Supplementary Tutorial
How to synthesize a deletion allele and make a knockout strain

This tutorial is aimed at the novice geneticist and provides anticipated outcomes for key steps of the genome engineering protocol. Here we provide an example of how to design primers and build an in-frame deletion allele. To do this, we focus on creating a deletion in the *Pseudomonas aeruginosa pelF* gene, which encodes a cytosolic glycosyltransferase essential for the biosynthesis of the extracellular polysaccharide PEL[1]. We subsequently use this deletion allele, as well as other deletion alleles, to illustrate the capacity of the non-mucoid *P. aeruginosa* PAO1 strain to produce two different biofilm structural extracellular polysaccharides, PEL and PSL[2].

Primer design (Steps 1-3)
We retrieved the information for *pelF* from the Pseudomonas Genome Database[3]. Using the guidelines presented in this protocol, we designed primers to delete an in-frame, 1473 bp fragment of coding sequence from the 1524 bp *pelF* ORF (illustrated in Supplementary Fig. 2A). In the example below, the desired T_m of the primers was 60 ± 2 °C. The target sequences in the genome are underlined, regions of reverse complementarity are *italicized*, and the targets of sequencing primers are highlighted in yellow:

Upstream sequence (500 bp):

GCCCTGCGCGACCCCGCCGACGACGTCCGCCTGCTGGCCTACTCGATGCTCGACCAGAAGGAAAGCCGGATCAACCAGCGCATCGAGGCCGCCCTCGGCC
GCCTCGCCGGCGCCACGCCGGCGCGACGCGGCGCGCTGCACGGAACCCTGGCGCGCTGGTACTGGGAACTGGCCTACCTGGGGTTGGCCCAGGGCAGCGT
GCTGGAGCACATCCTCGAGCAGGCGCGCGAGCATACCGACCAGGCGCTGCGCGGCACACCCTCGGCCGACCTGCATCTGCTGGCCGGGCGCATCGCCCTC
GAACAAGGCCGCCTGGAGGATGCCGGACGCGCCCTCCAGGCCGCCGAGGAGGCGGGAATCGATAGTGCCCAGCTGGCGCCGTTCCGCGCCGAGGTGGCGT
TCTTCCAGCGGCGCTACCGGGACATCCCCCGGCTTCTCGCCGGAATGCCCGACGACATGCTGCAACGGCCGCCCTTCGCGGCCCTGGCGAGATACTGGAC

pelF ORF:

ATGACCGAACACACCGCTCCGACGGCGCCCGTCGCCGATGTCTGCCTGCTGCTGGAGGGCACCTGGCCCTATGTCCGCGGCGGCGTCTCCAGCTGGGTCA
ACCAGTTGATCCTCGGTCTCCCCGACCTGACCTTCTCGGTGTTCTTCATCGGCGGCCAGAAGGATGCCTACGGCAAGCGCCACTACCCGATCCCGGACAA
TGTGCTGCACATCGAGGAACACTTCCTGGAAACCGCCTGGAGTTCGCCGAACCCGCAGACGCGACAGGGCAGTAGCGAGACCGAAAAGGCGTTGCGCGAT
CTGCACCGTTTCTTCCACTACCCGGAGACGCCGGACGTGGAGGAGGGCGACGCGCTGCTCGACCTGCTCGCCGAGGGCCGCATCGGCCGCGAGGACTTTC
TCCACAGCAAGGCCAGTTGGGAGGCGATCACCGCAGGCTACGAGCGCTATTGCACCGATCCGTCCTTCGTCAATTACTTCTGGACCCTGCGCTCGATGCA
GGCGCCGGTGTTCATGCTCGCCGAGGCGGCCCGGCGGATGCCGCGGGCGCGCATGCTGCACTCGATCTCCACCGGCTACGCCGGCCTGCTGGGCTGCATC
CTGCAACGTCGCTGGGGCTGCCGCTACCTGCTCAGCGAGCACGGCATCTACACCAAGGAGCGCAAGATCGACCTGGCCCAGGCCAACTGGATCGCGGAGA
ACCCCGACGAGCAGCTGAGTACCGGACTGGATGCCGAGGTCAGCTACATCCGTCGCTTGTGGATCCGCTTCTTCGAGCGTGTCGGCCTGCTCACCTATCG
CGCCGCCAATCCGATCGTCGCCCTCTACGAAGGCAACCGCCAGCGCCAGGTACTCGATGGCGCCGAGCCACGGCGCACCCGGGTGATCCCCAACGGCATC
GACCTCGATGCCTGGACCGGCGCCCTCGAACGGCGGCCGCCGGGGATTCCGCCGGTGGTCGGGCTGGTCGGCCGGGTAGTGCCGATCAAGGACGTGAAGA
CCTTCATCCGCGCCATGCGCGGGGTGGTCAGCGCGATGCCGGAGGCGGAGGGCTGGATCGTCGGTCCGGAGGAGGAAGATCCGGACTATGCCAGCGAATG
CCGCAGCCTGGTGGCCAGCCTCGGCCTGCAGGACAAGGTGAAGTTCCTCGGTTTCCGTCGGATCGGCGAGGTCCTGCCGCAACTCGGCCTGATGGTCCTC
ACCTCGATCAGCGAAGCGCAGCCGCTGGTGATCCTCGAAGCCTGGGCTGCCGGCGCCCCGGTGGTGAGCAGCGACGTCGGCTCCTGCCGCGAACTGATCG
AAGGCGCCGACGCCGAAGATCGCGCCCTGGGTCGCGCCGGGGAGGTGGTGGCGATCGCCGACCCGCAGGCCACTTCGCGGGCGATCCTCGCCCTGCTGCG
CAATCCGCAGCGCTGGCAGGCGGCCCAGGCGGTCGGCCTGCAACGGGTCGAACGCTACTACACCGAGGCGCTGATGCTCGGACGTTACCGCGGG*CTGTAC*
*CGCGAAGCCACGG*AGATTGCATGA

