Supplementary Information to:

Evolution of Dual Transcription Repression of a Putative Efflux System and of Global Control of the Integrative and Conjugative Element ICE*clc* by the TetR-type MfsR Protein

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Supplementary Figures S1-S4

Supplementary Tables S1-S4

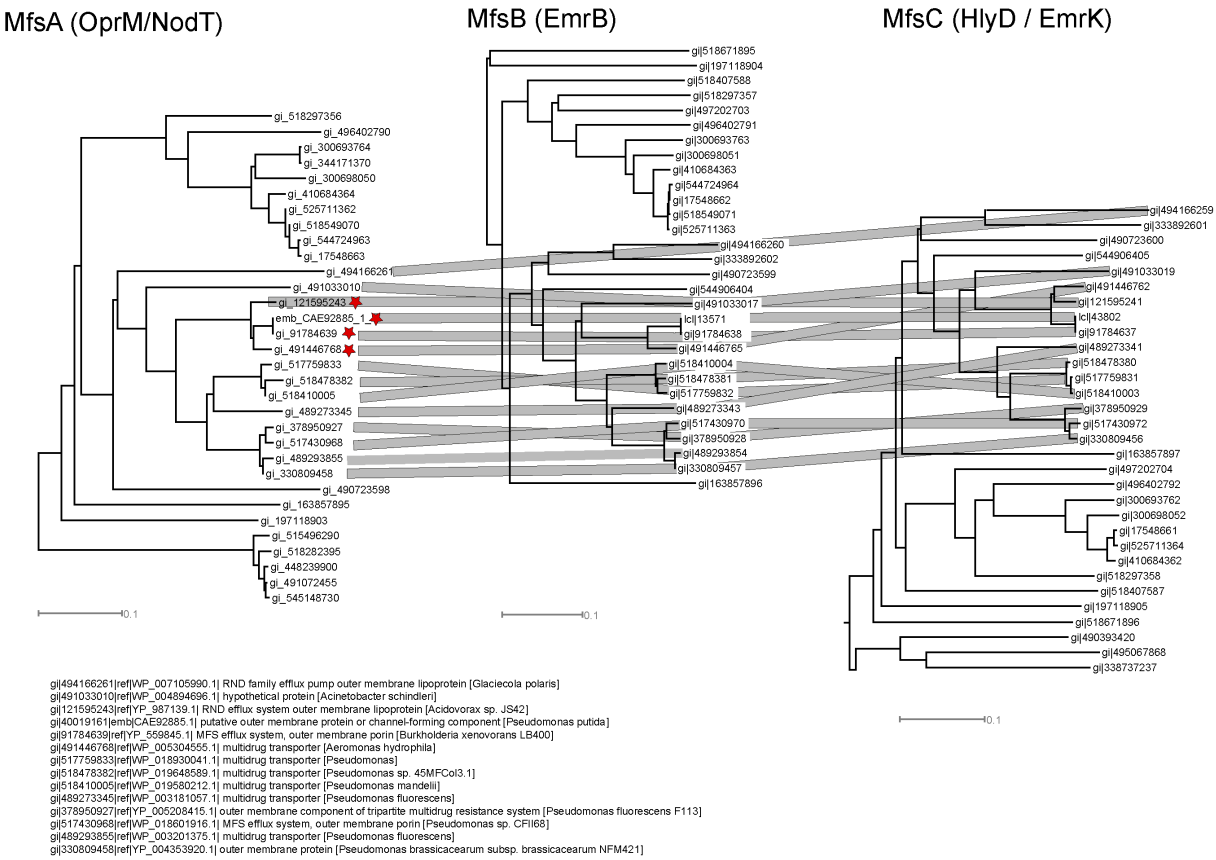

FIG S1 Phylogenetic trees of major superfamily facilitator tripartite protein subunits, outer membrane component (MfsA, OprM), multiple drug resistance efflux pump (MfsB, EmrB) and membrane fusion protein component MfsC (HlyB, EmrK), from a variety of closely related systems in *Gammaproteobacteria*. Tree tips indicate protein reference numbers, whereas corresponding organism names are displayed below the graphs. Thick grey lines relate the three MFS components of individual systems across the different trees. Those having red asterisks point to MFS systems present on mobile integrative DNA elements such as ICE*clc*, suggesting their coherent and separate branching. Trees produced by using Clustal Omega (www.ebi.org) on a COBALT alignment of BlastP hits to MfsA, MfsB and MfsC of ICE*clc*, displayed using Dendroscope.

