**Supplemental materials for**

**"The cAMP-Vfr signaling pathway in *Pseudomonas aeruginosa* is inhibited by c-di-GMP."**

**Figure S1**

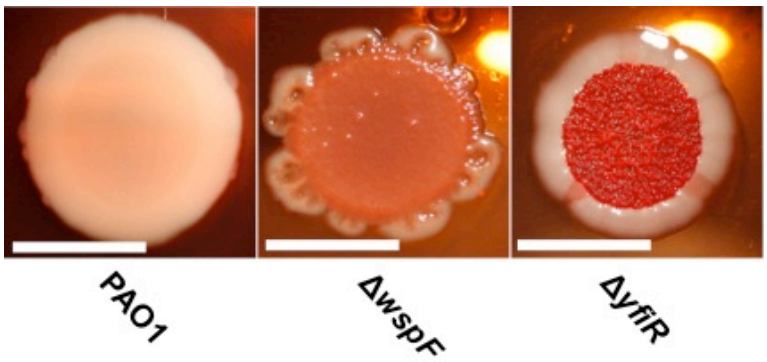


**Figure S1. Congo Red Binding of RSCVs *P. aeruginosa***
PAO1 wild type, and Δ*wspF* and Δ*yfiR* spotted onto tryptone-Congo red agar plates with 1% arabinose. Cells were grown for 5 days at RT. Bar = 5mm.

**Figure S2**

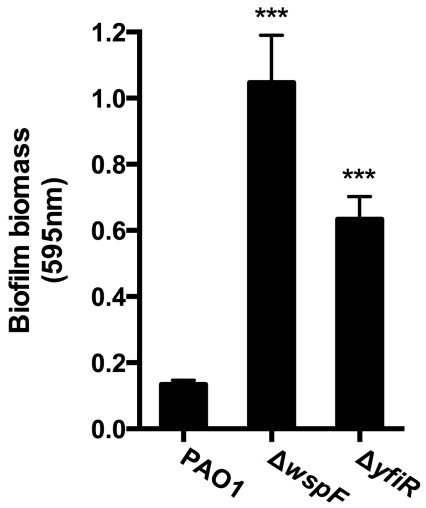


**Figure S2. Crystal violet assay for biofilm quantification.**
*P. aeruginosa* strain PAO1wild type, Δ*wspF,* and Δ*yfiR* grown in a 96-well microtiter dish in VBMM. Biofilm formation was quantified after 8 hours by Crystal Violet staining. All measurements represent at least 2 biological experiments with at least 8 technical replicates Error bars indicate SD. Statistical significance ($P \leq 0.001$) is indicated with three asterisks.

