Interaction of ArmZ with the DNA-binding domain of MexZ induces expression of *mexXY* multidrug efflux pump genes and antimicrobial resistance in *Pseudomonas aeruginosa*

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Supplementary figures, tables and data sets.

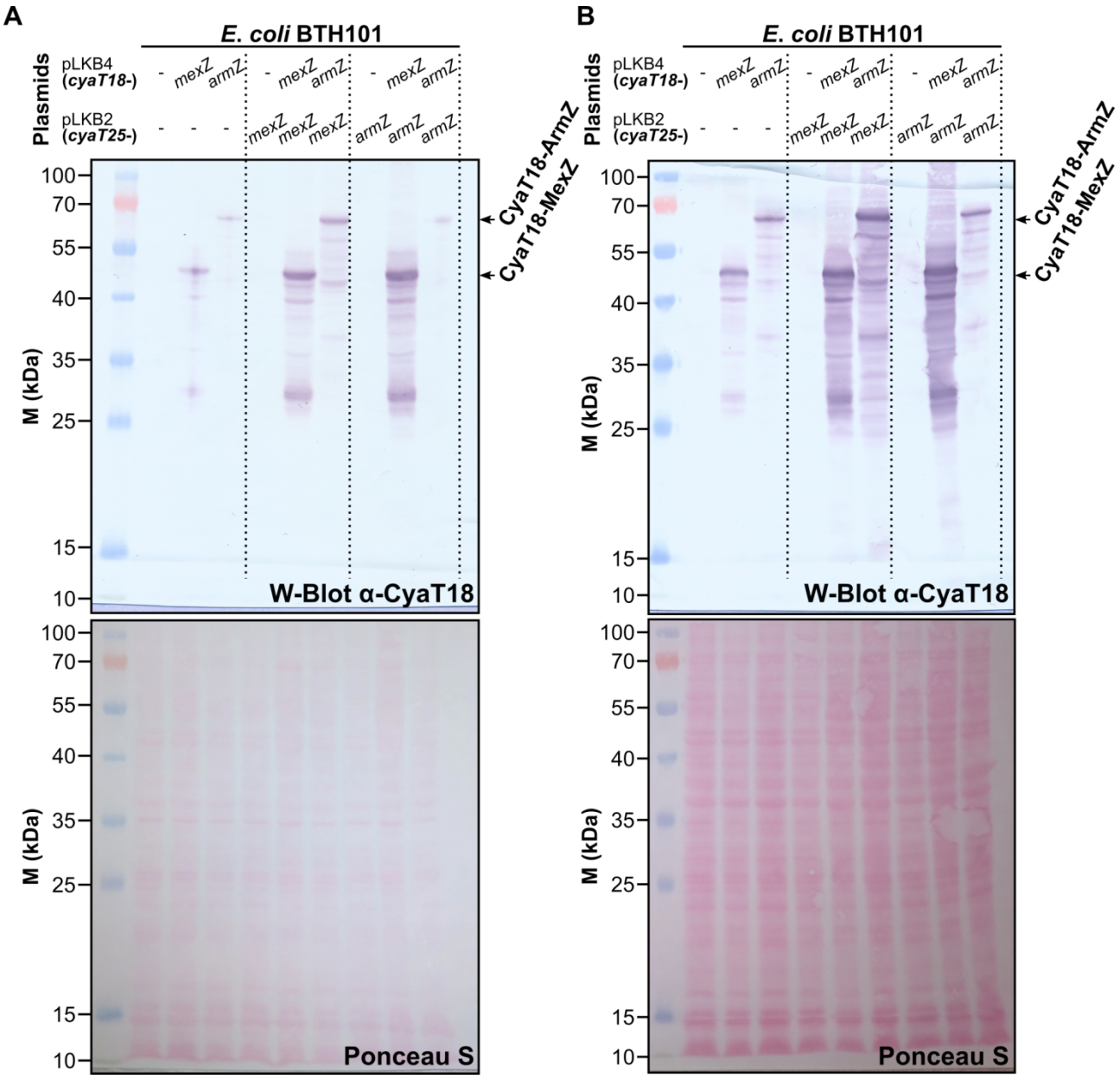

Figure S1. Impact of CyaA complementation on the cellular level of CyaT18- fused proteins. Western blot analysis of CyaT18, CyaT18-MexZ and CyaT18-ArmZ levels in *E. coli* BTH101 double transformants (as on Fig. 1A). Cells from cultures used for β-galactosidase assays were diluted in fresh medium, grown to OD_{600} = 0.6, harvested and boiled in the loading buffer. Cell extracts corresponding to 300 µl (**A**) or 1.2 ml of cultures (**B**) were separated on 10% polyacrylamide gels and analyzed by Western blot using anti CyaT18 antibodies. PonceauS staining of membranes after transfer was shown as a loading control (bottom panels).