Downstream sequence (500 bp):

CATGGCCGGCATCGGCTTCGAACTGCGGAAGATCCTTTCCCGCGATTCCTATACGGCGACCCTGCGCGCCTACCTCTACGCCGGGCTGATCAGCTCCGGT
CCCTGGGTGCTGTCGATCGTCAGCGTGATGCTGATCGGCGTGCTGAGCCTCGGCGTGGTGGTGCCGGACGTGCTGGTCCGGCAGTTCCTGATCACGGTGA
CCTACCTGATGGCGCTGTCGCTGATCTTCACCGGGGGACTGCAACTGTTCTTCACCCGCTTCATTTCCGATCGCCTGTTCGAGCGCAAGCACGAGGCGAT
CCTGCCCAACCTGGTGGGCGTGCTGTTGCTGGTGACGTTGGCGGCGGGCCTGCTCTCGGCGATATTGCTGGCGACCCTGTTCGATGAGCCGTTCGCCTAC
CGCCTGCTGGTGATGGCCAACTTCGTGGTGCTCTGCAACCTGTGGCTGGTGATCATCTTCCTGTCGGGAATGAAGGCCTACAAACGCATCCTGCTGGTGA

The primers were synthesized as follows (note that *attB*1 and *attB*2 sequences for Gateway® recombination are shaded, see Box 2 for design guidelines):

pelFupF01-GWB1 (Primer "Up-F")

GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA CGC TGG TAC TGG GAA CTG GC

pelFupR01 (Primer "Up-R")

*GCA ATC TCC GTG GCT TCG CGG TAC AG*C GGA GCG GTG TGT TCG GTC

pelFdownF01 (Primer "Down-F")

CTG TAC CGC GAA GCC ACG G

pelFdownR01-GWB2 (Primer "Down-R")

GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA CAG GGT CGC CAG CAA TAT CG

pelFF01-SEQ (Primer "Seq-F")

CTA CTC GAT GCT CGA CCA GAA G

pelFR01-SEQ (Primer "Seq-R")

GAT GCG TTT GTA GGC CTT CAT TC

PCR to synthesize the mutant allele (Steps 4-12)

Primer melting temperature (T_m) calculated with Oligocalc[4] or Primer3[5] are usually accurate; however, these Tm values do not always correspond to those that give the best amplification *in vitro*. Here, temperature-gradient PCR was used to amplify the upstream and downstream fragments (Supplementary Fig. 2B). Typically, we use four wells on the gradient, set at 70.8, 68.7, 65.6 and 61.7 °C (Supplementary Fig. 2B) with a high fidelity polymerase such as Phusion (NEB). Using the guidelines for primer design in Box 3, we estimate that we have historically captured >95% of the target amplicons cloned using this approach on the first attempt (for difficult templates, see troubleshooting in Table 3). Individual PCR fragments (424 and 439 bp for the "Up" and "Down" fragments, respectively, Supplementary Fig. 2B) were cut from the agarose and gel purified. The fragments were then joined using SOE-PCR to produce the Δ*pelF* allele using temperature-gradient PCR (Supplementary Fig. 2B).

Cloning efficiency and sequencing of the allelic exchange vector (Steps 13-35)

The gel purified SOE-PCR product (837 bp, Supplementary Fig. 2B) was recombined with pDONRPEX18Gm, incubated for 1 h at room temperature with BP Clonase®, and transformed into chemically competent *E. coli* Zymo5α Mix & Go cells using the 5 minute protocol provided by the manufacturer (Zymogen). One fifth of the outgrown, transformed cells were spread on selective agar, which yielded ~50 colonies after overnight incubation at 37 °C. A yield of 50-100 clones (or ~250-500 clones for the entire transformation mixture) is typical of the BP clonase reaction in our hands. Four of these colonies we examined by colony PCR, and all of them contained an insert (Supplementary Fig. 2C). The expected size of the PCR product from the pDONRPEX18 vectors is equal to the size of the SOE-PCR product plus 416 bp (1253 bp in this example), which accounts for the distance between M13F and M13R priming sites and the cloned insert. Based on a sample of 50 allelic exchange vectors we have built using Gateway® technology, on the average ~80% of the screened colonies contained an insert. Both of the colonies sent for Sanger sequencing were a perfect match to the predicted sequence. We have found that ~10% of the vectors created using this protocol contain an error that has arisen during the cloning process, which is the impetus for sending two clones for Sanger sequencing at a time.

Biparental mating and merodiploid selection (Step 36)

The pENTRPEX18Gm::Δ*pelF* vector was introduced into *P. aeruginosa* PAO1 via biparental mating with *E. coli* S17.1. After selection on VBMM agar containing 60 μg ml^{-1} Gm, ~40 colonies were recovered from a 500 μl aliquot of the cells (Step 36A). This was much lower than usual number of merodiploids given the lengths of the regions of homology between the Δ*pelF* allele and the PAO1

chromosome (~776 bp). Based on a sample of 42 biparental matings with pEX18-based allelic exchange vectors, we recovered on average ~450 colonies per mating per 1 kb of homology to the recipient chromosome (Fig. 6A).

