		yhaV	mqsR	tse2
Alphaproteobacteria	Ehrlichia	o	o	o
	Anaplasma	o	o	o
	Wolbachia	o	o	o
	Rickettsia	o	o	o
	Brucella	o	o	o
	Bartonella	o	o	o
Betaproteobacteria	Burkholderia	o	x	o
	Neisseria	x	o	o
	Bordetella	x	x	o
Gammaproteobacteria	Legionella	x	o	o
	Francisella	o	o	o
	Moraxella	o	o	o
	Acinetobacter	o	o	x
	Pseudomonas	o	o	x
	Stenotrophomonas	o	o	o
	Shewanella	o	o	o
	Aeromonas	o	o	o
	Vibrio	o	o	o
	Haemophilus	o	o	o
	Pasteurella	o	o	o
	Hafnia	o	x	o
	Pantoea	x	o	o
	Yersinia	o	x	o
	Serratia	o	o	o
	Klebsiella	x	x	o
	Raoultella	o	o	o
	Enterobacter	x	o	o
	Citrobacter	x	o	o
	Salmonella	x	o	o
	Escherichia	x	x	o
	Shigella	x	x	o
	Proteus	x	o	o
	Morganella	o	o	o
	Providencia	o	o	o
Epsilonproteobacteria	Helicobacter	o	o	o
	Campylobacter	o	o	o

Fig S1 Toxin(s) predicted to be useful (green open circle) in diverse pathogens based on absence of toxin homolog by BLASTP in high confidence genomes deposited in NCBI. Red X's denote presence of toxin gene.