**Figure S3**

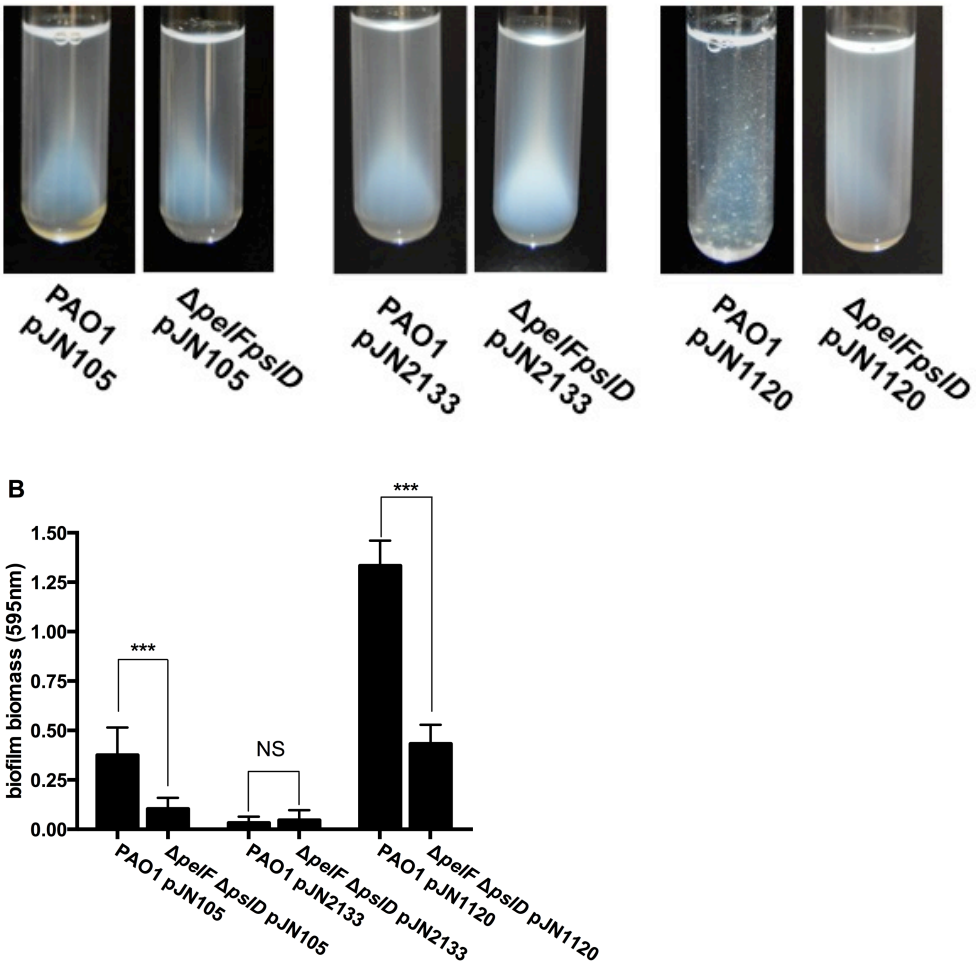


**Figure S3. Loss of aggregation by *pel* and *psl* deletion.**
*P. aeruginosa* PAO1 wild type and the double mutant strain **Δ***pelF***Δ***pslD*. Cells were transformed with the three plasmids: pJN105 (vector control), pJN2133 (PDE) and pJN1120 (DGC). Cell-cell aggregation was visualized during planktonic growth (A). Biofilm formation was quantified after 8 hours by Crystal Violet staining (B). All measurements represent at least 2 biological experiments with at least 8 technical replicates Error bars indicate SD of the mean.
Statistical significance ($P \leq 0.001$) is indicated with three asterisks.

**Figure S4**

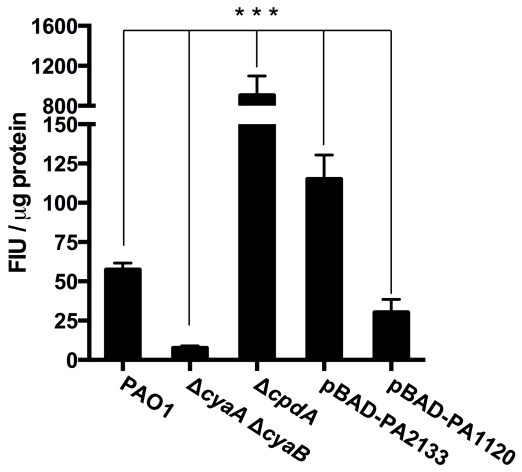


**Figure S4. Verification of fluorescent cAMP reporter.**
A new GFP based transcriptional reporter was created to follow cAMP-Vfr signaling in real time. The cAMP monitor (pUCp22GW::lacP1-GFP(ASV) was verified experimentally in a series of knock-out mutants. Reporter activity was tested in a wild type PAO1, Δ*cyaAcyaB* (no cAMP), Δ*cpdA* (high cAMP), pBAD-PA2133 (chromosomal expression of PA2133 PDE), and pBAD-PA1120 (chromosomal expression of PA1120 DGC). All measurements represent at least 2 biological experiments with at least 8 technical replicates Error bars indicate SD of the mean. Statistical significance ($P \leq 0.001$) is indicated with three asterisks.

**Figure S5**

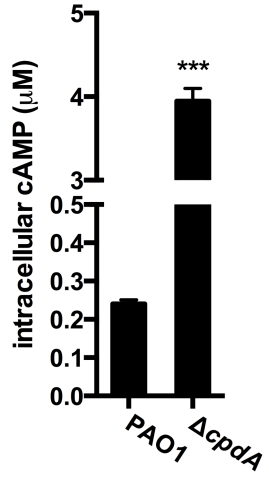


**Figure S5. Intracellular cAMP concentration of cpdA mutant**
Intracellular cAMP levels of a Δ*cpdA* strain were measured using EIA. All measurements were standardized to the total protein content. Bars indicate SD of the mean.
Statistical significance ($P \leq 0.001$) is indicated with three asterisks.