Counter-selection and mutant detection (Steps 37-49)
A merodiploid colony was smeared across the surface of NSLB + 15% sucrose agar and incubated for ~36 h. The growth on this plate was scant (usually no more than 200 colonies are recovered during counter-selection). Note that the growth of a bacterial lawn on NSLB + 15% sucrose agar indicates a problem with selection or counter-selection (see troubleshooting in Table 3). Four colonies were picked from the NSLB + 15% sucrose agar plate, transferred onto selective media and screened by PCR. Given the target amplicon of the Seq-F and Seq-R primers, it was expected that the wild type allele would produce a PCR product of 2476 bp, and the deletion allele 1009 bp. We found that two of these colonies had the desired mutation (Supplementary Fig. 2D), as well as antibiotic sensitivity and sucrose resistance (Supplementary Fig. 2E). We did not observe any sucrose resistant merodiploids. The PCR products were sent for sequencing and both 100% matched the predicted deletion sequence.

Proof-of-principle
We used the Δ*pelF* construct, as well as previously described constructs for Δ*pslD*[6] and Δ*wspF*[7], to show that *P. aeruginosa* PAO1 relies on two extracellular polysaccharides, Pel and Psl, for biofilm development[2]. Production of Pel and Psl is controlled by an intracellular second messenger called cyclic-di-GMP. WspF is a repressor of a diguanylate cyclase, WspR, which synthesizes cyclic-di-GMP[7]. Inactivation of *wspF* artificially increases the intracellular cyclic-di-GMP, causing *P. aeruginosa* PAO1 to overexpress Pel and Psl and overproduce biofilm[8,9]. These biofilms are easily visible in culture tubes and may be stained with crystal violet (Supplementary Fig. 3).

After generating the Δ*pelF* mutant above, we used this protocol to introduce Δ*pslD* into the wild type and Δ*pelF* strains, generating a Δ*pslD* and Δ*pelF*Δ*pslD* single and double mutant cell lines, respectively. Next, we used this protocol again to introduce the Δ*wspF* allele into wild type, Δ*pelF*, Δ*pslD* and Δ*pelF*Δ*pslD* backgrounds in parallel. This produced Δ*wspF*, Δ*wspF*Δ*pelF*, Δ*wspF*Δ*pslD* and Δ*wspF*Δ*pelF*Δ*pslD* strains. Consistent with previous descriptions[9], loss-of-function mutations in *pelF* or *pslD* decreased the ability of the Δ*wspF* mutant to produce biofilm, whereas the double Δ*pelF* and Δ*pslD* mutations abolished biofilm production in a Δ*wspF* background (Supplementary Fig. 3). Because these mutations could be made in parallel, the construction of the deletion allele as well as the single, double and triple mutants took <5 weeks.

Supplementary Table 1. Primers.

Oligonucleotide	DNA sequence[a]
Plasmid sequencing (includes sequences deposited in Genbank, see Table 1)[b]	
JJH367_M13-Universal-F(-21)	TGT AAA ACG ACG GCC AGT
JJH368_M13-Universal-R	CAG GAA ACA GCT ATG AC
LRH28	GCC CGA GGC ATA GAC TGT ACA AAA
LRH30	GGC GGT ACT TGG GTC GAT ATC A
LRH41	CAA AAG GCC AGG AAC CGT AAA A
LRH109	CTC TAG AGG ATC CCC GGG
LRH110	CGA CCG CTG CGC CTT ATC C
LRH111	CAG CGG TCC AGT GAT CGA AG
LRH112	CAG CAA CCG CAC CTG TGG C
LRH113	GCA GAC AAG CCC GTC AGG
LRH114	GAT TCT TCG CCT TGG TAG CC
LRH115	GCT TTT GGT TCG TTT CTT TCG
LRH116	CAT AGT CCA CGA CGC CCG TG
LRH117	TGT AGA AAC GCA AAA AGG CCA TC
LRH118	GTT CAG CTG GAT ATT ACG GCC
LRH119	GAT GTC AAT ATC TCC GGT CTG G
LRH161	CTA CGG GGT CTG ACG CTC
LRH162	GCG ACC TGA GCA ACA ACA TG
LRH163	GAA ACT CAA CGA GCT GGA CG
LRH164	CTT TCG CTT GAG GTA CAG CG
LRH165	GCT GAA CCT GAC CAT TCT TGT G
LRH166	GCA GAC TAC GGG CCT AAA G
LRH167	CGC TAT AAT GAC CCC GAA GC
LRH169	GTG CAC CAT AAT CGG CAT TTT C
LRH170	CGT TCA GCT GGA TAT TAC GGC C
LRH171	GTT GCT CAA GGC ATA TAT GAT G
LRH172	GAT TTA ATA CGG CAT TGA GGA C

Sequencing primers for verification of deletion or insertion mutations[c]