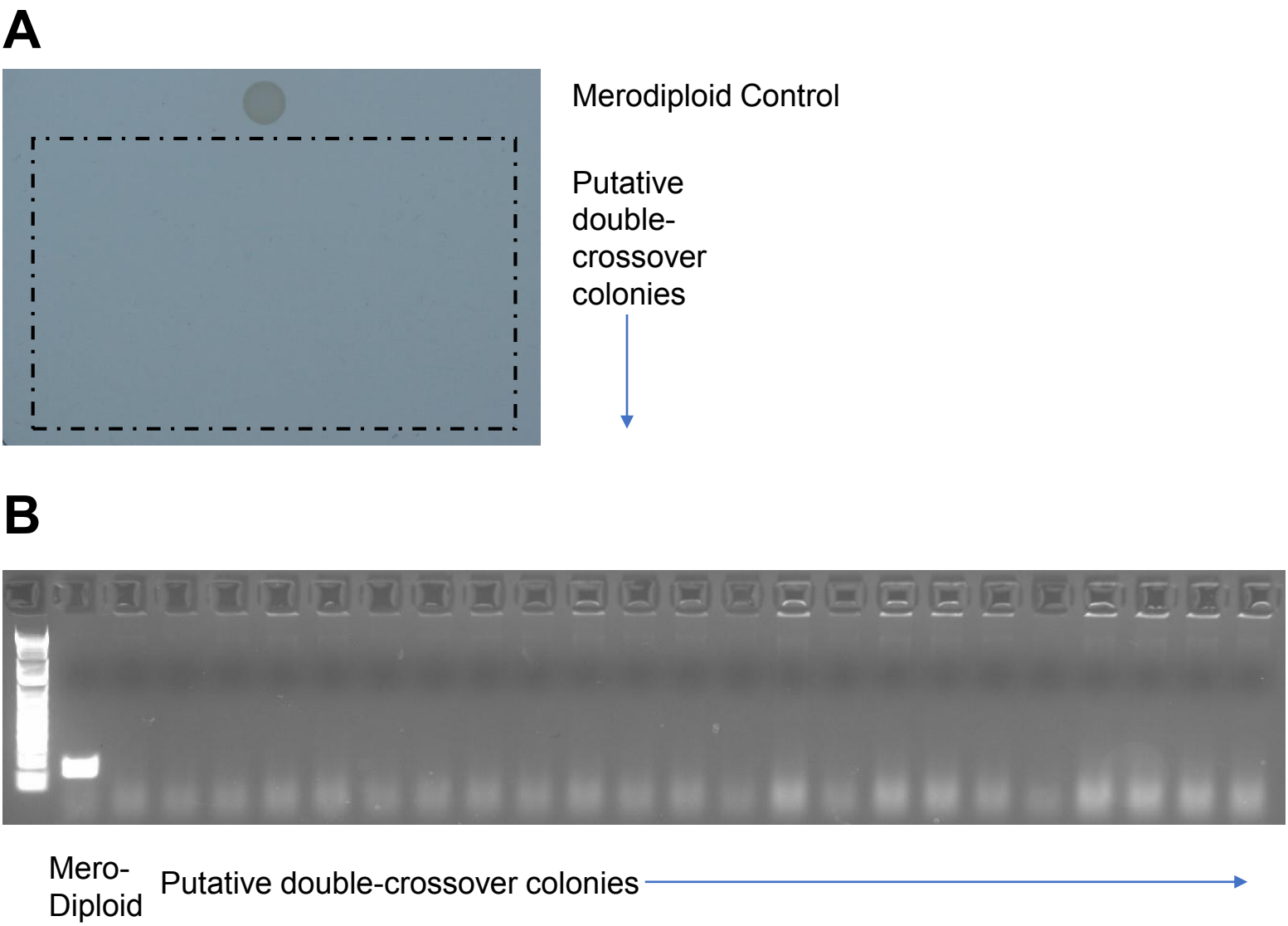

Fig S2 Putative double-crossover *S. marcescens* colonies (for each toxin, 23 colonies from 3 different crossovers) were randomly selected for characterization. A) A colony was resuspended in non-selective LB and then spotted onto LB+chloramphenicol. The top colony is the merodiploid positive control. There is no growth where the putative double-crossover colonies were spotted (hatched box). B) Colony PCR was performed for a small intergenic amplicon (between *rhaS* and the chloramphenicol resistance promoter) to test for presence of retained pTOX. The first lane is the merodiploid positive control.