**Figure S6**

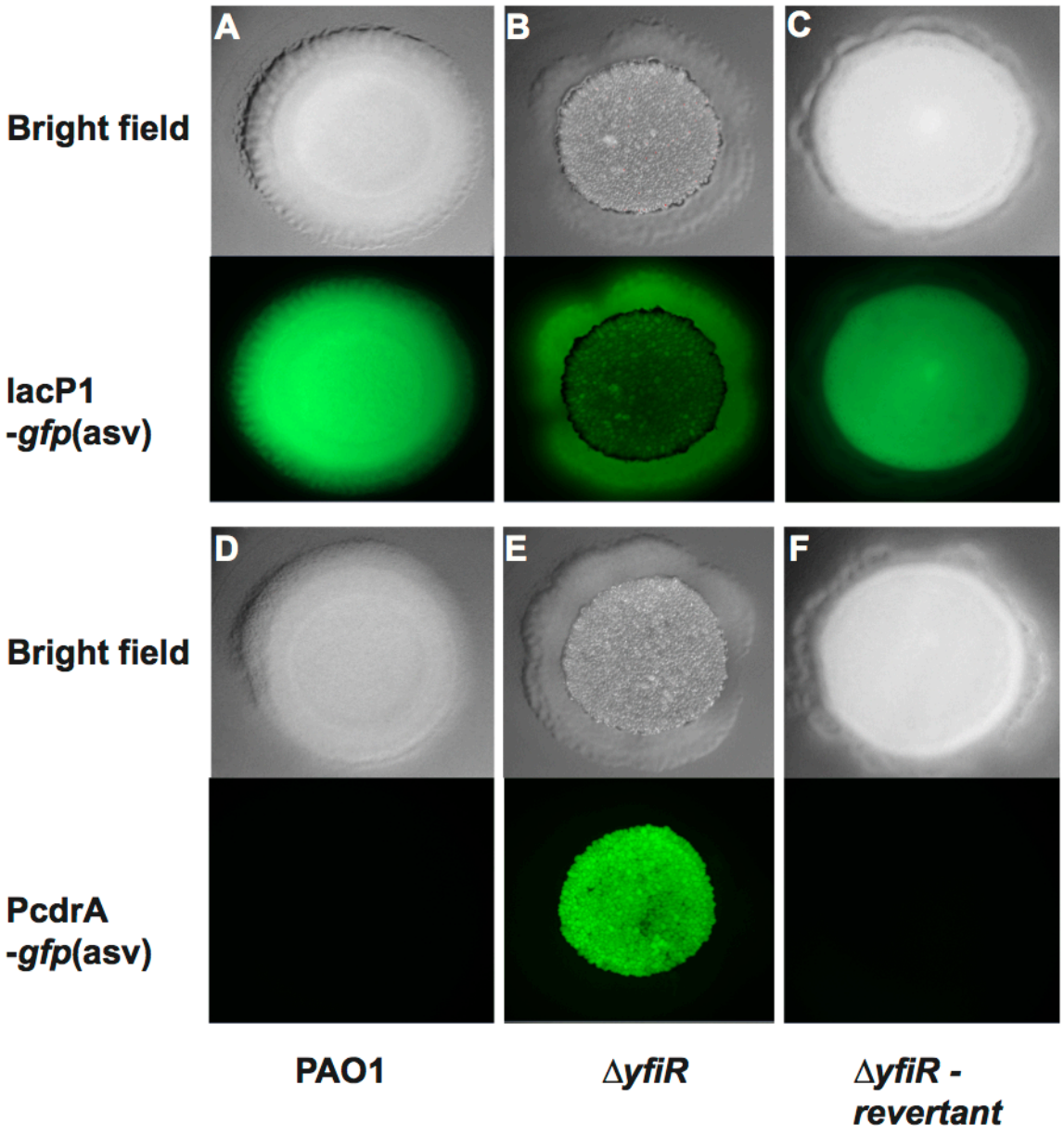


**Figure S6. Phenotypic reversion of RSCVs cause a regain of cAMP-Vfr signaling. (GFP)**

c-di-GMP signaling and Vfr-cAMP signaling were monitored for several days on CR agar plates using fluorescent transcriptional reporters. Images were obtained after 4 days of growth at RT. Vfr-cAMP signaling was monitored using the cAMP GFP biosensor for wild type PAO1 (A), Δ*yfiR* (B) and a reverted Δ*yfiR* strain (C). C-di- GMP signaling was also monitored using the c-di-GMP GFP biosensor for wild type PAO1 (D), Δ*yfiR* (E) and a reverted Δ*yfiR* strain (F).