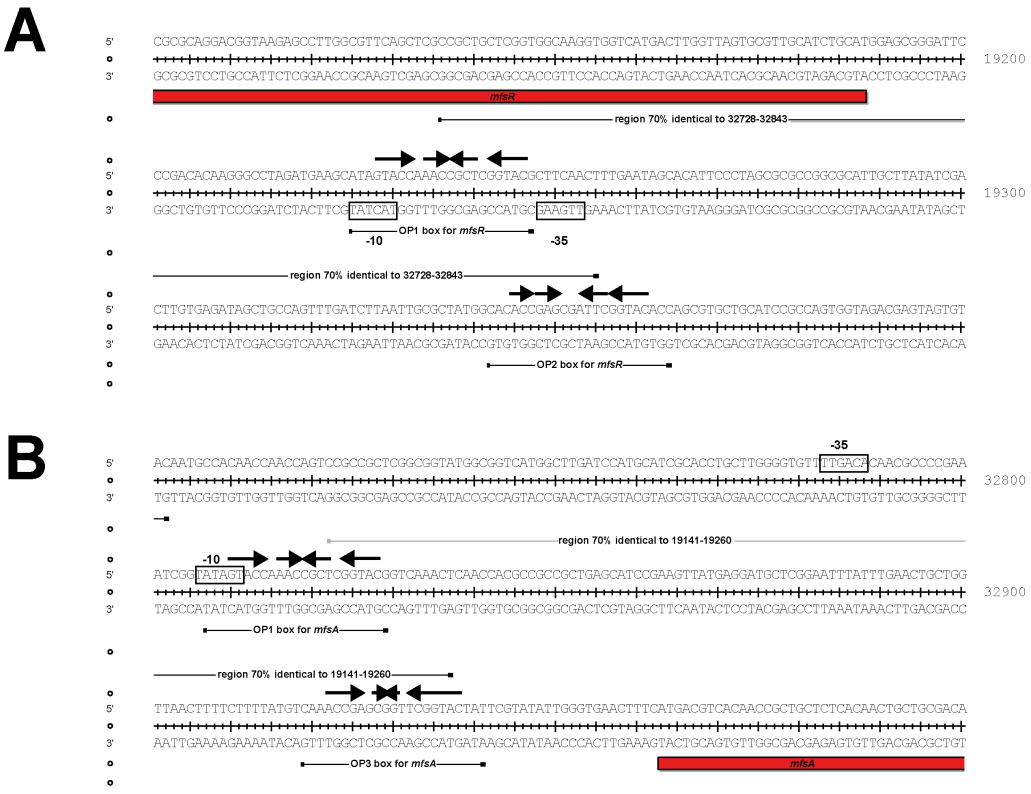

FIG S2 Overview of the *mfsR* (A) and *mfsA* (B) upstream regions with the MfsR operator (OP) sites, and predicted -35 and -10 motifs overlapping with the OP1 sites. Thick arrows point to palindromic sequences within OP1, OP2 and OP3. Thin underlinings indicate further regions of sequence similarity between *mfsA* and *mfsR* promoters. Red bars show the coding regions of *mfsR* and *mfsA*. Note how sequences immediately surrounding the OP1 sites diverged, putatively changing the original bidirectional promoters to being active in single transcription direction only.