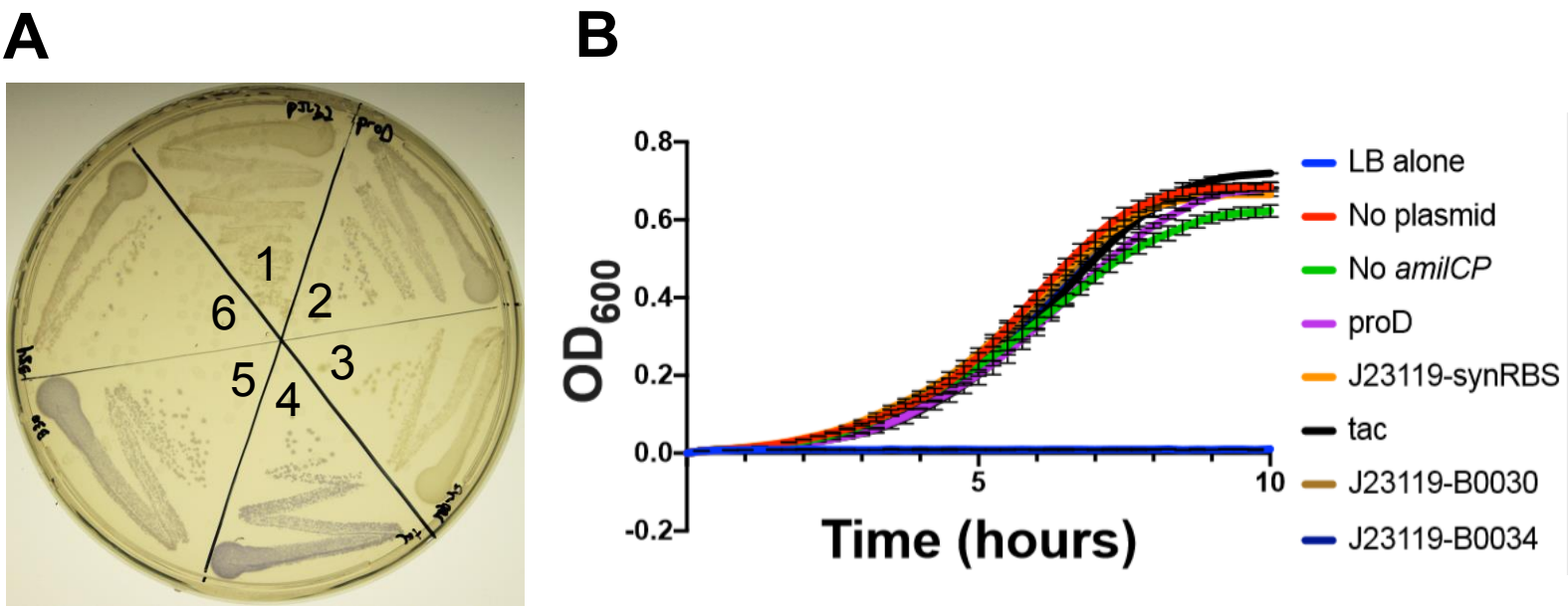

Fig S3 A) *E.* coli donors containing pTOX vectors with *amilCP* and various promoters and ribosome binding sites (RBS) at 24h at 37^{O}C. From top, clockwise: 1) pTOX with no *amilCP;* 2) pTOX-*amilCP* with proD promoter; 3) pTOX-*amilCP* with J23119 promoter and synthetic RBS; 4) pTOX-*amilCP* with tac promoter; 5) and 6) pTOX-*amilCP* with J23119 promoter with the B0030 or B0034 RBS, respectively. B) Growth curves for *E. coli* donor strains (or LB alone) containing pTOX with *amilCP* driven by indicated promoter/RBS.