**Table S1. Bacterial strains.**

| Strain | Genotype, description or relevant characteristics | Source |
|---|---|---|
| *Escherichia coli* | | |
| NEB5α | fhuA2Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17 | New England Biolabs |
| ccdB Survival 2™ T1R | $Str^r$, F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araΔ139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG fhuA::IS2 | Invitrogen |
| DB3.1™ | F- gyrA462 endA1 glnV44 Δ(sr1-recA) mcrB mrr hsdS20($r_B^-$, $m_B^-$) ara14 galK2 lacY1 proA2 rpsL20($Sm^r$) xyl5 Δleu mtl1 | Invitrogen |
| S17.1 | $Str^r$, $Tet^r$, F– RP4-2-Tc::Mu aphA::Tn7 recA λpir lysogen | [57] |
| *Pseudomonas aeruginosa* | | |
| JJH0 | PAO1 wild type strain originating from the laboratory of Dr. Colin Manoil (MPAO1) | [58] |
| PAO1 **Δ***wspF* | MPAO1 **Δ***wspF* | [17] |
| PAO1 **Δ***yfiR* | PAO1 **Δ***yfiR* | [18] |
| JJH402 | JJH0 **Δ***cyaA* | This study |
| JJH405 | JJH0 **Δ***cyaB* | This study |
| JJH421 | JJH0 **Δ***cyaA* **Δ***cyaB* | This study |
| JJH412 | JJH0 **Δ***cpdA* | This study |
| JJH498 | JJH0 **Δ***pslD* | [59] |
| JJH502 | JJH0 **Δ***pelF* **Δ***pslD* | This study |
| JJH628 | JJH0 **Δ***chpA* | This study |
| HA67 | JJH0 **Δ***pelF* **Δ***pslD* **Δ***chpA* | This study |
| JJH469 | JJH0 **Δ***vfr* | This study |
| JJH431 | JJH0 attCTX::lacP1-lacZ | This study |
| JJH436 | PAO1 **Δ***wspF* attCTX::lacP1-lacZ | This study |
| HA225 | PAO1 **Δ***yfiR* attCTX::lacP1-lacZ | This study |
| JJH439 | JJH0 **Δ***cyaA* attCTX::lacP1-lacZ | This study |

| | | |
|---|---|---|
| JJH440 | JJH0 **Δ***cyaB* attCTX::lacP1-lacZ | This study |
| JJH525 | JJH0 **Δ***cyaA* **Δ***cyaB* attCTX::lacP1-lacZ | This study |
| JJH441 | JJH0 **Δ***cpdA* attCTX::lacP1-lacZ | This study |
| HA24 | JJH0 **Δ***pelF* **Δ***pslD* attCTX::lacP1-lacZ | This study |
| HA89 | JJH0 **Δ***chpA* attCTX::lacP1-lacZ | This study |
| HA345 | JJH0 **Δ***cyaA* **Δ***cyaB* attCTX::lacP1-lacZ attTn7::araC-pBAD-RBSII-cyaA | This study |
| HA346 | JJH0 **Δ***cyaA* **Δ***cyaB* attCTX::lacP1-lacZ attTn7::araC-pBAD-RBSII-cyaB | This study |
| HA165 | JJH0 attCTXT2.1.GW::*Ptrc*-GFP | This study |
| HA147 | JJH0 attCTXT2.1.GW::*Ptrc*-mCherry | This study |