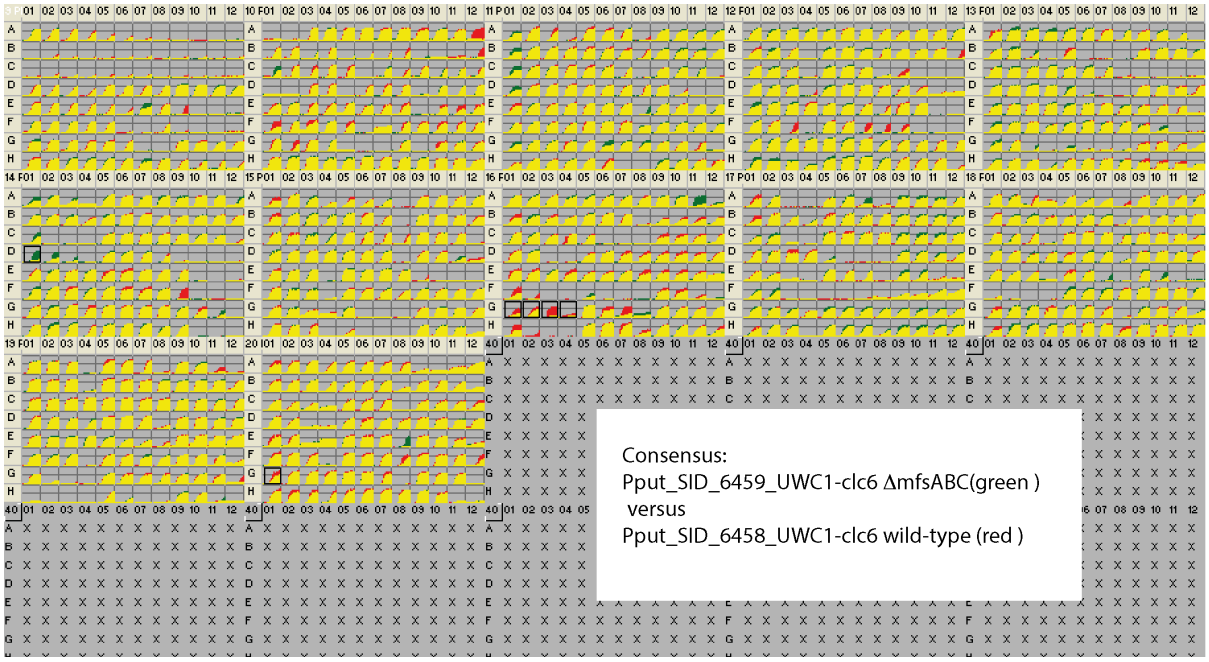

FIG S3 Consensus respiratory profiles from duplicate experiments in BIOLOG PM plates PM09-PM20, testing different chemical sensitivities of *P. putida* UWC1 (ICE*clc*-Δ*mfsABC*), in green, versus *P. putida* UWC1 (ICE*clc*), in red. Yellow profiles show overlap between the profiles of both strains. Black squares point to consistently different behaviour in both replicates.

Phenotypes Gained by *P. putida* UWC1 (ICE*clc*-Δ*mfsABC*):

PM14A	D01	107	Cadmium chloride	toxic cation

Phenotypes Lost by *P. putida* UWC1 (ICE*clc*-Δ*mfsABC*):

PM20B	G01	-64	Captan fungicide, carbamate	
PM16A	G01,G02,G03,G04	-303	Chromium (III) chloride	toxic cation