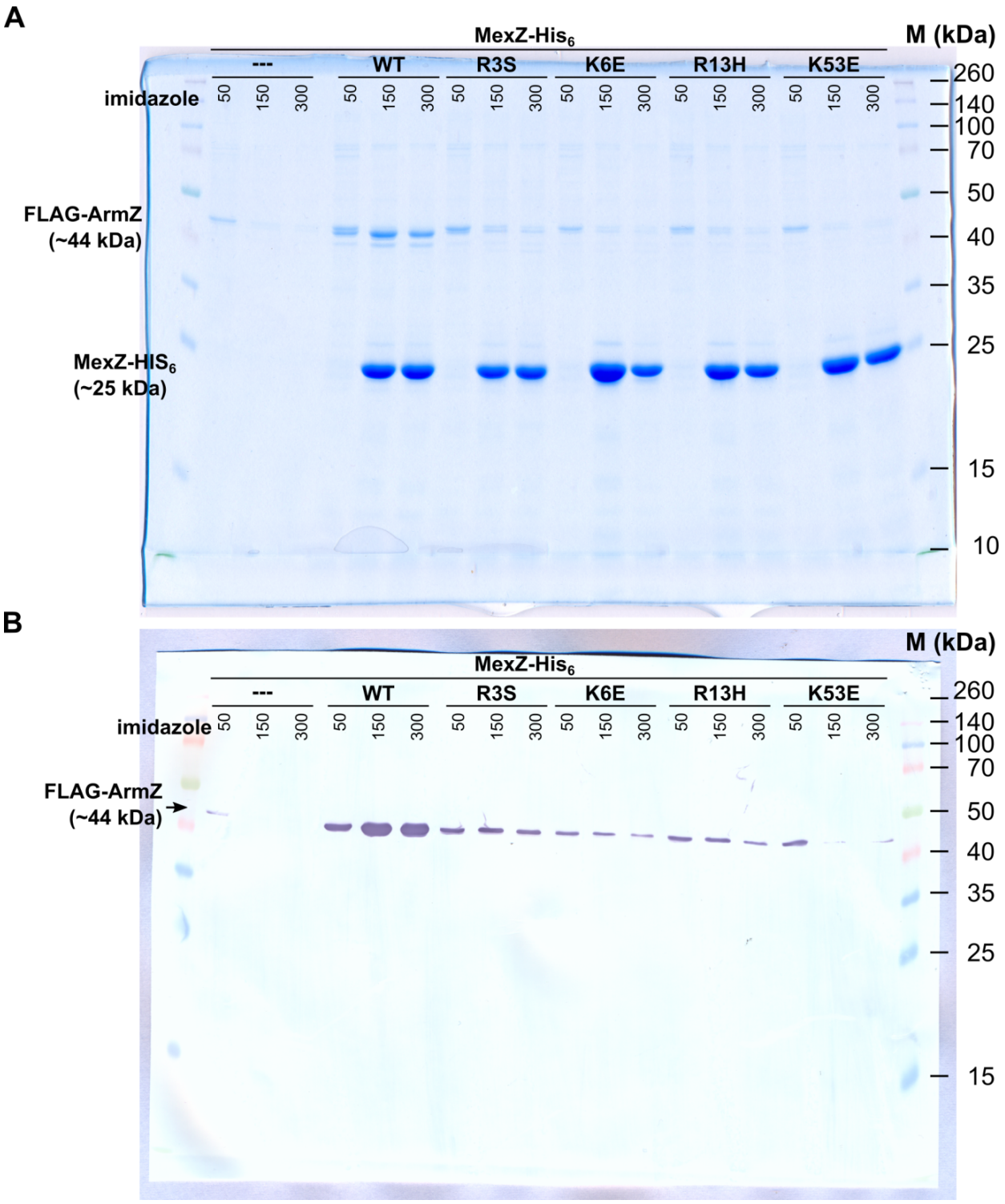

Figure S2. ArmZ interactions with MexZ WT and R3S, K6E, R13H, K53E mutants. Purified MexZ-His_6 (WT or mutant, 150 µg) was mixed with 1 mg of extract from *E. coli* BL21 (DE3) pKAB28.6 cells overproducing FLAG-ArmZ and transferred to a Ni-NTA column. After washing the bound proteins were eluted using imidazole gradient. Eluates were separated by SDS-PAGE, followed by Coomassie staining (**A**) or Western blot analysis using anti-FLAG antibodies (**B**).