KMC179_pslDF1-SEQ	CCG AGG TCT ACC ATT CCC ACG
KMC180_pslDR1-SEQ	GAA CTT GGT GCG CTT CCA CAG
MEL82_pilBF01-NESTED-SEQ	GTC GCA GTA GAA GCA GTA G
MEL88_pilCF01-NESTED-SEQ	CTC TGT TCG CAC TGC AAG
MEL94_pilUF01-NESTED-SEQ	CCT GAT CAA GAA GAT CGG C
MEL100_pilNF01-NESTED-SEQ	CAG CTC ACC GAG GAG ATC
MEL112_pilSF01-NESTED-SEQ	CTT CGG TCT CTT CGA CTG AT
MEL118_pilRF01-NESTED-SEQ	GAC AGC CAA CTG CAC CTG
MEL124_pilIF01-NESTED-SEQ	GTT GAT GAC TCT CCG ACC
MEL130_ pilFF01-NESTED-SEQ	GAG AAG CGC GTG CTG AC
MEL154_ fimTF01-NESTED-SEQ	GTA GAA GTC CTG CGA CCA G
JJH764_chpBup-SEQ	AAA TGG CGG CGA GCG AA
JJH765_chpBdown-SEQ	CAG CAG GGC GAG GAA TT
JJH770_pilDup-SEQ	TTA TGC ACG GAC CTT GT
JJH771_pilDdown-SEQ	GAT GGC ATA GGG CGA TT
JJH776_pilHup-SEQ	CCA TCC GAA CAT CAT TT
JJH777_pilHdown-SEQ	TTG CAG TGC CGC TTC TA
JJH782_pilGup-SEQ	TGT TCC ATG TAG AAC AG
JJH783_pilGdown-SEQ	ACC GCA TTG ATG GTT TT
JJH788_pilJup-SEQ	ATG GAC CTC TGC GGT TT
JJH789_pilJdown-SEQ	GGG GAT GAC GGA AGA AA
JJH794_pilZup-SEQ	TGA AGA AGC TGC TCA AG
JJH795_pilZdown-SEQ	TTC GTA GTG GTA GTC GA
JJH800_pilY2up-SEQ	GCT GCT GCA AAC CAT CA
JJH801_pilY2down-SEQ	GAG GGG CTC TTT CGT TT
JJH806_pilTup-SEQ	CTC CTG CAG GTA GTT TT
JJH807_pilTdown-SEQ	ATG GTT CTC GGC GAA AT
JJH812_fimUup-SEQ	CGC TTG CAA AGA AGG AAA
JJH813_fimUdown-SEQ	GTT CGT TCT TCA CCT GTT

JJH818_pilVup-SEQ	GAG GAA CTC AAT GCG AT
JJH819_pilVdown-SEQ	GAT AGC GGT AGC AGA AT
JJH1183_wspFforward2-SEQ	CTG CGC TCG GAG CAT TTC G
JJH1184_wspFreverse2-SEQ	CAG TGA GCG ACG CAC AGC
JJH1481_pelFF01-SEQ	CTA CTC GAT GCT CGA CCA GAA G
JJH1482_pelFR01-SEQ	GAT GCG TTT GTA GGC CTT CAT TC
rjs_pilA_F-SEQ	CCA AAT CGA GGA AAT CCA GCT GTC
rjs_pilA_R-SEQ	GAA GCG GGG CTT TTT TAT GCG

Construction of allelic exchange vectors with in-frame deletion alleles

MEL78_pilBupF01-GWB1	GGG G**AC AAG TTT GTA CAA AAA AGC AGG CTA C**CT TGT TGG CAT CCG GCT CG
MEL79_pilBupR01	*TTA ATC CTT GGT CAC GCG GTT GAC* GGA CAG GCC GCT CAG TTG
MEL80_pilBdownF01	GTC AAC CGC GTG ACC AAG
MEL81_pilBdownR01-GWB2	GGG G**AC CAC TTT GTA CAA GAA AGC TGG GTA** GGT CGC GAC TCT ATC CAG C
MEL84_pilCupF01-GWB1	GGG G**AC AAG TTT GTA CAA AAA AGC AGG CTA C**GT TCA ACC TGG CGA CCT C
MEL85_pilCupR01	*TTA TCC GAC GAC GTT GCC GAG TTG* CAG ATG GGC CTT CAC CAG
MEL86_pilCdownF01	CAA CTC GGC AAC GTC GTC
MEL87_pilCdownR01-GWB2	GGG G**AC CAC TTT GTA CAA GAA AGC TGG GTA** GCA GTT GGT GAT CGG CAT C
MEL90_pilUupF01-GWB1	GGG G**AC AAG TTT GTA CAA AAA AGC AGG CTA C**CA TGG TTC GCT CGA TGC TC
MEL91_pilUupR01	*GGC AGG GTC GTC GTC GGT GAT TTC* CAG GCG CAG CAG CTT TTC
MEL92_pilUdownF01	GAA ATC ACC GAC GAC GAC C
MEL93_pilUdownR01-GWB2	GGG G**AC CAC TTT GTA CAA GAA AGC TGG GTA** CAG GGC AAC AGC CTG AAC
MEL96_pilNupF01-GWB1	GGG G**AC AAG TTT GTA CAA AAA AGC AGG CTA C**CG ACA CCG ACC AGT TGA C
MEL97_pilNupR01	*TCA TTT CTT GGC TCC TTG CGC AAC* GAT CCG TGC CAT CAG TCG
MEL98_pilNdownF01	GTT GCG CAA GGA GCC AAG
MEL99_pilNdownR01-GWB2	GGG G**AC CAC TTT GTA CAA GAA AGC TGG GTA** GTC GCC AAG TCG TGG TAG
MEL108_pilSupF01-GWB1	GGG G**AC AAG TTT GTA CAA AAA AGC AGG CTA C**GA TCA CAG CGC CGA CGA C
MEL109_pilSupR01	*TCA GCT GAG TTT GCG TGG GTG GGC* GCT CAG CCG TAG CCG TTC
MEL110_pilSdownF01	GCC CAC CCA CGC AAA CTC
MEL111_pilSdownR01-GWB2	GGG G**AC CAC TTT GTA CAA GAA AGC TGG GTA** CAG CTT GCC GAT CTG GTT G
MEL114_pilRupF01-GWB1	GGG G**AC AAG TTT GTA CAA AAA AGC AGG CTA C**CA GCA GCT CGA CCT GAA G