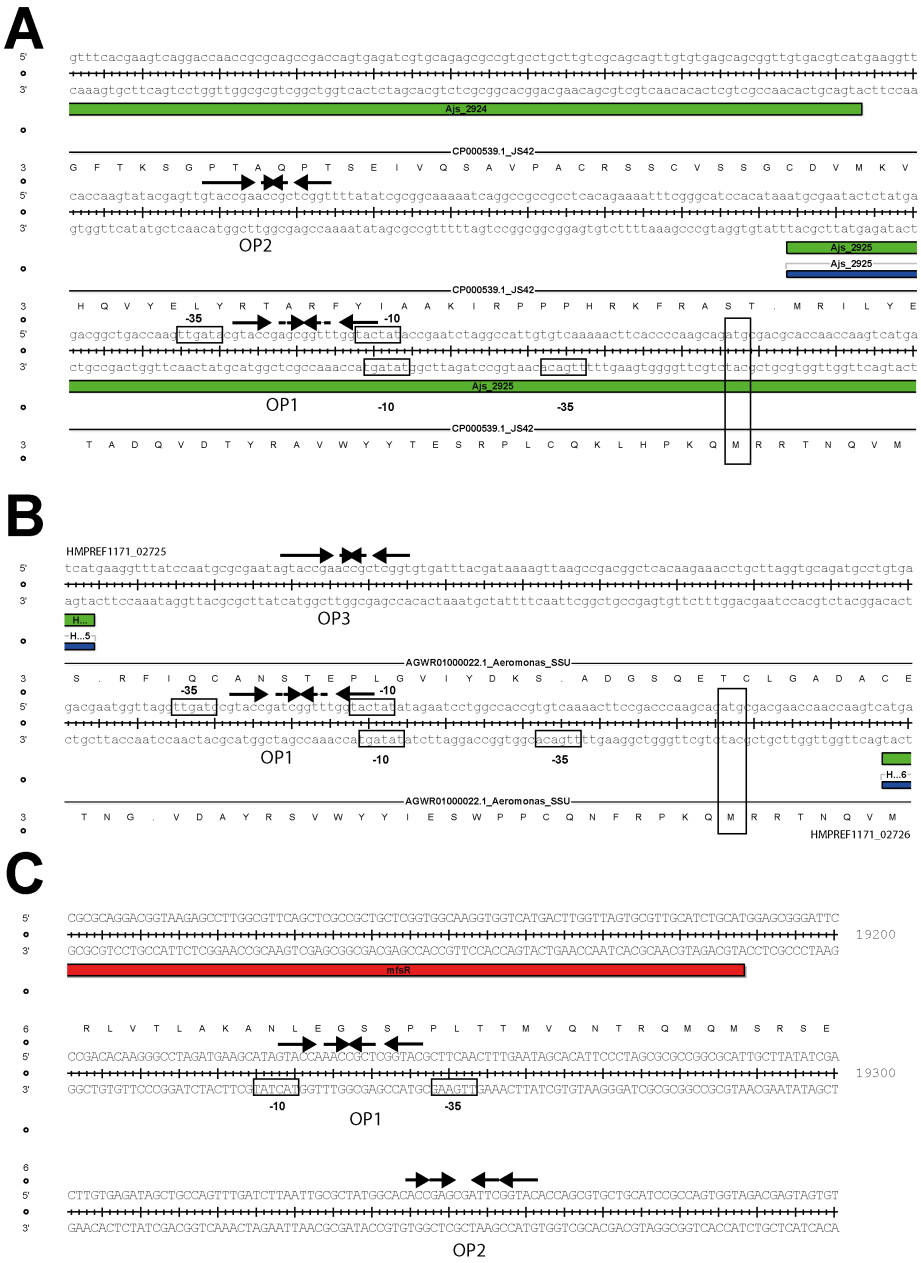

FIG S4 Detailed sequence comparison of operator and promoter motifs among the analogous *mfs* systems of *Acidovorax* sp. strain JS42 (A), *Aeromonas hydrophilia* SSU (B), and ICE*clc* (C). Equivalent of *mfsR* in JS42 is Ajs_2925 and in strain SSU HMPREF1171_02726. Palindromic sequences within OP1 and OP2 boxes are indicated by thick arrows, coding regions in color with amino acid translation below, and putative -10 and -35 motifs are boxed on the appropriate strands. Numbers within thin lines represent GenBank accession numbers. Note how gene orientation in (C) is displayed opposite to (A) and (B). Further note how bidirectional promoters are still present in the JS42 and SSU *mfs* analogous systems, but no longer in ICE*clc*, suggesting its divergence from an ancestor similar to JS42 or SSU. Finally, note how the reading frames of Ajs_2925 and HMPREF1171_02726 should likely start at different positions (long rectangles indicating the likely correct start codon).

Table S1. Strains used in this study.