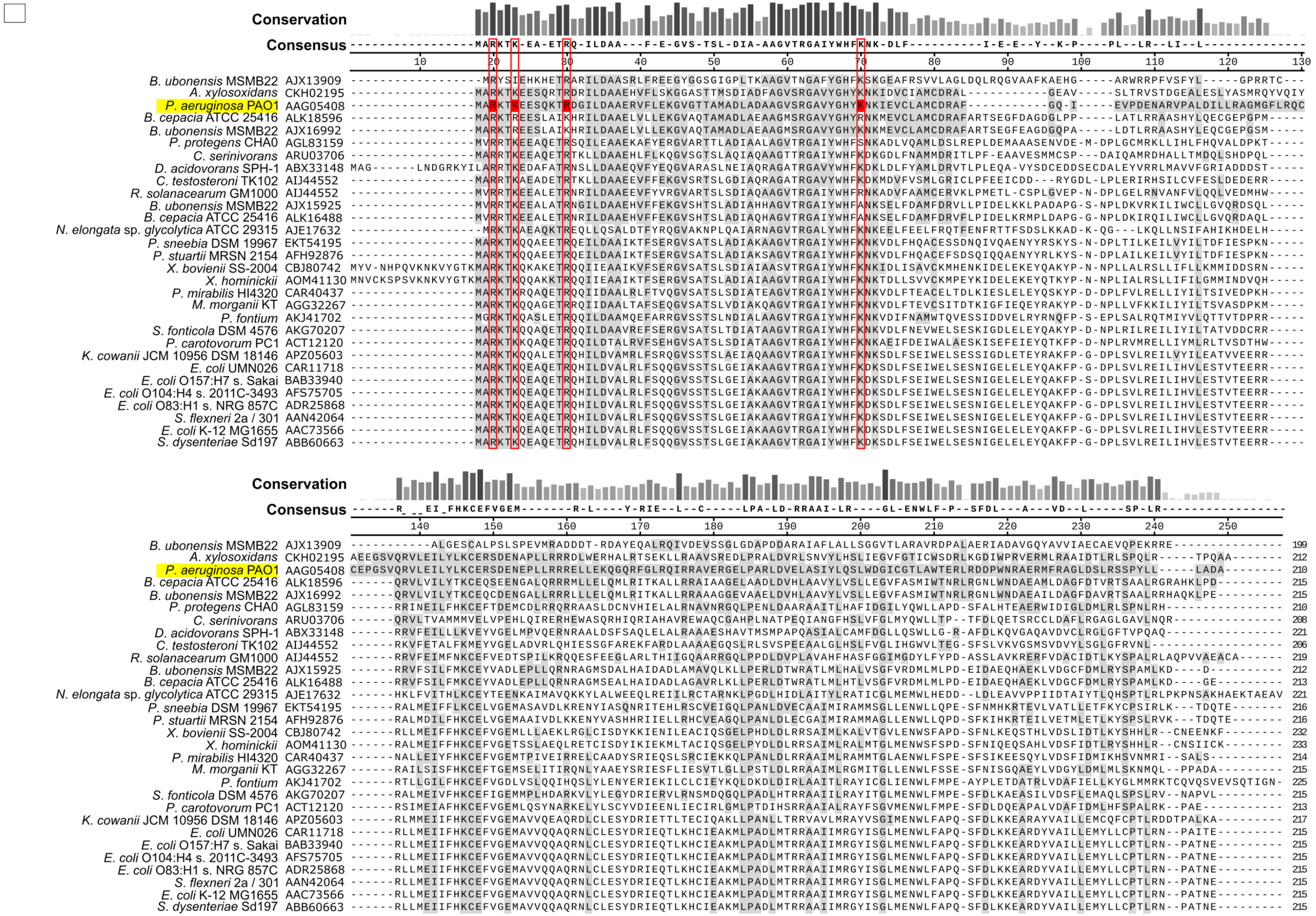

Figure S3. Sequence alignment of the MexZ orthologs identified in genomes encoding ArmZ/PA5470 orthologs. Gene groups encoding clustered orthologs of *P. aeruginosa* MexZ/MexX/MexY or ArmZ/PA5470 were identified in bacterial genomes (Refseq 91, reference and representative genomes) using MultiGeneBlast. All accessions are included in Data set S3.