MEL115_pilRupR01	*GTA GCG CAT CGA GCG GAA CGT CAG* GAC GAT CAG GGC TTT TTG TCG
MEL116_pilRdownF01	CTG ACG TTC CGC TCG ATG
MEL118_pilRdownR01-GWB2	GGG G**AC CAC TTT GTA CAA GAA AGC TGG GTA** CTG GAA CTG CCC GTG GTA C
MEL120_pilIupF01-GWB1	GGG G**AC AAG TTT GTA CAA AAA AGC AGG CTA C**GA CGC CGT TTC CTG ATA TCC
MEL121_pilIupR01	*TAC GGC GAC GTC GAG GAA GCC CTG* GAA GGG GGT CTG AAC GTC
MEL122_pilIdownF01	CAG GGC TTC CTC GAC GTC
MEL123_pilIdownR01-GWB2	GGG G**AC CAC TTT GTA CAA GAA AGC TGG GTA** CTG TTC GCT GGC GAG GAT G
MEL126_pilFupF01-GWB1	GGG G**AC AAG TTT GTA CAA AAA AGC AGG CTA C**CG CAC CGA ACG ACG AGT TG
MEL127_pilFupR01	*TTC CTG ATA TTC GAG AGA GCC TGG* CAG CCC AAC TGC CAA CAG
MEL128_pilFdownF01	CCA GGC TCT CTC GAA TAT C
MEL129_pilFdownR01-GWB2	GGG G**AC CAC TTT GTA CAA GAA AGC TGG GTA** GTA TGG ATC TCG GTG GTG C
MEL150_fimTupF01-GWB1	GGG G**AC AAG TTT GTA CAA AAA AGC AGG CTA C**CT CCT CGA TCG GCA CTT CC
MEL151_fimTupR01	*TCC GGA AGT GCT GCA TAG CTC ACG* CGC TCT CTG CGA CCT TTC
MEL152_fimTdownF01	CGT GAG CTA TGC AGC ACT TC
MEL153_fimTdownR01-GWB2	GGG G**AC CAC TTT GTA CAA GAA AGC TGG GTA** GCC GTG AAG GTC AGA TGT TC
JJH736_pilY1upF01-GWB1	GGG G**AC AAG TTT GTA CAA AAA AGC AGG CTC A**GT ACT GGA AAG CCG CAT CAC
JJH737_pilY1upR01	*TCA GTT CTT TCC TTC GAT GGG GCG* GAT CTG GTG GAG TAC CGA TTT
JJH738_pilY1downF01	CGC CCC ATC GAA GGA AAG A
JJH739_pilY1downR01-GWB2	GGG G**AC CAC TTT GTA CAA GAA AGC TGG GTA** GAT CAC GTA GTT CTG GTA ACT G
JJH740_pilQupF01-GWB1	GGG G**AC AAG TTT GTA CAA AAA AGC AGG CTC A**CA GTC CTA CAT GGA CGA GG
JJH741_pilQupR01	*TCA GCG ACC GAT TGC GAT GGC CTG* GCG CGA GAG GCC ACT GTT
JJH742_pilQdownF01	CAG GCC ATC GCA ATC GGT C
JJH743_pilQdownR01-GWB2	GGG G**AC CAC TTT GTA CAA GAA AGC TGG GTA** CGG TCT CCA CCA CCA CAT C
JJH951_pilWupF02-GWB1	GGG G**AC AAG TTT GTA CAA AAA AGC AGG CTC A**CA TGC TCG GCA GCA ACC TG
JJH745_pilWupR01	*TGG CAC GAG ATT CCT GAG TGT CTG* GGA GCG GTT GTT CAT GCT C
JJH746_pilWdownF01	CAG ACA CTC AGG AAT CTC GTG
JJH952_pilWdownR02-GWB2	GGG G**AC CAC TTT GTA CAA GAA AGC TGG GTA** GGT CGC GCT GCT GAT GTC
JJH748_pilMupF01-GWB1	GGG G**AC AAG TTT GTA CAA AAA AGC AGG CTC A**CA CAT CGA CGC CAT AAT GGT T
JJH749_pilMupR01	*TCA GTC GAA ACT CCT CAA CGC CAG* CGC TTT CTT CTT TAT GAG CCC
JJH750_pilMdownF01	CTG GCG TTG AGG AGT TTC G

