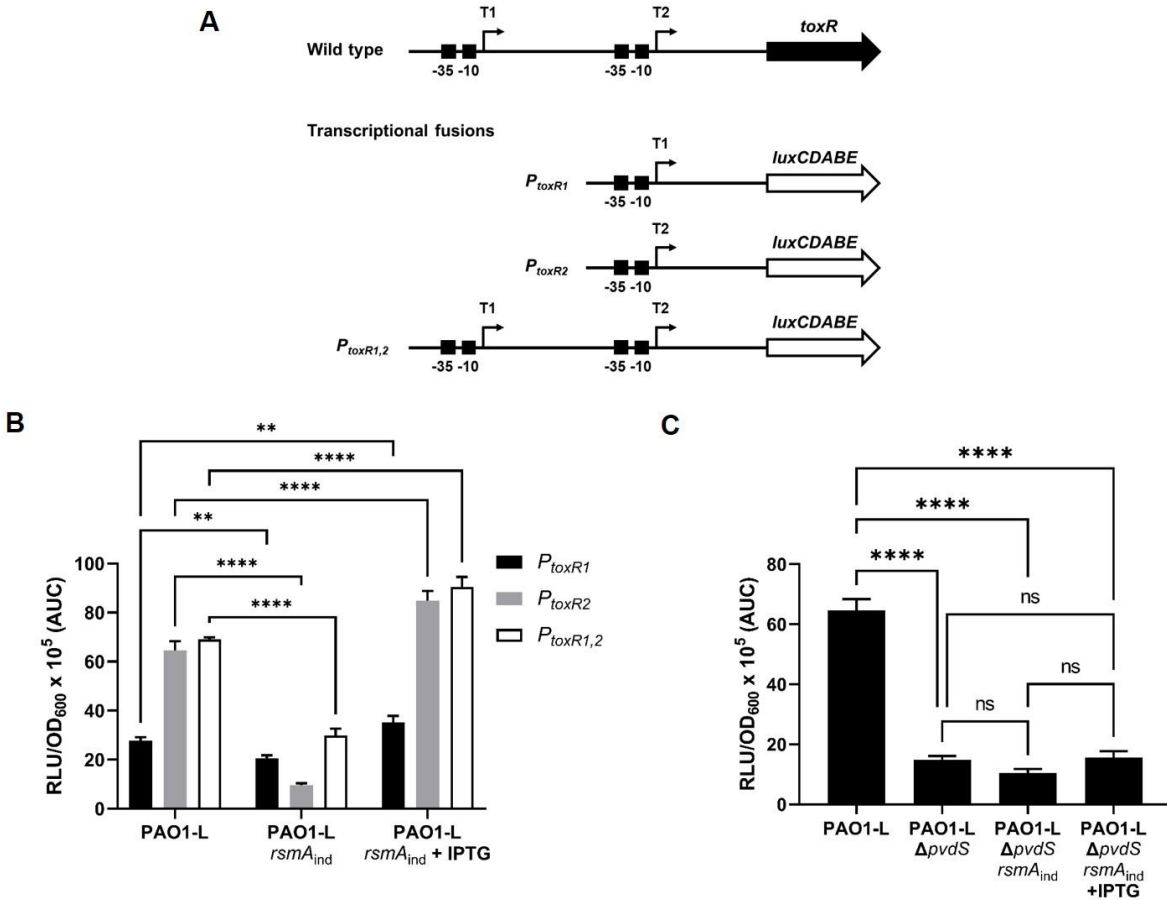
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Supplementary Figure 2. The 2.2-Mb inversion is not present in the PAO1 sublines used in this study. Alignment of the optical map of PAO1-UW reference sequence (Genbank Accession Number: NC 002516) produced *in silico* with the optical maps of PAO1 sublines PAO1-L or PAO1-N obtained experimentally. Optical maps are shown in blue, and the alignment of restriction enzyme cut sites between optical maps are shown in black.