Str, streptomycin; Tet, tetracycline

**Table S2. Plasmids**

| Plasmid | Description or relevant characteristics | Source |
|---|---|---|
| **Helper plasmids** | | |
| pFLP2 | $Ap^r$, site-specific excision vector | [55] |
| pTNS1 | $Ap^r$, helper plasmid expressing *tnsABCD* | [60] |
| **Cloning and shuttle vectors** | | |
| pJN105 | $Gm^r$, araC-$P_{BAD}$ cassette cloned into pBBR1MCS-5 | [61] |
| pJN1120 | $Gm^r$, pJN105 with PA1120 inserted in MCS | [62] |
| pJN2133 | $Gm^r$, pJN105 with PA2133 inserted in MCS | [17] |
| pJNcyaB | $Gm^r$, pJN105 with RBSII-*cyaB* inserted in MCS | This study |
| pJNcyaA | $Gm^r$, pJN105 with RBSII-*cyaA* inserted in MCS | This study |
| miniCTX2 | $Tet^r$, integration proficient vector | [63] |
| pQE30::cyaB217-463 | $Amp^r$, $Cm^r$ , pQE30 plasmid with the catalytic domain of CyaB (residues 217-463) in the MCS. | [41] |
| **Gateway donor and destination vectors** | | |
| pDONR221 | $Kn^r$, $Cm^r$, donor vector for Gateway® cloning | Invitrogen |
| pDONR221P1P5r | $Kn^r$, $Cm^r$, donor vector for multisite Gateway® cloning | Invitrogen |
| pDONR221P2P5 | $Kn^r$, $Cm^r$, donor vector for multisite Gateway® cloning | Invitrogen |
| pDONRPEX18Gm | $Gm^r$, $Cm^r$, allelic exchange vector with Gateway® donor site | [54]) |
| pEX18GmGW | $Gm^r$, $Cm^r$, allelic exchange vector with Gateway® destination site | [27] |
| pUC18-miniTn7T2.1-Gm-GW | $Gm^r$, $Cm^r$, $Ap^r$, *aacC1* on miniTn7 based vector with transcriptional terminators at the left and right ends of the Tn7 transposon; Gateway® destination vector | [59] |
| pMH487 | $Gm^r$, $Ap^r$, pUCp22Not containing RNase III splice site and GFP (mut3*) | [36] |
| pUCP22T2.1-GW | $Gm^r$, $Cm^r$, $Ap^r$, *colE1* origin of replication, *bla* and *rep* from pUCP22, ligated to the Gateway destination fragment from pUC18-miniTn2T2.1-Gm-GW; contains M13 priming sites flanking | This study |

| | | |
|---|---|---|
| | *attR1* and *attR2* | |
| miniCTX2T2.1-GW | Tet$^r$, Cm$^r$, Ap$^r$, miniCTX2 with transcriptional terminators at the left and right ends of the CTX element; Gateway® destination vector | This study |
| **Reporters and fluorescent protein labels** | | |
| miniCTX::lacP1- lacZ | miniCTX:*lacZ* with the *E.coli* lacP1 promoter cloned into the MCS | [30] |
| pUCP22Not::P*cdrA*-GFP | Gm$^r$ pUCP22Not-P*cdrA*-RBSII-*gfp*(ASV)-T0-T1 | [36] |
| pMKB1::mCherry | Carb$^r$, pMKB1::mCherry | [64] |
| pHA36 | Kn$^r$ , pENTR221L5L2::*PexoT* | This study |
| pHA46 | Kn$^r$ , pENTR221L1L5r::*mCherry*, RBSII inserted upstream of the mCherry start codon. | This study |
| pHA50 | Kn$^r$ , pENTR221L5L2::*Ptrc* | This study |
| pHA51 | Tet$^r$, miniCTX2T2.1-GW::*Ptrc*-mCherry | This study |
| pHA60 | Kn$^r$, pENTR221L1L5r::GFP(ASV), RBSII inserted upstream of the GFP start codon. | This study |
| pHA110 | Kn$^r$ , pENTR221L5L2::*lacP1* | This study |
| pHA67 | Gm$^r$, pUCP22T2.1-GW::*lacP1*-GFP(ASV) | This study |
| pHA69 | Kn$^r$ , pENTR221L5L2::*PptxR* | This study |
| pHA75 | Gm$^r$, pUCP22T2.1-GW::*PexoT*-GFP(ASV) | This study |
| pHA76 | Gm$^r$, pUCP22T2.1-GW::*PptxR*-GFP(ASV) | This study |
| **Allelic exchange vectors** | | |
| pEX18Gm | Gm$^r$, allelic exchange vector | [55] |
| pEX18Gm::Δ*pelF* | Gm$^r$, pEX18Gm with an in-frame deletion construct for *pelF* | [65] |
| pEX19Gm::Δ*wspF* | Gm$^r$, pEX19Gm with an in-frame deletion construct for *wspF* | [17] |
| pJJH55 | Gm$^r$ , pEX18Gm with an in-frame, 678 bp deletion construct for *cpdA* inserted in MCS (EcoRI/HindIII) | This study |
| pJJH57 | Gm$^r$ , pEX18Gm with an in-frame, 678 bp deletion construct for *cyaB* inserted in MCS | This study |