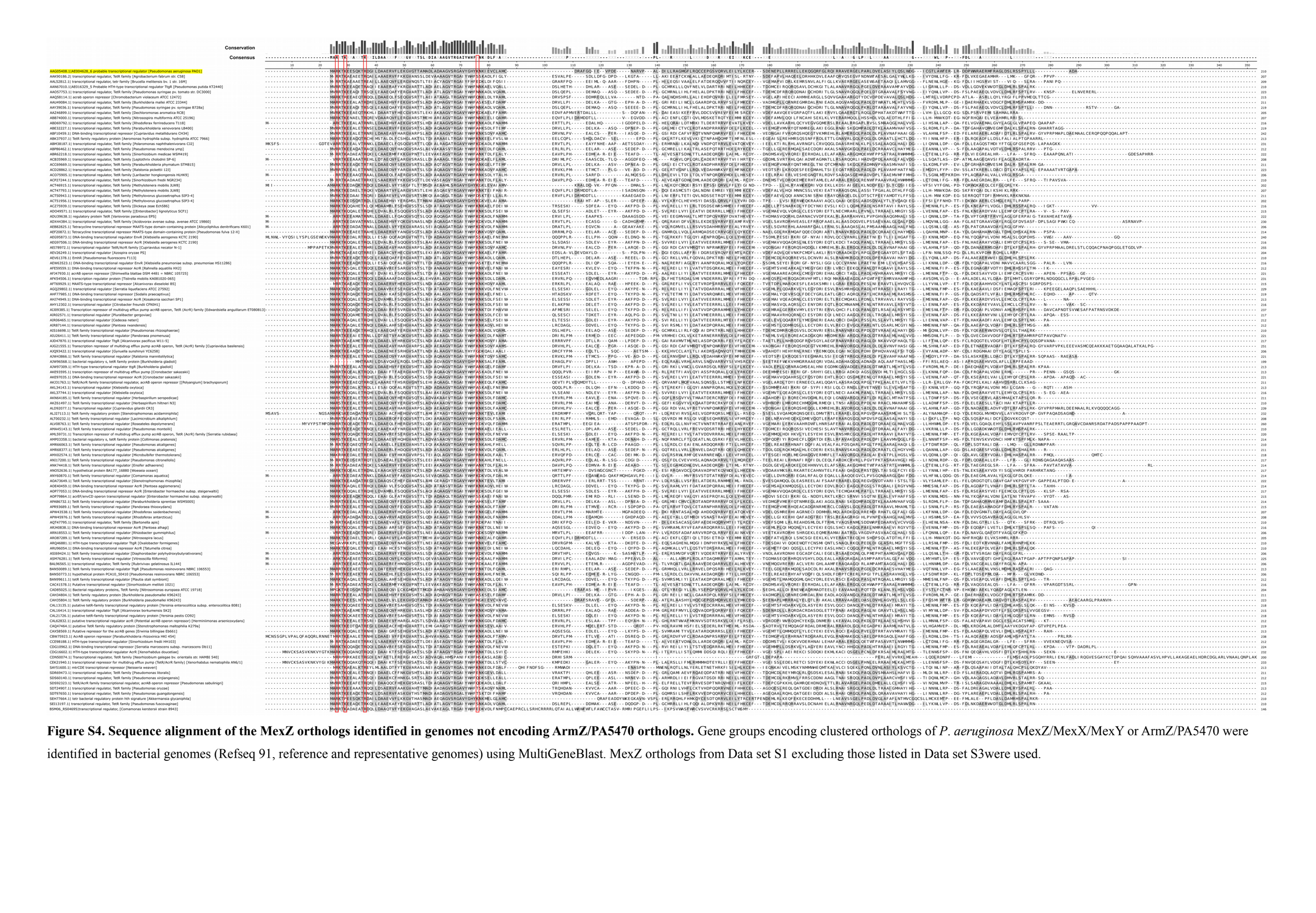

Figure S4. Sequence alignment of the MexZ orthologs identified in genomes not encoding ArmZ/PA5470 orthologs. Gene groups encoding clustered orthologs of *P. aeruginosa* MexZ/MexX/MexY or ArmZ/PA5470 were identified in bacterial genomes (Refseq 91, reference and representative genomes) using MultiGeneBlast. MexZ orthologs from Data set S1 excluding those listed in Data set S3were used.

Table S1 Plasmids used in this study.

Name	Relevant features	Origin
pABB28.2	pET28a based expression vector with *T7*p-*flag*-*mcs*[1], Kan^R	(1)
pAKE600	suicide vector, $oriV_{MB1}$, *sacB* cassette, Amp^R	(2)
pET28mod	$oriV_{MB1}$, *T7*p, *lacO*, his_6-*tag*, pET28a modified to remove the T7 tag, Kan^R	(3)
pGBT30	$oriV_{MB1}$, $lacI^q$, *tac*p, expression vector, Amp^R	(4)
pKAB240	pBAD24 derivative; *araC*-*araBADp*-*mcs*-*rrnBt* cassette flanked by NsiI restriction sites, Amp^R	(1)
pKAB600	pAKE600 with *PA5412*/*PA5413* intergenic region of PAO1161 with internal PstI site, Amp^R	(1)
pKAB601	pKAB600 with *araC*-*araBAD*p-*mcs*-*rrnBt* cassette inserted into PstI site, Amp^R	(1)
pKGB8	broad host range expression vector, $ori_{IncA/C}$, *araC*-*araBAD*p, Chl^R,	(1)
pLKB2	pKT25 with modified *mcs, lac*p-*cyaT25*-*mcs*, Kan^R	(5, 6)
pLKB4	pUT18C with modified *mcs, lac*p-*cyaT18*-*mcs*, Amp^R	(5, 6)
pPT01	$oriV_{SC101}$, promoter-less *xylE* cassette, Kan^R	(7)
pUC19	$oriV_{MB1}$, *lacZ*α with *mcs*, Amp^R	(8)

[1] *mcs*- multiple cloning site

Table S2 Plasmids constructed in this study.