JJH751_pilMdownR01-GWB2	GGG G**AC CAC TTT GTA CAA GAA AGC TGG GTA** CTT GAC CTC GTT CAG GGT C
JJH766_pilDupF01-GWB1	GGG G**AC AAG TTT GTA CAA AAA AGC AGG CTC A**GT CGA TGC GCT CGA CTC
JJH767_pilDupR01	*TCA TTT GAA TCC GGC GAA TTG CAG* CAG GTA GTC GAG GAG GG
JJH768_pilDdownF01	CTG CAA TTC GCC GGA TTC AAA
JJH769_pilDdownR01-GWB2	GGG G**AC CAC TTT GTA CAA GAA AGC TGG GTA** GGT ACT GGA CGA TCT CCG
JJH772_pilHupF01-GWB1	GGG G**AC AAG TTT GTA CAA AAA AGC AGG CTC A**TC GTC GAC ATC ATG ATG CC
JJH773_pilHupR01	*TCA GCC CGC CAG CAC CGC ATT GAT* ATC AAC AAT CAA AAT ACG AGC CAT
JJH774_pilHdownF01	ATC AAT GCG GTG CTG GCG
JJH775_pilHdownR01-GWB2	GGG G**AC CAC TTT GTA CAA GAA AGC TGG GTA** GCA GAG GTC CAT GAT CGG
JJH778_pilGupF01-GWB1	GGG G**AC AAG TTT GTA CAA AAA AGC AGG CTC A**GC GAG CTG TCC TTC TTG AA
JJH779_pilGupR01	*TCA GGA AAC GGC GTC CAC CGG GGT* CAA ACC GTC GGA TTG CTG TT
JJH780_pilGdownF01	ACC CCG GTG GAC GCC
JJH781_pilGdownR01-GWB2	GGG G**AC CAC TTT GTA CAA GAA AGC TGG GTA** CTT GGT GGT GAC GAT GAT CA
JJH784_pilJupF01-GWB1	GGG G**AC AAG TTT GTA CAA AAA AGC AGG CTC A**GC TGG TGG TGG AGC ACC
JJH785_pilJupR01	*TCA GGC CTG CTC CAC GCC CTC CGG* ATT GCC TGC GTT GAT TTT CTT C
JJH786_pilJdownF01	CCG GAG GGC GTG GAG
JJH787_pilJdownR01-GWB2	GGG G**AC CAC TTT GTA CAA GAA AGC TGG GTA** GCA GGG TCG CCC ACT C
JJH790_pilZupF01-GWB1	GGG G**AC AAG TTT GTA CAA AAA AGC AGG CTC A**CA GCG TGC CGT TGC CAT T
JJH791_pilZupR	*TTA CAT CGT GTG GGT CGG CCG GTC C*CC CAG ATT GGG TGG CAA
JJH792_pilZdownF01	GAC CGG CCG ACC CAC
JJH793_pilZdownR01-GWB2	GGG G**AC CAC TTT GTA CAA GAA AGC TGG GTA** GTC CGG ATC GGC GAG AC
JJH797_pilY2upF01-GWB1	GGG G**AC AAG TTT GTA CAA AAA AGC AGG CTC A**GA ACG ATG ACC CCT GTG C
JJH798_pilY2upR	*TCA TCG GGG CTG CTC CTC CCC GTC* CAG CAG CAT AGG CAG CAC
JJH799_pilY2downF01	GAC GGG GAG GAG CAG C
JJH800_pilY2downR01-GWB2	GGG G**AC CAC TTT GTA CAA GAA AGC TGG GTA** TTG ATC GGC GTG GCG GT
JJH802_pilTupF01-GWB1	GGG G**AC AAG TTT GTA CAA AAA AGC AGG CTC A**TC GCC GAA GTC GCG AAG
JJH803_pilTupR	*TCA GAA GTT TTC CGG GAT CTT CGC* GGC GAG CAG GTC GGT AAT
JJH804_pilTdownF01	GCG AAG ATC CCG GAA AAC TT
JJH805_pilTdownR01-GWB2	GGG G**AC CAC TTT GTA CAA GAA AGC TGG GTA** CTT GGT CAC CGG CAT CAC
JJH808_fimUupF01-GWB1	GGG G**AC AAG TTT GTA CAA AAA AGC AGG CTC A**GG CTC CTC TGG AAC CGT T

JJH809_fimUupR	*TCA ATA GCA GTA CTG GGG CGC CTT G*GT CGA GTT GGA ACG ATA TG
JJH810_fimUdownF01	AAG GCG CCC CAG TCA TG
JJH811_fimUdownR01-GWB2	GGG G**AC CAC TTT GTA CAA GAA AGC TGG GTA** CGG TCC TTG ATC GCG TC
JJH814_pilVupF01-GWB1	GGG G**AC AAG TTT GTA CAA AAA AGC AGG CTC A**GA TCA GCA TCC AGG CGC T
JJH938_pilVupR02	*CAT GGC TCG ACC CTG AGG GTG TA*C CTG TGT CGC GAT TTC AAT AG
JJH816_pilVdownF01	TAC ACC CTC AGG GTC GAG
JJH817_pilVdownR01-GWB2	GGG G**AC CAC TTT GTA CAA GAA AGC TGG GTA** GTT GAG CCG GCT TTG AAT G
JJH1435_pelFupF01-GWB1	GGG G**AC AAG TTT GTA CAA AAA AGC AGG CTA C**GC TGG TAC TGG GAA CTG GC
JJH1436_pelFupR01	*GCA ATC TCC GTG GCT TCG CGG TAC AG*C GGA GCG GTG TGT TCG GTC
JJH1437_pelFdownF01	CTG TAC CGC GAA GCC ACG G
JJH1438_pelFdownR01-GWB2	GGG G**AC CAC TTT GTA CAA GAA AGC TGG GTA** CAG GGT CGC CAG CAA TAT CG

Construction of allelic exchange vectors with *aacC1*-marked deletion alleles[d]