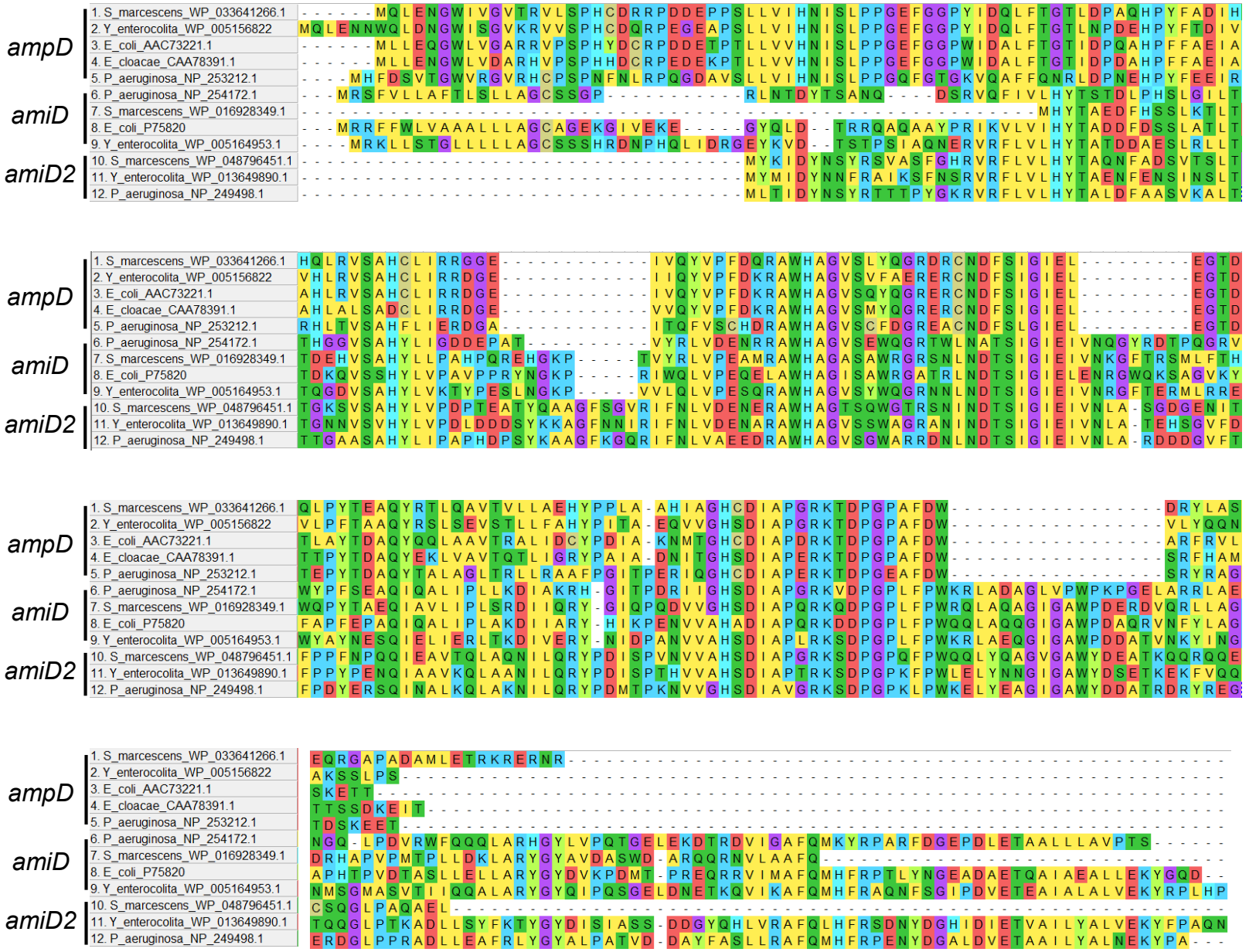

Fig S4 Indicated sequences were aligned in MEGA X using the MUSCLE algorithm (with the UPGMA method). The results are depicted as sequential (reading from left to right from the first through fourth panel) primary sequences.

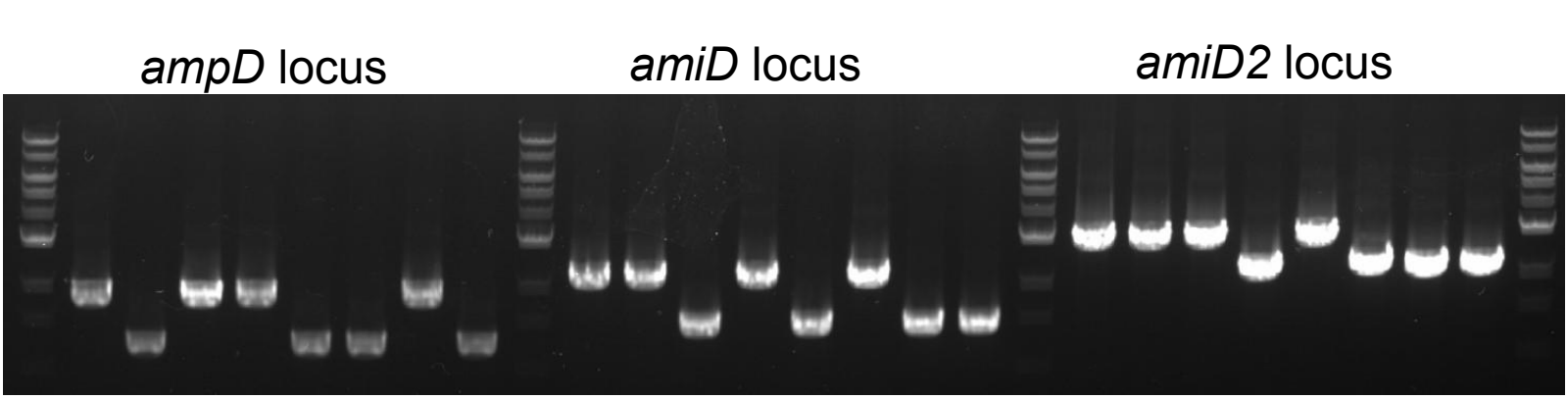

Fig S5 PCR-based genotyping of *S. marcescens* amidohydrolase mutations. Lanes from left to right: marker, Wt, Δ*ampD,* Δ*amiD,* Δ*amiD2,* Δ*ampD*Δ*amiD,* Δ*ampD*Δ*amiD2,* Δ*amiD*Δ*amiD2,* Δ*ampD*Δ*amiD*Δ*amiD2.* The *ampD* locus was amplified with prJL53 and prJL54; *amiD* with prJL55 and prJL56; and *amiD2* with prJL57 and prJL58