Name	Relevant features and construction
pLKB4 derivatives (BACTH vectors)	
pKAB18.1	*cyaT18-mexZ* translational fusion; PCR amplified *mexZ* (*PA2020*) using primers #3 and #4, digested with EcoRI and BglII and inserted between EcoRI and BamHI sites of pLKB4
pKAB18.2	*cyaT18-armZ* translational fusion; *armZ* (*PA5471*) amplified using primers #5 and # 6, digested with EcoRI and MfeI and inserted into EcoRI site of pLKB4
pKAB18.3	*cyaT18-mexZ* (ΔN8) translational fusion; 5' truncated *mexZ* allele amplified using primers #8 and #10, digested with EcoRI and MfeI and inserted into the EcoRI site of pLKB4
pKAB18.4	*cyaT18-mexZ* (ΔN26) translational fusion; 5' truncated *mexZ* allele amplified using primers #9 and #10, digested with EcoRI and MfeI and inserted into EcoRI site of pLKB4
pKAB18.5	*cyaT18-mexZ* (ΔC9) translational fusion; 3' truncated *mexZ* allele amplified using primers #11 and #12, digested with EcoRI and MfeI and inserted into EcoRI site of pLKB4
pKAB18.6	*cyaT18-mexZ* (ΔC31) translational fusion; 3' truncated *mexZ* allele amplified using primers #11 and #13, digested with EcoRI and MfeI and inserted into EcoRI site of pLKB4
pKAB18.7	*cyaT18-mexZ* (ΔC61) translational fusion; 3' truncated *mexZ* allele amplified using primers #11 and #14, digested with EcoRI and MfeI and inserted into EcoRI site of pLKB4
pKAB18.8	*cyaT18-mexZ*$_{R3S}$ translational fusion; library clone, 9G>C change in *mexZ*
pKAB18.9	*cyaT18-mexZ*$_{K6E}$ translational fusion; library clone, 16A>G change in *mexZ*
pKAB18.10	*cyaT18-mexZ*$_{R13H}$ translational fusion; library clone, 38G>A change in *mexZ*
pKAB18.11	*cyaT18-mexZ*$_{K53E}$ translational fusion; library clone, 157A>G change in *mexZ*
pKAB18.12	*cyaT18-mexZ*$_{K53M}$ translational fusion; library clone, 158A>T change in *mexZ*
pKAB18.13	*cyaT18-mexZ*$_{K53N}$ translational fusion; library clone, 159G>T change in *mexZ*
pKAB18.14	*cyaT18-mexZ*$_{K53Q}$ translational fusion; library clone, 157A>C change in *mexZ*
pKAB18.15	*cyaT18-mexZ*$_{K53E}$ translational fusion; library clone, 158A>C change in *mexZ*
pLKB2 derivatives (BACTH vectors)	
pKAB25.1	*cyaT25-mexZ* translational fusion; *mexZ* excised as EcoRI-SmaI fragment from pKAB18.1 and inserted into pLKB2
pKAB25.2	*cyaT25-armZ* translational fusion; *armZ* excised as EcoRI-BamHI fragment from pKAB18.3 and inserted into pLKB2
pKAB25C	pLKB2 with Kan^R marker replaced by Chl^R; PCR amplified *cat* from pKGB8 using primers #26 and #27 was digested with BglII and NsbI and inserted into BglII and NsbI digested pLKB2
pKAB25C.2	*cyaT25-armZ* translational fusion, Chl^R; *armZ* excised as EcoRI-KpnI fragment from pKAB18.3 and inserted into pKAB25C
pAKE600 based suicide vectors used in allele replacement	
pKAB610	*araC-araBAD*p-*armZ*-T$_{rrnB}$ flanked by sequences allowing insertion in *PA5412*/*PA5413* intergenic region; NsiI fragment from pKAB244 was inserted into PstI site of pKAB600
pKAB611	*mexZ* deletion cassette; Gibson assembly of HindIII digested pAKE600 and two PCR fragments corresponding to PAO1 coordinates 2212177-2212676 and 2213164–2213664, amplified using pairs of primers #28/ #29 and #30/ #31, respectively