JJH1636_Gm-F	CGA ATT AGC TTC AAA AGC GCT CTG A
JJH1637_Gm-R	CGA ATT GGG GAT CTT GAA GTT CCT
JJH1638_ GW-attB1	GGG G**AC AAG TTT GTA CAA AAA AGC AGG CT**
JJH1639_ GW-attB2	GGG G**AC CAC TTT GTA CAA GAA AGC TGG GT**
JJH1644_pilCupF01-GWL	*TAC AAA AAA GCA GGC T* GT TCA ACC TGG CGA CCT C
JJH1645_pilCupR01-Gm	*TCA GAG CGC TTT TGA AGC TAA TTC G* CAG ATG GGC CTT CAC CAG
JJH1646_pilCdownF01-Gm	*AGG AAC TTC AAG ATC CCC AAT TCG* CAA CTC GGC AAC GTC GTC
JJH1647_pilCdownR01-GWR	*TAC AAG AAA GCT GGG T* GCA GTT GGT GAT CGG CAT C
JJH1652_pilNupF01-GWL	*TAC AAA AAA GCA GGC T* CG ACA CCG ACC AGT TGA C
JJH1653_pilNupR01-Gm	*TCA GAG CGC TTT TGA AGC TAA TTC G* GAT CCG TGC CAT CAG TCG
JJH1654_pilNdownF01-Gm	*AGG AAC TTC AAG ATC CCC AAT TCG* GTT GCG CAA GGA GCC AAG
JJH1655_pilNdownR01-GWR	*TAC AAG AAA GCT GGG T* GTC GCC AAG TCG TGG TAG
JJH1664_pilRupF01-GWL	*TAC AAA AAA GCA GGC T* CA GCA GCT CGA CCT GAA G
JJH1665_pilRupR01-Gm	*TCA GAG CGC TTT TGA AGC TAA TTC G* GAC GAT CAG GGC TTT TTG TCG
JJH1666_pilRdownF01-Gm	*AGG AAC TTC AAG ATC CCC AAT TCG* CTG ACG TTC CGC TCG ATG
JJH1667_pilRdownR01-GWR	*TAC AAG AAA GCT GGG T* CTG GAA CTG CCC GTG GTA C

[a]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**.
[b]In addition to M13 universal primers, DNA sequences deposited in Genbank (see Table 1) were obtained using the following primers: pEX19Gm (KM887142) – LRH28, LRH30, LRH41, LRH161,

LRH164, LRH165, LRH166, LRH167; pEXG2 (KM887143) – LRH28, LRH30, LRH41, LRH114, LRH115, LRH161, LRH162, LRH163; pDONRPEX18Ap (KM880129) - LRH114, LRH115, LRH116, LRH161, LRH167, LRH169, LRH170, LRH171, LRH172; pDONRPEX18Gm (KM880128) and pEX18GmGW (KM880127) - LRH28, LRH30, LRH41, LRH114, LRH164, LRH165, LRH166, LRH167, LRH169, LRH170, LRH171, LRH172; pDONRPEX18Tc (KM880130) – LRH109, LRH110, LRH111, LRH112, LRH113, LRH114, LRH115, LRH116, LRH117, LRH118, LRH119.

[c]In absence of sequencing primers, mutations were identified and PCR products sequenced using the corresponding "Up-F" and "Down-R" primers used to build the in-frame deletion allele.

[d]Engineered according to the method of Choi and Schweizer[10].

Supplementary Table 2. Bacterial strains.

Strain	Genotype, description or relevant characteristics[a]	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
S17.1 λ*pir*	Str^r, Tp^r, F$^–$ RP4-2-Tc::Mu aphA::Tn7 recA λpir lysogen	[11]
SM10 λ*pir*	Km^r, F$^–$ RP4-2-Tc::Mu recA λpir lysogen	[11]
Pseudomonas aeruginosa		
JJH0	PAO1 wild type strain originating from the laboratory of Dr. Colin Manoil (MPAO1)	[12]
PAO1 Δ*wspF*	MPAO1 Δ*wspF*	[7]
JJH485	JJH0 Δ*pelF*	[13]
JJH492	JJH0 Δ*pilA*	[6]
JJH498	JJH0 Δ*pslD*	[7]
JJH502	JJH0 Δ*pelF* Δ*pslD*	[14]
JJH524	JJH0 Δ*pelF* Δ*pslD* Δ*wspF*	This study
JJH628	JJH0 Δ*chpA*	[14]
JJH587	JJH0 Δ*pilQ*	This study
JJH588	JJH0 Δ*pilD*	This study
JJH591	JJH0 Δ*pilG*	This study
JJH592	JJH0 Δ*pilM*	This study
JJH594	JJH0 Δ*pilJ*	This study
JJH595	JJH0 Δ*pilH*	This study
JJH596	JJH0 Δ*chpB*	This study
JJH606	JJH0 Δ*pilY1*	This study
JJH608	JJH0 Δ*pilY2*	This study
JJH610	JJH0 Δ*pilT*	This study
JJH631	JJH0 Δ*pilV*	This study
JJH635	JJH0 Δ*pilW*	This study
JJH645	JJH0 Δ*pilZ*	This study
JJH647	JJH0 Δ*fimU*	This study

JJH739	JJH0 Δ*pslD* Δ*wspF*	This study
JJH741	JJH0 Δ*pelF* Δ*wspF*	This study
MEL12	JJH0 Δ*pilB*	This study
JJH873	JJH0 Δ*pelF* (created for the Supplementary Tutorial)	This study
JJH899	JJH0 Δ*pilC*	This study
JJH901	JJH0 Δ*pilN*	This study
JJH903	JJH0 Δ*pilS*	This study
JJH904	JJH0 Δ*pilR*	This study
JJH906	JJH0 Δ*fimT*	This study
JJH907	JJH0 Δ*pilF*	This study
JJH909	JJH0 Δ*pilI*	This study
JJH911	JJH0 Δ*pilU*	This study
JJH913	Gm^r, JJH0 Δ*pilC::aacC1*	This study
JJH915	Gm^r, JJH0 Δ*pilN::aacC1*	This study
JJH917	Gm^r, JJH0 Δ*pilR::aacC1*	This study