Strain	Plasmid or Genotype	Characteristics	Reference or source	Lab collection strain number	Primers used to amplify gene fragment (s)
E. coli BL21 (DE3)	pET22b(+)-*mfsR-His*$_6$	Overexpression of MfsR	this study	3787	110705 + 110706
E. coli DH5α	pGEM-T-easy-P$_{mfsR-1}$	Promoter region of *mfsR*	this study	4453	120824 + 120825
E. coli DH5α	pGEM-T-easy-P$_{mfsR-2}$	Promoter region of *mfsR*	this study	4454	120826 + 120827
E. coli DH5α	pGEM-T-easy-P$_{mfsR-3}$	Promoter region of *mfsR*	this study	4455	120828 + 120829
E. coli DH5α	pGEM-T-easy-P$_{mfsR-4}$	Promoter region of *mfsR*	this study	4456	120830 + 120831
E. coli DH5α	pGEM-T-easy-P$_{mfsR-5}$	Promoter region of *mfsR*	this study	4457	120832 + 120833
E. coli DH5α	pGEM-T-easy-P$_{mfsR-6}$	Promoter region of *mfsR*	this study	4458	120824 + 100210
E. coli DH5α	pGEM-T-easy-P$_{mfsR-7}$	Promoter region of *mfsR*	this study	4459	121106 + 121107
E. coli DH5α	pGEM-T-easy-P$_{mfsR-8}$	Promoter region of *mfsR*	this study	4460	121107 + 121109 for the fusion: 121211 + 121212
E. coli DH5α	pGEM-T-easy-P$_{mfsR-9}$	Promoter region of *mfsR.* OP1 region replaced by *Xho*I.	this study	4461	121106+121107 *Xho*I-ligated with 121108+121109 for the fusion: 121211 + 121212
E. coli DH5α	pGEM-T-easy-P$_{mfsR-10}$	Promoter region of *mfsR*	this study	4462	121108 + 121109
E. coli DH5α	pGEM-T-easy-P$_{mfsR-11}$	Promoter region of *mfsR*	this study	3859	121211 + 121213
E. coli DH5α	pGEM-T-easy-P$_{mfsA-1}$	Promoter region of *mfsA*	this study	4463	120513 + 121105
E. coli DH5α	pGEM-T-easy-P$_{mfsA-2}$	Promoter region of *mfsA*	this study	4464	121104 + 120512
E. coli DH5α	pGEM-T-easy-P$_{mfsA-3}$	Promoter region of *mfsA*	this study	4465	121102 + 120512 for the fusion : 121214 + 120512
E. coli DH5α	pGEM-T-easy-P$_{mfsA-4}$	Promoter region of *mfsA*	this study	4466	121102 + 121103
E. coli DH5α	pGEM-T-easy-P$_{mfsA-5}$	Promoter region of *mfsA*	this study	4467	121104 + 121103
E. coli DH5α	pGEM-T-easy-P$_{mfsA-6}$	Promoter region of *mfsA*	this study	3918	120513 + 120512

E. coli DH5α	pGEM-T-easy-$P_{85934\text{-}88400}$	Contains promoter region of ICE*clc* $P_{85934\text{-}88400}$	this study	4000	120203 + 120204
P. putida UWC1	wild-type	Plasmid-less derivative of *P. putida* mt-2 (TOL), used as recipient for ICE*clc*, Rif^R	(1)	1291	
P. putida UWC1	mini-Tn*7*-*mfsR*	1291-derivative with integrated single copy mini-Tn*7*-*mfsR*, Gm^R	(2)	4301	100208 + 120222
P. putida UWC1	ICE*clc* wt	One copy of ICE*clc* integrated in *tRNAGly*-gene #5	(3)	2737	
P. putida UWC1	ICE*clc*-Δ*mfsR(-Δ'marR)*	2737-derivative with a deletion in *mfsR* plus small part of *orf17984.*	(2)	3543	101105 +101106, 101107 + 101108
P. putida UWC1	ICE*clc*-Δ*mfsR* + mini-Tn*7*-*mfsR*	3543-derivative complemented in trans with mini-Tn*7*-*mfsR* with its native promoter	(2)	4161	
P. putida UWC1	ICE*clc*-Δ*mfsABC*	2737-derivative with deletion in *mfsABC.*	this study	4165	120716 + 120717, 120718 + 129719
P. putida UWC1	ICE*clc*-Δ*mfsR*	2737-derivative with a deletion of *mfsR* only	(2)	4322	
P. putida UWC1	mini-Tn*5*-$P_{mfsR\text{-}11}$-m*cherry*	Derivative of 1291 with single copy *mfsR*-11 promoter fragment fused to *mcherry*	this study	3482-3487	
P. putida UWC1	mini-Tn*7*-*mfsR* + mini-Tn5-$P_{mfsR\text{-}11}$-m*cherry*	Derivative of 4301 with single copy *mfsR-mcherry* fusion	this study	4302-4307	
P. putida UWC1	ICE*clc* wt + mini-Tn5-$P_{mfsR\text{-}11}$-m*cherry*	Derivative of 2737 with single copy *mfsR-mcherry* fusion	this study	3497-3502	
P. putida UWC1	ICE*clc*-Δ*mfsR* + mini-Tn5-$P_{mfsR\text{-}11}$-m*cherry*	Derivative of 3543 with single copy *mfsR-mcherry* fusion	this study	3606-3611	
P. putida UWC1	ICE*clc*-Δ*mfsR* + mini-Tn*7*-*mfsR* + mini-Tn5-$P_{mfsR\text{-}11}$-m*cherry*	Derivative of 4161 with single copy *mfsR-mcherry* fusion	this study	4282-4287	
P. putida UWC1	mini-Tn5-$P_{mfsA\text{-}6}$-m*cherry*	Derivative of 1291 with single copy *mfsA-mcherry* fusion (fragment A-6)	this study	4272-4277	
P. putida UWC1	mini-Tn*7*-*mfsR* + mini-Tn5-$P_{mfsA\text{-}}$	Derivative of 4301 with single copy	this study	4308-4313	