pKAB612	*armZ* deletion cassette; Gibson assembly of SmaI digested pAKE600 and two PCR fragments corresponding to PAO1 coordinates 6161187–6160688 and 6159714– 6159214 amplified using pairs of primers #32/ #33 and #34/ #35, respectively
pKAB613	*mexZ*$_{R3S}$ mutagenesis cassette; PCR fragments amplified with primers #28/ #36 and #37/ #14 were used as a template in overlap PCR (primers #28 and #14). MunI and XbaI digested product was inserted between EcoRI and XbaI sites in pAKE600. Introduction of a NheI site facilitated the identification of mutagenic allele in the genome.
pKAB614	*mexZ*$_{K6E}$ mutagenesis cassette; PCR fragments amplified with primers #28/ #38 and #39/ #14 were used as a template in overlap PCR (primers #28 and #14). MunI and XbaI digested product was inserted between EcoRI and XbaI sites in pAKE600. Introduction of a PauI site facilitated the identification of mutagenic allele in the genome.
pKAB615	*mexZ*$_{R13H}$ mutagenesis cassette; PCR fragments amplified with primers #28 / #40 and #41 / #14 were used as a template in overlap PCR (primers #28 and #14). MunI and XbaI digested product was inserted between EcoRI and XbaI sites in pAKE600. Introduction of a PagI site facilitated the identification of mutagenic allele in the genome.
pKAB616	*mexZ*$_{K53E}$ mutagenesis cassette; PCR fragments amplified with primers #1 / #42 and #43 / #14 were used as a template in overlap PCR (primers #1 and #14). MunI and BamHI digested product was inserted between EcoRI and BamHI sites in pAKE600. Elimination of the XmiI site facilitated the identification of mutagenic allele in the genome.
pET28 based expression vectors	
pKAB28	pET28mod with deletion of *his*$_6$-tag and EcoRI site adjacent to RBS; PCR product obtained using primers #21/ #22 and pET28mod as a template, digested with BglII and EcoRI, replaced the corresponding fragment in pET28mod
pKAB28.1	*mexZ-his*$_6$ translational fusion; EcoRI-BlpI *mexZ-his*$_6$ fragment from pKAB20.4 was ligated with pKAB28
pKAB28.2	*mexZ*$_{R3S}$-*his*$_6$ translational fusion; 218 bp EcoRI-KpnI *mexZ* fragment of pKAB18.9 replaced the corresponding fragment in pKAB28.1
pKAB28.3	*mexZ*$_{K6E}$-*his*$_6$ translational fusion; 218 bp EcoRI-KpnI *mexZ* fragment of pKAB18.10 replaced the corresponding fragment in pKAB28.1
pKAB28.4	*mexZ*$_{R13H}$-*his*$_6$ translational fusion; 218 bp EcoRI-KpnI *mexZ* fragment of pKAB18.11 replaced the corresponding fragment in pKAB28.1
pKAB28.5	*mexZ*$_{K53E}$-*his*$_6$ translational fusion; 218 bp EcoRI-KpnI *mexZ* fragment of pKAB18.12 replaced the corresponding fragment in pKAB28.1