Supplemental Text 1

Sequence 1 – Codon-optimized *rhaS* with promoter

cgcggaacccctatttgtttatttttctaaatacattcaaatatgtatccgctcatgagacaataaccctgataaatgcttcaataatattgaa
aaaggaagagtATGACGGTGCTGCACTCGGTTGACTTCTTCCCTAGCGGCAATGCCAGCGTTG
CCATTGAGCCGCGCCTGCCTCAAGCCGACTTCCCGGAGCACCACCACGACTTCCACGAGA
TCGTTATCGTGGAGCACGGTACCGGCATCCACGTTTTCAACGGCCAACCGTACACGATTAC
GGGCGGTACCGTGTGCTTCGTTCGTGATCACGACCGCCACTTATACGAGCACACGGACAA
CTTATGCTTAACCAACGTTTTATACCGTAGCCCTGACCGCTTCCAATTCCTGGCGGGCTTA
AACCAACTGTTACCGCAGGAATTAGACGGCCAATACCCTAGCCATTGGCGTGTGAATCATT
CGGTGCTGCAACAAGTTCGCCAATTAGTGGCGCAAATGGAGCAACAAGAGGGCGAGAACG
ACCTGCCGAGCACGGCGAGCCGTGAAATTCTGTTCATGCAGCTGTTACTGCTGTTACGCA
AGTCGAGCCTGCAAGAAAATTTAGAGAATTCGGCGAGCCGCCTGAATCTGCTGTTAGCGT
GGTTAGAAGATCACTTCGCGGACGAAGTTAACTGGGATGCGGTTGCCGACCAGTTCAGCC
TGAGCTTACGCACCCTGCACCGCCAACTGAAACAACAGACCGGCTTAACCCCGCAACGCT
ATTTAAACCGTTTACGCTTAATGAAGGCGCGCCACTTACTGCGCCATTCGGAAGCGTCGGT
GACCGATATTGCGTACCACTGTGGCTTTTCGGATAGCAATCATTTCAGCACCCTGTTCCGT
CGCGAATTCAATTGGAGCCCTCGCGACATCCGCCAAGGCCGCGACGGTTTCTTACAGTGA

Sequence 2 – Forward terminator and expanded polylinker

AGCTTCGAGCTAATCGcgcaaaaaaccccgcttcggcggggttttttcgcTGATCACGTACGATATCTTCGA
ACCGGTGCACATGGTGTACAGGGCCCTAGGATAGGACGTCTTAAGGTTTAAACCAGGTTA
ATTAATTTAAATGCATCCCGGGACGTCTCGAGCTCGATCGGACCGCGGCCGCTAGCACGT
ATACCAAGTGTCCTGT

Sequence 3 – Synthetic strong ribosome binding site

ttatttttctaaatacattcaaatatgtatccgctcatgagacaataaccctgataaatgcttcaataatattgaaaaaggaGCTTATC
ACCGATAAGGAGGTTTTTTAATGACGGTGCTGCACTCGGTTGACTTCTTCCCTAGCGGCAA
TGCCAGCGTTGCCATTGAGCCGCGCCTGCCTCAAGCCGACTTCCCGGAGCAC

Sequence 4 – *amilCP* with tac promoter

ttgacaattaatcatcggctcgtataatgtgtggaaaggcggttcaccgccgttttcacacaggaaacagaattctttaagaaggagata
tacatATGTCAGTGATAGCAAAGCAGATGACATACAAAGTATATATGAGCGGTACTGTAAATG
GTCACTACTTCGAAGTAGAAGGTGATGGCAAAGGGAAGCCGTATGAGGGTGAACAAACAG
TGAAGCTTACAGTTACGAAGGGTGGCCCTTTGCCGTTCGCGTGGGACATTTTGTCGCCAC
AGTGCCAGTACGGGAGCATACCATTCACCAAGTATCCAGAGGACATACCAGACTACGTGA
AACAGTCCTTTCCCGAAGGCTATACCTGGGAGCGCATAATGAACTTTGAAGACGGTGCGG
TTTGTACGGTATCGAACGATTCATCAATCCAAGGAAATTGCTTTATTTATCATGTTAAATTTT
CGGGGCTTAACTTTCCGCCAAATGGCCCCGTGATGCAGAAGAAAACTCAGGGCTGGGAAC
CAAATACCGAGCGCCTTTTCGCTCGGGACGGAATGCTGTTGGGAAACAATTTTATGGCGTT
GAAGCTGGAAGGGGGTGGCCACTATCTCTGTGAATTCAAGACAACATACAAAGCCAAGAA
GCCTGTCAAGATGCCCGGTTATCACTATGTGGACCGGAAGCTCGATGTCACTAATCACAAC
AAGGATTATACTTCAGTTGAACAGTGCGAAATTTCGATCGCTCGCAAACCTGTCGTAGCAT
AA