	$_{6}$-m*cherry*	*mfsA-mcherry* fusion			
P. putida UWC1	ICE*clc* wt + mini-Tn5-P_{mfsA-6}-m*cherry*	Derivative of 2737 with single copy *mfsA-mcherry* fusion	this study	4254-4259	
P. putida UWC1	ICE*clc*-Δ*mfsR* + mini-Tn5-P_{mfsA-6}-m*cherry*	Derivative of 3543 with single copy *mfsA-mcherry* fusion	this study	4260-4265	
P. putida UWC1	ICE*clc*-Δ*mfsR* + mini-Tn7-*mfsR* + mini-Tn5-P_{mfsA-6}-m*cherry*	Derivative of 4161 with single copy *mfsA-mcherry* fusion	this study	4266-4271	
P. putida UWC1	ICE*clc* wt + mini-Tn5-P_{mfsR-8}-m*cherry*	Derivative of 1291 with single copy *mfsR-mcherry* fusion (fragment R-8)	this study	4410-4413	
P. putida UWC1	ICE*clc*-Δ*mfsR* + mini-Tn5-P_{mfsR-8}-m*cherry*	Derivative of 3543 with single copy *mfsR-mcherry* fusion	this study	4414-4417	
P. putida UWC1	ICE*clc*-Δ*mfsR* + mini-Tn7-*mfsR* + mini-Tn5-P_{mfsR-8}-m*cherry*	Derivative of 4161 with single copy *mfsR-mcherry* fusion	this study	4418-4421	
P. putida UWC1	mini-Tn5-P_{mfsR-8}-m*cherry*	Derivative of 1291 with single copy *mfsR-mcherry* fusion	this study	4402-4405	
P. putida UWC1	mini-Tn7-*mfsR* + mini-Tn5-P_{mfsR-8}-m*cherry*	Derivative of 4301 with single copy *mfsR-mcherry* fusion	this study	4406-4409	
P. putida UWC1	mini-Tn5-P_{mfsR-9}-m*cherry*	Derivative of 1291 with single copy *mfsR-mcherry* fusion (fragment R-9)	this study	4392-4395	
P. putida UWC1	mini-Tn7-*mfsR* + mini-Tn5-P_{mfsR-9}-m*cherry*	with single copy *mfsR-mcherry* fusion	this study	4396-4399	
P. putida UWC1	mini-Tn5-P_{mfsA-3}-m*cherry*	with single copy *mfsA-mcherry* fusion	this study	4424-4427	
P. putida UWC1	mini-Tn7-*mfsR* + mini-Tn5-P_{mfsA-3}-m*cherry*	with single copy *mfsA-mcherry* fusion	this study	4428-4431	
P. putida UWC1	mini-Tn5-P_{mfsA-6}rev-m*cherry*	with single copy *mfsA-mcherry* fusion	this study	4434-4437	
P. putida UWC1	mini-Tn7-*mfsR* + mini-Tn5-P_{mfsA-6}rev -m*cherry*	with single copy *mfsA-mcherry* fusion	this study	4438-4441	
P. aeruginosa PAO509	ICE*clc*	PAO509 into which ICE*clc* was introduced by conjugation from strain 2737	this study	4731-4734	
P. aeruginosa PAO509	ICE*clc* Δ*mfsABC*	idem the *mfsABC* deletion mutant, using strain 4165 as donor	this study	4735-4748	