pKAB28.6	*flag-armZ* translational fusion; *armZ* excised as EcoRI-KpnI fragment from pKAB18.3 was inserted between EcoRI and KpnI sites in pABB28.2
pKAB28.7	pET28mod with BamHI and ScaI sites in *mcs*; synthetic DNA fragment obtained by annealing of oligonucleotides #24 and #25 inserted between EcoRI and SalI sites in pET28mod
pKAB28.8	*his*$_6$-*armZ* translational fusion; *armZ* excised as EcoRI-BamHI fragment from pKAB18.3 was inserted between EcoRI and BamHI sites of pKAB28.7
pKAB28.9	*flag-mexZ* translational fusion; *mexZ* (EcoRI-SalI fragment from pKAB18.1) inserted between EcoRI and SalI sites of pABB28.2.
Other plasmids	
pKAB8	expression vector with *araC-araBAD*p;. pKGB8 digested with XhoI, treated with Klenow fragment (filling-in 3' recessed ends) and self-ligated
pKAB10.1	*mexXY*p-*xylE* transcriptional fusion, *mexX-mexZ* intergenic region amplified using primers #1 and #2 and PAO1161 genomic DNA digested with BamHI and BglII and inserted into BamHI site,

	upstream of promoterless *xylE* in pPT0I
pKAB19.1	cloning intermediate; pUC19 digested with BamHI and HindIII, blunted by filling-in 3' recessed ends using Klenow fragment, and self-ligated
pKAB19.2	cloning intermediate; Three fragments amplified with primer pairs #15/ #16, #17/ #18 and #19/ #20 and pKAB28.9 as template were used in overlap PCR using primers #15 and #20 to yield a 992 bp product containing *flag-mexZ-his*$_6$. PCR product was inserted as EcoRI, SmaI fragment into pKAB19.1.
pKAB20	pUC19 derivative with *flag-mcs* (MunI, HindIII, NotI, XhoI, BamHI)-*his*$_6$; allows in frame attachment of *flag* to 5' and/or *his*$_6$ to the 3' of a gene. The *mexZ* stuffer removed from pKAB19.2 by HindIII digestion and vector self-ligation.
pKAB20.4	*mexZ-his*$_6$ translational fusion; *mexZ* gene without stop codon amplified using primers #3/ #23, digested with EcoRI and BamHI and inserted between EcoRI and BamHI sites of pKAB20.
pKAB244	*araC-araBADp-armZ-T*$_{rrnB}$ cassette; *armZ* excised as EcoRI-KpnI fragment from pKAB18.3 inserted between EcoRI-KpnI sites in pKAB240
pKAB301	*tac*p-*armZ* transcriptional fusion; *armZ* excised as EcoRI-KpnI fragment from pKAB18.3 inserted between EcoRI-KpnI sites in pGBT30
pKAB302	*tac*p-*armZ/PA5470* transcriptional fusion; *armZ-PA5470* operon amplified using primers #5 and #47, digested with EcoRI and KpnI and inserted between EcoRI-KpnI sites in pGBT30
pKAB801	*araC-araBAD*p-*mexZ* transcriptional fusion; *mexZ* excised as EcoRI-SacI fragment from pKAB18.1 cloned into pKAB8

PCR was performed using PAO1161Rif^R genomic DNA as a template, unless stated otherwise.