Sequence 5 – *tsPurple* with apFAB46 promoter

AGGCCTtctagagtcgacctgcaaaaaagagtattgacttcgcatctttttgtacctataatagattcattactagagaaagaggaga
aatactagATGGCGTCCCTTGTAAAGAAGGATATGTGTGTTAAGATGACAATGGAGGGAACCG
TCAACGGGTATCACTTTAAGTGTGTTGGCGAGGGAGAAGGCAAACCGTTCGAGGGTACAC
AGAATATGCGCATTCGTGTCACCGAAGGGGGCCCTTTGCCCTTTGCTTTCGATATTTTGGC
CCCGTGCTGTATGTATGGTTCGAAGACTTTTATCAAACACGTAAGCGGAATCCCTGACTAC
TTCAAGGAGAGCTTTCCCGAAGGCTTTACTTGGGAACGTACGCAGATTTTTGAAGACGGAG
GAGTTTTGACTGCCCATCAAGATACATCACTTGAGGGAAACTGTTTAATCTACAAAGTTAAG
GTGTTAGGGACAAATTTTCCTGCCAATGGTCCGGTTATGCAGAAAAAGACAGCAGGCTGG
GAACCGTGTGTAGAGATGTTGTACCCCCGCGACGGGGTTCTTTGCGGGCAATCCCTTATG
GCCTTGAAATGTACTGATGGAAATCACTTGACATCCCATTTACGCACCACTTATCGCTCGC
GTAAGCCAAGTAATGCGGTGAACATGCCGGAATTTCACTTCGGAGACCATCGTATTGAAAT
CTTGAAAGCCGAACAAGGAAAATTTTATGAACAATACGAATCCGCAGTGGCACGTTATTCA
GATGTTCCAGAAAAGGCGACTTGATAAggcatgcaagcttggctgtt

Sequence 6 – J23119 promoter with synthetic ribosome binding site

TTGACAGCTAGCTCAGTCCTAGGTATAATGCTAGCTGAGAAAAGAAAGGGAAACTAAGGAG
GTATTTT

Supplemental Text 2

Those working with species intrinsically resistant to or strains with acquired resistance to chloramphenicol and gentamicin might want to replace the antibiotic selection cassette.

A general one step isothermal assembly approach is to linearize the appropriate pTOX vector with:

Forward primer – acagtactgcgatgagtggc

Reverse primer – agatccttggcggcaagaaa

A high fidelity polymerase such as Q5 (NEB) should be used. The PCR reaction should be optimized due to the high GC content of the mobRP4. For example, with Q5, we used 10% GC enhancer in the reaction, doubled the amount of recommended polymerase, and extended the elongation time to 45 seconds per kB.

The desired resistance cassette should then be amplified from an appropriate template. To facilitate isothermal assembly, we have found the following overlaps (complementary to the linearized vector above) to be effective:

Forward primer overlap – gccctgccactcatcgcagtactgt

Reverse primer overlap - CAAGTGTCCTGTtttcttgccgccaaggatct

SUPPLEMENTAL TABLE 1 Plasmids used in this study

pDS132	From Philippe *et al.*
pON.mCherry	From Gebhardt *et al.*
PGR-Blue	From Bradshaw *et al.*
pSB3C5-proD-B0032-E0051	From Davis *et al.*
pSLC-239	From Khetrapal *et al.*
pSLC-241	"
pSLC-246	"
pTOX1	This work. Encodes the YhaV toxin. CAM^R
pTOX2	This work. Encodes the MqsR toxin. CAM^R
pTOX3	This work. Encodes the Tse2 toxin. CAM^R
pTOX4	This work. Encodes the YhaV toxin and *amilCP*. CAM^R
pTOX5	This work. Encodes the MqsR toxin and *amilCP*. CAM^R
pTOX6	This work. Encodes the Tse2 toxin and *amilCP*. CAM^R
pTOX7	This work. Encodes the YhaV toxin and *tsPurple*. CAM^R
pTOX8	This work. Encodes the MqsR toxin and *tsPurple*. CAM^R
pTOX9	This work. Encodes the Tse2 toxin and *tsPurple*. CAM^R
pTOX10	This work. Encodes the YhaV toxin. $Gent^R$
pTOX11	This work. Encodes the MqsR toxin. $Gent^R$
pTOX12	This work. Encodes the Tse2 toxin. $Gent^R$

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Khetrapal V, Mehershahi K, Rafee S, Chen S, Lim CL, Chen SL. 461 2015. A set of powerful negative selection systems for unmodified Enterobacteriaceae. Nucleic Acids Res 43:e83.

Philippe N, Alcaraz J-P, Coursange E, Geiselmann J, Schneider D. 2004. Improvement of pCVD442, a suicide plasmid for gene allele exchange in bacteria. Plasmid 51:246–255.