[a]Gm, gentamicin; Km, kanamycin; Str, streptomycin; Tp, trimethoprim

Supplementary Table 3. Additional plasmids

Plasmid	Description or relevant characteristics[a]	Source
Helper plasmids		
pFLP2	Ap^r, vector for expressing Flp recombinase	[15]
Allelic exchange vectors with unmarked deletion alleles		
pCYL1	Gm^r, pENTRPEX18Gm with an *attL* flanked, in-frame deletion allele for *P. aeruginosa* PAO1 *pilB*	This study
pHA11	Gm^r, pENTRPEX18Gm with an *attL* flanked, in-frame deletion allele for *P. aeruginosa* PAO1 *chpB*	This study
pHA12	Gm^r, pENTRPEX18Gm with an *attL* flanked, in-frame deletion allele for *P. aeruginosa* PAO1 *pilD*	This study
pHA13	Gm^r, pENTRPEX18Gm with an *attL* flanked, in-frame deletion allele for *P. aeruginosa* PAO1 *pilH*	This study
pHA14	Gm^r, pENTRPEX18Gm with an *attL* flanked, in-frame deletion allele for *P. aeruginosa* PAO1 *pilG*	This study
pHA15	Gm^r, pENTRPEX18Gm with an *attL* flanked, in-frame deletion allele for *P. aeruginosa* PAO1 *pilJ*	This study
pHL129	Gm^r, pEX18Gm with an in-frame deletion allele for *P. aeruginosa* PAO1 *pslD*	[6]
pEX18Gm:: Δ*pelF*	Gm^r, pEX18Gm with an in-frame deletion allele for *P. aeruginosa* PAO1 *pelF*	[13]
pEX19Gm:: Δ*wspF*	Gm^r, pEX19Gm with an in-frame deletion allele for *P. aeruginosa* PAO1 *wspF*	[7]
pJJH130	Gm^r, pEX18GmGW with an *attB* flanked, in-frame deletion allele for *P. aeruginosa* PAO1 *pilA*	[6]
pJJH189	Gm^r, pENTRPEX18Gm with an *attL* flanked, in-frame deletion allele for *P. aeruginosa* PAO1 *pilY1*	This study
pJJH190	Gm^r, pENTRPEX18Gm with an *attL* flanked, in-frame deletion allele for *P. aeruginosa* PAO1 *pilQ*	This study
pJJH192	Gm^r, pENTRPEX18Gm with an *attL* flanked, in-frame deletion allele for *P. aeruginosa* PAO1 *pilM*	This study
pJJH196	Gm^r, pENTRPEX18Gm with an *attL* flanked, in-frame deletion allele for *P. aeruginosa* PAO1 *pilY2*	This study
pJJH197	Gm^r, pENTRPEX18Gm with an *attL* flanked, in-frame deletion allele for *P. aeruginosa* PAO1 *pilT*	This study
pJJH205	Gm^r, pENTRPEX18Gm with an *attL* flanked, in-frame deletion allele for *P. aeruginosa* PAO1 *pilZ*	This study
pJJH206	Gm^r, pENTRPEX18Gm with an *attL* flanked, in-frame deletion allele for *P. aeruginosa* PAO1 *chpA*	[14]
pJJH207	Gm^r, pENTRPEX18Gm with an *attL* flanked, in-frame deletion allele for *P. aeruginosa* PAO1 *fimU*	This study
pJJH210	Gm^r, pENTRPEX18Gm with an *attL* flanked, in-frame deletion allele for *P. aeruginosa* PAO1 *pilV*	This study
pJJH214	Gm^r, pENTRPEX18Gm with an *attL* flanked, in-frame deletion allele for *P. aeruginosa* PAO1 *pilW*	This study
pJJH302	Gm^r, pENTRPEX18Gm with an *attL* flanked, in-frame deletion allele for *P. aeruginosa* PAO1 *pelF* (created for the Supplementary Tutorial)	This study

pJJH337	Gm^r, pENTRPEX18Gm with an *attL* flanked, in-frame deletion allele for *P. aeruginosa* PAO1 *pilC*	This study
pJJH338	Gm^r, pENTRPEX18Gm with an *attL* flanked, in-frame deletion allele for *P. aeruginosa* PAO1 *pilN*	This study
pJJH340	Gm^r, pENTRPEX18Gm with an *attL* flanked, in-frame deletion allele for *P. aeruginosa* PAO1 *pilS*	This study
pJJH341	Gm^r, pENTRPEX18Gm with an *attL* flanked, in-frame deletion allele for *P. aeruginosa* PAO1 *pilR*	This study
pJJH342	Gm^r, pENTRPEX18Gm with an *attL* flanked, in-frame deletion allele for *P. aeruginosa* PAO1 *fimT*	This study
pJJH343	Gm^r, pENTRPEX18Gm with an *attL* flanked, in-frame deletion allele for *P. aeruginosa* PAO1 *pilF*	This study
pJJH344	Gm^r, pENTRPEX18Gm with an *attL* flanked, in-frame deletion allele for *P. aeruginosa* PAO1 *pilI*	This study
pJJH345	Gm^r, pENTRPEX18Gm with an *attL* flanked, in-frame deletion allele for *P. aeruginosa* PAO1 *pilU*	This study
Allelic exchange vectors with marked deletion alleles		
pJJH331	Gm^r, pENTRPEX18Ap:: Δ*pilC*::*aacC1*	This study
pJJH334	Gm^r, pENTRPEX18Ap:: Δ*pilR*::*aacC1*	This study
pJJH336	Gm^r, pENTRPEX18Ap:: Δ*pilN*::*aacC1*	This study

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

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