| | | |
|---|---|---|
| | (EcoRI/HindIII) | |
| pJJH61 | $Gm^r$ , pEX18Gm with an in-frame, 907 bp deletion construct for *cyaA* inserted in MCS (EcoRI/HindIII) | This study |
| pJJH107 | $Gm^r$, pEX18GW with an in-frame, 516 bp deletion construct for *vfr* | This study |
| pJJH206 | $Gm^r$, pDONRPEX18Gm with an in-frame, 1170 bp deletion construct for *chpA* | This study |
| **miniTn7 vectors** | | |
| pUC18-miniTn7T-Gm | $Ap^r$, $Gm^r$, cloning vector for gene insertion in $Gm^s$ bacteria | [60] |
| pHA59 | $Ap^r$, $Gm^r$, pUC18-miniTn7T-Gm with araC-pBAD-RBSII-cyaB fragment inserted in MCS (KpnI/PstI) | This study |
| pHA104 | $Ap^r$, $Gm^r$, pUC18-miniTn7T-Gm with araC-pBAD-RBSII-cyaA fragment inserted in MCS (KpnI/SacI) | This study |

Ap, ampicillin; Cm, chloramphenicol; Kn, kanamycin; Gm, Gentamicin; Tet, tetracycline

**Table S3. Primers.**

| Oligonucleotide | DNA sequence* |
|---|---|
| **DNA sequencing and verification of deletion or insertion mutations** | |
| M13F(-21) | TGT AAA ACG ACG GCC AGT |
| M13R | CAG GAA ACA GCT ATG AC |
| HA61_pelFseqUp | TAC TGG GAA CTG GCC TA |
| HA62_pelFseqDown | AAA TGA AGC GGG TGA AGA |
| JJH881_pUCP22repF01-SEQ | CAA GGT CAC ACA TCT GTT G |
| JJH894_DestGWR01readout-SEQ | CTG CTG TCA GAT AAA GTC TCC |
| JJH895_DestGWF01readout-SEQ | GCT TCC TTA GCT CCT GAA AAT C |
| JJH926_pUCP22T1R01-SEQ | GGC GAT TAA GTT GGG TAA CG |
| JJH927_pUCP22oriF01-SEQ | CAC TCA AAG GCG GTA ATA CG |
| JJH928_pUCP22blaF01-SEQ | CGA TCG TTG TCA GAA GTA AG |
| JJH929_pUCP22aacC1F01-SEQ | GTT AGG TGG CGG TAC TTG G |
| JJH930_pUCP22backboneF01-SEQ | CTT CAG GGG CAC AAA TGC G |
| JJH931_GWDestFragcatF01-SEQ | GTG ATG GCT TCC ATG TCG G |
| **Construction of shuttle vectors (pJNcyaA and pJNcyaB)** | |
| cyaB-up-EcoRI-RBSII | ATC CGG **GAA TTC** *AAA GAG GAG AAA* ATG AAG CCT ACC CTC CC |
| cyaB-dn-pstI | ATC CGG **CTG CAG** TTA GAG GAT GAC CTTG TCG |
| cyaA-RBSII-NheI | CGC C**GC TAG C***AA AGA GGA GAA A*AT GAA CCG ACA CCC CGC A |
| cyaA-SacI | CGT C**GA GCT CT**C ATT GTT CCA GCA GCG CC |
| **Construction of destination vectors (pUCP22T2.1-GW and miniCTX2T2.1-GW)** | |
| JJH876_pMH487-T1-F01 | ATC CGG **AAG CTT** GCA GTT ATT GGT GCC CTT AAA C |
| JJH878_pMH487-rep-M13R-R01 | ATC CGG **AAG CTT** gtc ata gct gtt tcc tgT CAG TTG CGC AGC CTG AAA G |
| JJH879_miniTn72.1-attR1-F01 | ATC CGG **AAG CTT** GTT GGC CTG CAA GGC CTT C |