P. aeruginosa PAO509	ICE*clc* Δ*mfsR*	idem the *mfsR* deletion mutant, using strain 4322 as donor	this study	4739-4742	

Table S2. List of Primers used in this study

primer number	sequence 5'-3'	Comment
100208	ATCATGATCGGGAGCGTTTACT	FWD, ANNEALS AT THE END OF ORF18502, POSITION 18501
100210	GCTAGGGAATGTGCTATTCAAAG	rev, anneals before the RBS of orf 18502, position 19254
101105	GAATTCTGCTCCAGGACGGTGAACAA	Fwd primer in the region upstream of orf18502
101106	CTCGAGGGCTGCTCCATTTGGTTTGACT	Rev primer in the region upstream of orf18502
101107	CTCGAGGACTTGGTTAGTGCGTTGCATCT	Fwd primer in the region downstream of orf18502
101108	GGATCCGCAGTGCGAGAGTTCCTTTTAGAG	Rev primer in the region downstream of orf18502
110705	TTTTCATATGCAGATGCAACGCACTA	Fwd primer to clone orf18502 into pET22b for His-tagging.
110706	TTTTCTCGAGTGATCGGGAGCGTTTAC	Rev primer to clone orf18502 into pET22b for His-tagging.
120204	TTTTTTCTAGAGACCCTCATTACATCGACATGAC	Rev primer promoter region ORF 85934-88400
120222	TTTTGGATCCCCACCTTCGTCGGTGCAATTC	flank orf18502+native promoter, adds a BamH1 site
120512	TTTTGGATCCAGCAGCGGTTGTGACGTCAT	Rev primer for intergenic region upstream of orf32963
120513	TTTTTCTAGAGGAGCTGATGGATGGATGA	Fwd primer for intergenic region upstream of orf32963
120203	TTTTTGGATCCGGACGGGCTCCTTGGAAAAG	Fwd primer promoter region ORF 85934-88400
120716	TTTTTCTAGACTGCCTTTGCCGATGC	Fwd primer from 32204 of ICEclc, amplifying upstream of orf32963
120717	GAAAGTTCACCCAATATACGAATAG	Rev primer from 32968 of ICEclc, amplifying upstream of orf32963
120718	ACTAGTCGCCATTCGTCCAGCATG	Fwd primer from 37118 of ICEclc, amplifying upstream of orf36077
120719	TTTTGGATCCAATGGCCACCCCATAG	Rev primer from 37813 of ICEclc, amplifying upstream of orf36077
120824	ATGACTTGGTTAGTGCGTTGCAT	Fwd1, to amplify the intergenic region upstream of orf18502 for EMSA.
120825	TCACAAGTCGATATAAGCAATGCG	Rev1, to amplify the intergenic region upstream of orf18502 for EMSA.
120826	CGCATTGCTTATATCGACTTGTGA	Fwd2, to amplify the intergenic region upstream of orf18502 for EMSA
120827	GGCAGGAATGAACTGCGCC	Rev2, to amplify the intergenic region upstream of orf18502 for EMSA .
120828	GGCGCAGTTCATTCCTGCC	Fwd3, to amplify the intergenic region upstream of orf18502 for EMSA .
120829	TGCCTGCCAATCTCGGCATCTC	Rev3, to amplify the intergenic region upstream of orf18502 for EMSA .
120830	AAGAGCTGCTGCGACAGCAG	Fwd4, to amplify the intergenic region upstream of orf18502 for EMSA .
120831	CCACCTTCGTCGGTGCAATTC	Rev4, to amplify the intergenic region upstream of orf18502 for EMSA .
120832	CGCTTCAACTTTGAATAGCAC	Fwd5, to amplify the intergenic region upstream of orf18502 for EMSA .
120833	CACGCTGGTGTACCGAAT	Rev5, to amplify the intergenic region upstream of orf18502 for EMSA .
121102	GTCAAACTCAACCACGCCGC	Fwd primer to produce fragm A or B if used with 120512 or 121103
121103	GGTTTGACATAAAAGAAAAG	Rev primer to produce fragm B if used with 121102
121104	TTGACACAACGCCCCGAAATCGGT	Fwd primer to produce fragm C or E if used with 121103 or 120512
121105	AACACCCCAAGCAGGTGCGA	Rev primer to produce fragm D if used with primer 120513
121106	TTTTCTCGAGGCTTCATCTAGGCCCTTGT	Rev primer to produce the upper part of fragm XI