68

69 **Supplementary Figure 3. Effect of RsmA and PvdS on *toxR* expression. (A)**

70 Schematic of the *toxR* promoter region used to build mini-CTX:: P_{toxR1} , P_{toxR2} and $P_{toxR1,2}$

71 - *luxCDABE* transcriptional fusions. (B) Effect of *rsmA* mutation on the transcriptional

72 activity of the *toxR* promoters measured in PAO1-L and the IPTG-inducible conditional

73 *rsmA* mutant of PAO1-L (*rsmA*_{ind}) under iron limiting conditions in CAA medium. (C)

74 Transcriptional activity of mini-CTX:: P_{toxR2} - *luxCDABE* in *P. aeruginosa* PAO1-L wild type

75 and its corresponding mutants $\Delta pvdS$ and $\Delta pvdS$ *rsmA*_{ind} measured under iron starvation

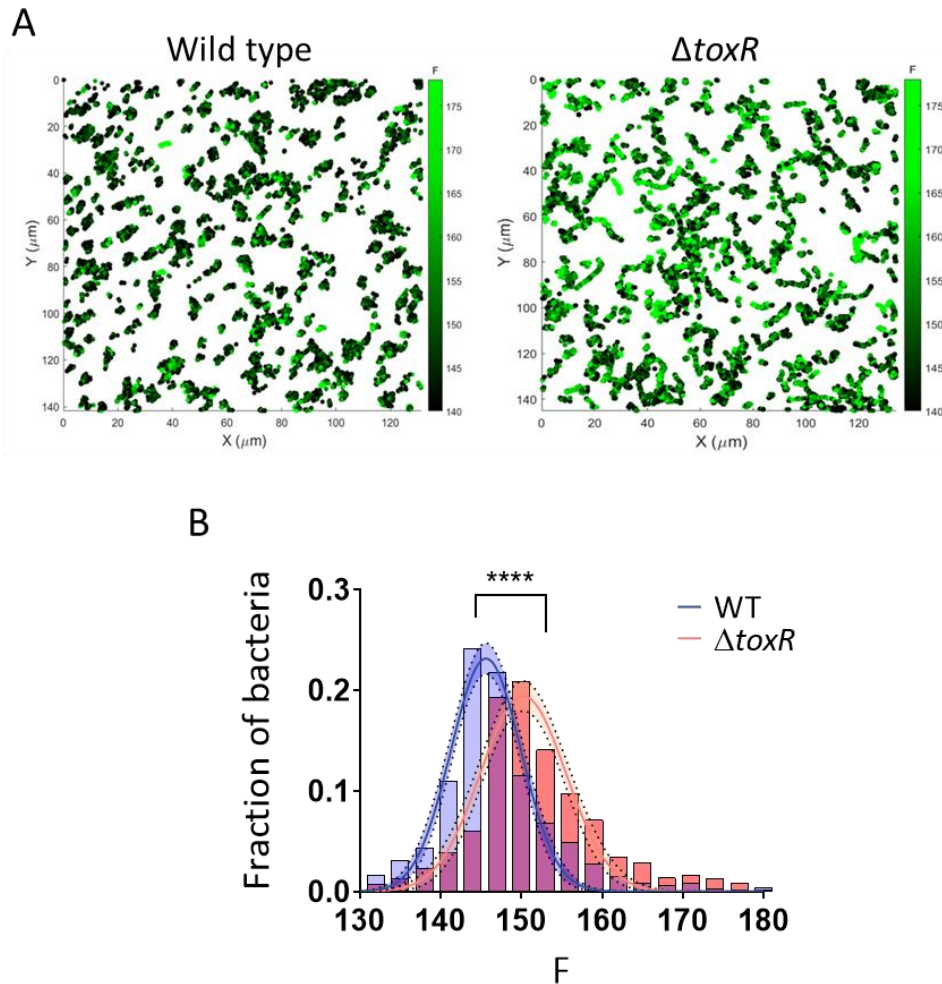
76 in CAA medium. Cells were grown in a 96-well microplate. Bioluminescence and

77 absorbance (OD₆₀₀) were measured using an automated luminometer-spectrometer

78 (TECAN Genios Pro). Reported values are averages from three different cultures \pm

79 standard deviation and correspond to the area under the curve (AUC) derived from
80 plotting relative luminescence units normalized to culture density (RLU/OD₆₀₀) over time
81 (24 h). Statistical differences between group means were determined by two-way ANOVA
82 tests. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.0001$).