Table S3 Oligonucleotides used in this study

Number	Sequence (5'-3')
#1	GTCAGGATCCGGGTGTCCCTCGATTCG
#2	TATAAGATCTTGAACGTCCTCACAAGGGAA
#3	TATAGAATTCATGGCCAGGAAAACCAAAG
#4	CGAGATCTGTCGACTGCACATCAGCGAGGAAGA
#5	TATAGAATTCATGGGCAACTACATCAAGCC
#6	TATACAATTGTCATCGGCAGCACTCCC
#7	TGTGCTGCAAGGCGATTAAG
#8	ATAAAGCTTGAATTCATGTCCCAGAAAACCCGCGA
#9	TATAGAATTCATGAAGGGCGTGGGCACC
#10	ATATCAATTGTCAGGCGTCCGCCAGC
#11	TATAGAATTCATGGCCAGGAAAACCAAAG
#12	ATATCAATTGTCAACTGCGCAGGCTGTCG
#13	ATATCAATTGTCAGCGCTCGGTCCAGG
#14	ATATCAATTGTCATTCGCCGCGTTCCAC
#15	GTCAGAATTCATGGACTACAAGGACGACGA
#16	GCCATAAGCTTCAATTGCGATCCGCGACCCATTT
#17	TCGCAATTGAAGCTTATGGCCAGGAAAACCAA
#18	ACCGGATCCCTCGAGTGCGGCCG
#19	ACTCGAGGGATCCGGTTCCGGTCACCACCACCACCACCACT
#20	TATACCCGGGTCGACCCGTTTAGAGGCCCCAAG
#21	GTCCGGCGTAGAGGATCG
#22	GCATGAATTCCTCCTTCTTAAAGTTAAACAAAATTATTTC
#23	GTATGGATCCGGCGTCCGCCAGCAA
#24	AATTCGGATCCGAGCTCAGTACTG
#25	TCGACAGTACTGAGCTCGGATCCG
#26	CGATCAGATCTTTTGGCGAAAATGAGACGTT
#27	GTACTGCGCATTAATTGCGTTGCGCTCAC
#28	CGGTGAATTCTCTAGAACAGGGCGCCGCGGCTGAT
#29	GCGATTGCAAGCTTTGAACGTCCTCACAAGGGAAAGGCG
#30	GACGTTCAAAGCTTGCAATCGCTCTGGGACGG
#31	GACCATGGCTGCAGATCTCTGCGGACGATTGCAG
#32	AGCCATGGTCGACCCCCTGCGCGAGGTCTACG

#33	GGTCGCCACCCGGGGTAGTTGCCCATAATCCAATCC
#34	GCAACTACCCCGGGTGGCGACCGCCAGCTGA
#35	TAATCCCGGATCCCCGAAGAGCGCAGGGTCTCGAAAC
#36	TTGGTTTTGCTAGCCACTGAACGTCC
#37	TCAGTGGCTAGCAAAACCAAAGAGG
#38	CTTCGGTTTTGCGCGCCACTGAACGTCCTCAC
#39	AGTGGCGCGCAAAACCGAAGAGGAATCCCA
#40	ATGCCGTCATGAGTTTTCTGGGATTCCTC
#41	CAGAAAACTCATGACGGCATACTCGATG
#42	TTCTCGTAGTGGCCATAGACCGCACCGCGAGAAAC
#43	GTCTATGGCCACTACGAGAACAAGATCGAGGTCTG
#44	CGGATGTACTGGAAACGGTG
#45	CGGGGCTGGCTTAACTATG
#46	TCGTATGTTGTGTGGAATTGTG
#47	ATAGGTACCGAAGCTGCGCAAGGAAGTG

qPCR primers

Number	Name	Sequence (5'-3')
#48	NADB_F	CTACCTGGACATCAGCCACA
#49	NADB_R	GGTAATGTCGATGCCGAAGT
#50	qPA5471_F	CGGCGGACAAACTGGAAAAG
#51	qPA5471_R	TAGATCCAGGGCTTCGATGC
#52	qMexY1_F	TGCCCAACGACATCTACTTC
#53	qMexY1_R	ATGCCTTCCTGGTAATGGTC

Restriction sites are underlined.

Supporting References:

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