| | |
|---|---|
| JJH880_miniTn72.1-attR2-R01 | ATC CGG **AAG CTT** GCA GGA ATT CCT CGA GAA GC |

**Construction of promoter-reporter and labeling vectors**

| | |
|---|---|
| Ptrc-attB2-fwr | GGG G**AC CAC TTT GTA CAA GAA AGC TGG GT**A GAC GGA TCC GGG GAA TT |
| Ptrc-attB5-rev | GGG G**AC AAC TTT GTA TAC AAA AGT TGC C**CT CCT CTA GAC TGC AGC |
| mCherry-RBSII-attB5r-fwr | GGG G**AC AAC TTT TGT ATA CAA AGT TGT** CAG TCT AGA GGA GAA AAA AAA ATG |
| mCherry-attB1-rev | GGG G**AC AAG TTT GTA CAA AAA AGC AGG CT**C TTA CTT GTA CAG CTC GTC C |
| attB2-lacP1-fwr2 | GGG G**AC CAC TTT GTA CAA GAA AGC TGG GT**A GCC CAA TAC GCA AAC CG |
| attB5-lacP1-rev2 | GGG G**AC AAC TTT GTA TAC AAA AGT TGC CC**G AAA GGG GGA TGT GC |
| attB2-PexoT-fwr | GGG G**AC CAC TTT GTA CAA GAA AGC TGG GT**A GGC GAC GTG CCT GTG C |
| attB5-PexoT-rev | GGG G**AC AAC TTT GTA TAC AAA AGT TGC CC**A TGA TTG ACG TCT CCT GAT G |
| attB2-PptxR-fwr | GGG G**AC CAC TTT GTA CAA GAA AGC TGG GTA** GCT GAA ACT TAT GCG CG |
| attB5-PptxR-rev | GGG G**AC AAC TTT GTA TAC AAA AGT TGC CC**C TGT CAT TCC TTA GCC |

**Construction of allelic exchange vectors with in-frame deletion alleles**

| | |
|---|---|
| JJH297_PA4969upF1 | ATC CCG **AAG CTT** GCT GAT GGC GAT CTA TCC |
| JJH298_PA4969upR1 | *GGC ATG TCA GTA TCC GGC GGT GTC* GTT CGA ATG GCG TGA CAA |
| JJH299_PA4969downF1 | GAC ACC GCC GGA TAC TG |
| JJH300_PA4969downR1 | ATC CCG **GAA TTC** CCT GCC GTG GGT GAT G |
| JJH329_cyaBupF1 | ATC CCG **AAG CTT** CAA GTA CCC GCT GTG GTC |
| JJH330_cyaBupR1 | *AAC TTA GAG GAT GAC CTT GTC GCG* GAC GGA CCG GCT CGT C |
| JJH331_cyaBdownF1 | CGC GAC AAG GTC ATC CTC |
| JJH332_cyaBdownR1 | ATC CCG **GAA TTC** CTC GGT GCT GGT GTT CC |

| | |
|---|---|
| JJH341_cyaAupF2 | ATC CCG **AAG CTT** CTA GTC CAA CTA CAC TCA C |
| JJH342_cyaAupR2 | *CAG CGC CTG GTT CAG CGC CGT TTC* GAC CTT GCG GTC GAT GC |
| JJH343_cyaAdownF2 | GAA ACG GCG CTG AAC CAG |
| JJH344_cyaAdownR2 | ATC CCG **GAA TTC** GCG TAT GGT GGA GGA AGC |
| JJH381_vfrupF3-GWB1 | GGG G**AC AAG TTT GTA CAA AAA AGC AGG CTC G**CA AAG GGC GCC AGC TTA G |
| JJH334_vfrupR2 | *TGC TGT TCA GCG GGT GCC GAA GAC* GTG GGT AAT AGC TAC CAT G |
| JJH345_vfrdownF2 | GTC TTC GGC ACC CGC T |
| JJH382_vfrdownR3-GWB2 | GGG G**AC CAC TTT GTA CAA GAA AGC TGG GT**G GGT ATT CTC AAC CGG GC |
| JJH892_chpAupF04-GWB1 | GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CCT ACT CGC TGG CGA TGT G |
| JJH845_chpAupR02 | *CGC GCT CAC TCA TGC TGG CCG AC*C TCG CCT TTC ACC CAC TCC |
| JJH846_chpAdownF02 | GTC GGC CAG CAT GAG TGA G |
| JJH893_chpAdownR04-GWB2 | GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA CAT GCC ACT GGC TGT GCC |

* Regions of homology to the target amplicons are underlined, regions of reverse complementarity are *italicized*, and restriction sites and Gateway attB1 and attB2 sequences are in **bold**.