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84

85 **Supplementary Figure 4. Impact of ToxR on the levels of *cdrA* expression at**
 86 **individual cell level within the bacterial population.** (A) Representative coloured
 87 maps of PAO1-L WT and PAO1-L $\Delta toxR$ mutant cells expressing the $P_{cdrA} - gfp(ASV)^C$
 88 transcriptional fusion reporter. Colours show instantaneous fluorescence intensity
 89 measured (F) according to the intensity scale shown. Tracks were obtained from
 90 combined images acquired using DIC and widefield epifluorescence microscopy every
 91 30 s for 60 min and then processed through a custom single-cell tracking algorithm. (B)
 92 Histogram of F for all tracks on PAO1-L WT and $\Delta toxR$ mutant (N > 1,000). Unpaired t-

93 test with Welch's correction was used to compare curves ($***p < 0.0001$). Dashed lines
94 represent data fit to Gaussian functions. Shaded areas represent 95% CI of the fits.

95

	Sid	1	80
96	Cd01948	100%	ADLRRALERGEFELYYPQIVDLRTGRIVGY--KALLWRHPEEGL-ISPAEFIPLAEEETGLIVELGRWVLEEACRQLARW
97	PA2133	27.2%	RRFQAALARGRVRLDWQAVRHAGHPEWEPLYR--TLLVTAASGEPPLPTQELILALERLGLVRLDDRCVLGTVLDRLN--
98	PA2200	36.4%	ARLLRALKREALEVHYQPIVRLASGRCAGV--KALA--WDSSLGR-VSPDFVFI GALES GDIE LTRFVFRALKQLGPL
99	PA2818	39.2%	GDMYRAMRAREFHMVYQPIIHLDTGECRGV--KALVWQRPDRSQ-VRPDI FIP LA EDN GMIGDLTRHIFGLVAADLAQL
100	PA3825	41.7%	SELRRALEANEFIPYYPQLSPGQGGRWIGV--KALMWRHPREGL-IRPDLFIPFAERSGLIVPMTRALMRQVAEDLGGH
101	RocR	38.6%	ADVVRGLDNGEFEAYYQPKVALDGGGLIGA--KVLAVNHNPHLGV-LPPSHFLYVMEYTNLVDKLFWQLFSQGLATRKKL
102	FimX	33.5%	AILQQALETNSFRLLFQPVISLRGDSHENY--KVLVLLNPNQQQE-VPPAEFLHAAKEAGLAEKIDRWVILNSIKLLAEH
103	LapD	30.5%	DWIDQALTEERRLLLYFQPVVDCLDTQRV-LHHKVLALLDLPQATA-IAAGRFLPWIERF GWAARM DLAMLEQSL EHLRR-
104	ToxR	20.0%	WLC LGNRDANDGFELFAHGIYARN GALVGS--KLSLERRQRVDLSAFLSGAPFLLAEEAVKHLLARLLCVHRHNT----
105			
106			
107		81	160
108	Cd01948	100%	Q-AGGPDLRLSVLSARQLRDPDFDLRLELLAETGLPPRRVLVIT--SALIDDLLEEALATLRLRALGVRIAL--FGTG
109	PA2133	27.2%	---AEPTLR LACLSRQSAAMD AWEAVCRWLAAR PQVARRLTLT--TAVGERVA-TREFIRRLREHGVR IAI--FGAA
110	PA2200	36.4%	L-REQRSFYVSVVTGKDIADPGFIDFAMRQMARESVRPEQVALTLT--RTT-EAQGC LLAGMNR LRELGLKIYV--FGTG