		(without operator) with primer 121107
121107	TTGGCGTTCAGCTCGCCGCTGCT	Fwd primer to produce fragm X or XI
121108	TTTCTCGAGCGCTTCAACTTTGAATAGCAC	Fwd primer to produce the lower part of fragm XI (without operator) with primer 121109
121109	GTGCCATAGCGCAATTAAGA	Rev primer to produce fragm X or XI
121211	TTTTGGATCCATGACTTGGTTAGTGCGTTGCAT	Fwd to produce “Fragm X or XI” into pJAMA39
121212	TTTTTCTAGAGTGCCATAGCGCAATTAAGA	Rev to produce “Fragm X or XI” into pJAMA39
121213	TTTTTCTAGACCACCTTCGTCGGTGCAATTC	Rev to produce “Fragm XII” into pJAMA39 (use with 121211)
121214	TTTTTCTAGAGTCAAACTCAACCACGCCGC	Fwd to produce “Fragm A” into pJAMA39 (use with 120512)

Table S3. MIC values for *P. putida* UWC1 (ICE*clc*), Δ*mfsR,* and Δ*mfsABC* mutants

Substance	***P. putida* (ICE*clc*)**				***P. putida* (ICE*clc*-Δ*mfsR*)**				***P. putida* (ICE*clc*-Δ*mfsABC*)**			
	MHB[a]		**3CBA**		**MHB**		**3CBA**		**MHB**		**3CBA**	
	Exp 1	**Exp 2**	**Exp 1**	**Exp 2**	**Exp 1**	**Exp 2**	**Exp 1**	**Exp 2**	**Exp 1**	**Exp 2**	**Exp 1**	**Exp 2**
3-chlorobenzoate	19.6[b]				39.1				19.6			
2-aminophenol	0.43				0.43				0.43			
4-chlorophenol	0.4				0.4				0.2			
Ethidium bromide	0.63				0.31				0.63			
Ciprofloxacine	0.064	0.03	0.06	2	0.125	0.03	0.25	0.25	0.064	0.06	0.06	0.5
Tetracycline	1	1	1		2	1	0.5		2	0.5	1	
Gentamycin	0.12		0.12		0.06		0.06		0.06		0.12	
Chloramphenicol	64		>256		64		>256		128		>256	
Piperacillin+Tazobactam	8		16	64	16		32	32	16		16	64
Meropenem	0.25		0.25		1		0.5		1		0.5	
Erythromycin	64		16		128		8		64		16	
Aztreonam	8		>64		32		32		32		64	
Imipenem	0.25		1		<0.125		1		<0.125		1	
Ofloxacin	0.25		0.5	4	0.25		1	1	0.25		0.5	1
Trimetoprim	>256		>256		>256		>256		>256		>256	
Ceftazidime	2		4		2		4		2		4	
Sparfloxacin				4				0.5				2
Trovafloxacin				1				0.25				0.5
Moxifloxacin				8				1				4
Levofloxacin				2				1				2

a) culture medium for test: MHB, Muller-Hinton broth; 3CBA, minimal medium with 3-chlorobenzoate (10 mM).
b) all concentrations in mg/ml

Table S4. MIC values for *P. aeruginosa* PAO1, PAO509 and ICE*clc* derivatives

Strain	Triclosan (mg/L)	Chromium (III) chloride hexahydrate (mg/L)
PAO509-ICE*clc* WT	16 ± 0	1250
PAO509-ICE*clc* *ΔmfsABC*	10 ± 4	1250
PAO509-ICE*clc* *ΔmfsR*	5 ± 2	1250
PAO1	>1024	ND
PAO509	16	625

1. **McClure NC, Weightman AJ, Fry JC.** 1989. Survival of *Pseudomonas putida* UWC1 containing cloned catabolic genes in a model activated-sludge unit. Appl. Environ. Microbiol. **55:**2627-2634.
2. **Pradervand N, Sulser S, Delavat F, Miyazaki R, Lamas I, van der Meer JR.** 2014. An operon of three transcriptional regulators controls horizontal gene transfer of the Integrative and Conjugative Element ICE*clc* in *Pseudomonas knackmussii* B13. PLoS Genet. **10:**e1004441.
3. **Sentchilo V, Czechowska K, Pradervand N, Minoia M, Miyazaki R, van der Meer JR.** 2009. Intracellular excision and reintegration dynamics of the ICE*clc* genomic island of *Pseudomonas knackmussii* sp. strain B13. Mol. Microbiol. **72:**1293-1306.