111	PA2818	39.2%	G--LGAGDHLGVVSGSHLASHGFVDDVRRLLGAI GSEGPQLVLEVT--REALPHDAQLQHNIQQ LRELGVQWAL--FGTG
112	PA3825	41.7%	AGKLEPGFHIGF--ISATHCHELALVDDCRELLA FPPGHITLVLELT--RELIESSEVTDRLFDELHALGVKIAI--FGTG
113	RocR	38.6%	A-QLGQPINLAFVHPSQLGSRALAEINISALLTEFHLPPSSVMF--IT--TGLISAPASSLENLVR LRMGCGGLAM--FGAG
114	FimX	33.5%	R-AKGHQTKLFVHLSASLQDPGLLPWLGVALKARLPPELVFQIS--ADATSYLKQAKQLTQGLATLHCQAAISQFGCS
115	LapD	30.5%	-----HPRPLALSLSAASVRNAQT FAPLLALLKAHPQEARQLTLDL--RHL-PAAAELERLSQV LRELGCGLGLQHFGR
116	ToxR	20.0%	--DLELLGKNFIP LHAS SLGNAGVCERILASARQLQHQVELCLLLAIDEQEPASAEYLTSLARLRDSGVRIALHPQRID
117			
118		161	240
119	Cd01948	100%	YSSL SYLKR LPV---DYLI--RSFVRD--IETDPEDRAIVRAIIALAHSLG LKVVAGV--TEEQLELLRELGC D--YV--GY
120	PA2133	27.2%	HNNLDFVLDARP---DVI--ICRYTRE--ARRSAKAEVLRHLLALCRELAPCVVLLGL--EDDAFARLPTGDV--YL--GN
121	PA2200	36.4%	HSNLVYLANLPV---DAI--IKVFTQS--IGDSSAVELIFDKLCSMAEHLEIGVVV--GIT--TQAQADHVLRRSPEALG--GW
122	PA2818	39.2%	QSSL SHLQKLHA---DFL--I--RSFVSS--VSGSGVNAVLETIIALAQRLDLAMTA--GIT--TREQEYQLCGHSIQ--WG--GY
123	PA3825	41.7%	HSSLAYLRKFQV---DCL--IQSFVAR--IGIDT LSGHILDSIVELSAKLDDLIVA--GV--TPEQRDYLAARGVD--YL--GY
124	RocR	38.6%	YSSLDRLCEFPF---SQI--L--RTFVQK--MKTQPRSCAVISSVVALAQALGISLVV--GV--SDEQRVRLIELGCS--IA--GY
125	FimX	33.5%	LNPFNALKHLTV---QFI--I--G SFVQD--LNQVENQEILKGLIAELHEQQKLSIV--PFV--SASV L ATLWQAGAT--YI--GY
126	LapD	30.5%	FSLIGNLTHLGL---AYL--L--GCYLHA---VDREGDKRLFIEAVYRTHSIDLPLIA--QV--TLGELEVLREMG L R--GAMGR
127	ToxR	20.0%	TDARQCF AEVDAGLC DY LGL--ARLLAPGLTRNLRQRKRSIEYLNRLVVAQDIQMLCLNV DNEELHQQANALPFA--FRHGR
128			
129			

130 **Supplementary Figure 5. Level of conservation between the ToxR EAL domain**
131 **and other c-di-GMP binding proteins in *P. aeruginosa*.** Clustal Omega Alignment of
132 the conserved active EAL site (cd01948) and EAL sequences from EAL-only proteins of
133 *Pseudomonas aeruginosa* PAO1 including ToxR. The residues that form the active site
134 of c-di-GMP PDEs are highlighted in red; conserved residues located in the vicinity of
135 the active site are highlighted in green.
136

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