An Operon of Three Transcriptional Regulators Controls Horizontal Gene Transfer of the Integrative and Conjugative Element ICEclc in Pseudomonas knackmussii B13

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Abstract

The integrative and conjugative element ICEclc is a mobile genetic element in Pseudomonas knackmussii B13, and an experimental model for a widely distributed group of elements in Proteobacteria. ICEclc is transferred from specialized transfer competent cells, which arise at a frequency of 3-5\% in a population at stationary phase. Very little is known about the different factors that control the transfer frequency of this ICE family. Here we report the discovery of a three-gene operon encoded by ICEclc, which exerts global control on transfer initiation. The operon consists of three consecutive regulatory genes, encoding a TetR-type repressor MfsR, a MarR-type regulator and a LysR-type activator TciR. We show that MfsR autoregulates expression of the operon, whereas TciR is a global activator of ICEclc gene expression, but no clear role was yet found for MarR. Deletion of mfsR increases expression of tciR and marR, causing the proportion of transfer competent cells to reach almost 100\% and transfer frequencies to approach 1 per donor. mfsR deletion also caused a two orders of magnitude loss in population viability, individual cell growth arrest and loss of ICEclc. This indicates that autoregulation is an important feature maintaining ICE transfer but avoiding fitness loss. Bioinformatic analysis showed that the mfsR-marR-tciR operon is unique for ICEclc and a few highly related ICE, whereas tciR orthologues occur more widely in a large variety of suspected ICE among Proteobacteria.

Introduction

Comparisons between ever-increasing numbers of sequenced genomes reveal the large extent to which prokaryotic genomes have undergone horizontal gene transfer (HGT) [1-5]. HGT has traditionally been viewed as the consequence of natural transformation, or of the action of mobile elements such as conjugative plasmids and phages [6,7]. During the last decade, however, other types of mobile genetic elements such as integrative and conjugative elements (ICEs) have been recognized, which are widespread and thus may significantly contribute to HGT [8-13]. In contrast to phages and plasmids, however, we still know little about the life styles of the diverse ICE types, their modes of self-transfer and regulatory pathways controlling self-transfer. Like temperate phages, ICEs mostly exist in an integrated form at one or more specific sites in the host’s chromosome (often in genes for tRNA), and are vertically transmitted to daughter cells by chromosome replication and segregation [8,10,14]. In order to transfer horizontally, ICEs excise themselves by site-specific recombination (attL and attR, Figure 1). This produces a circular double-stranded DNA molecule, which can transfer by conjugation to a new recipient cell, where it can reintegrate [14]. Autonomous plasmid-like replication of the excised form may occur [15-17], but is not required for the transfer itself.

The regulatory mechanisms that control the switch from integrated to excised state vary widely among different ICE types insofar as this has been studied. In several ICEs, this switch is the consequence of a cascade of a variety of regulatory factors, such as PhrI/RapI and ImmR/ImmA in ICEBsl [18], SetR/SetCD in ICEsxt [19,20], KorSA/Pra in pSAM2 [21-23] or QseM/TraR in ICEMelSym\textsuperscript{R7} [24,25]. Most wild-type ICEs transfer at low frequencies (i.e., less than 1 per 10\textsuperscript{5} donors), suggesting that the regulatory cascades keep extremely tight control and allow only a small subset of cells in a population to follow the path of ICE excision and transfer, but the need for such tight control is a priori unclear. This bistability is most pronounced and well-studied for a model ICE named ICEclc in Pseudomonas [26,27], which is evolutionary very distinct from the afore-mentioned ICEs [8,28]. ICEclc is originally found in two copies in Pseudomonas knackmussii B13 and is member of a family of ICEclc-like elements widely distributed among proteobacterial species [29]. ICEclc is integrated at the 3’-end of \textit{rRNA}\textsuperscript{23} genes
but can excise itself by the action of the IntB13 integrase encoded on the element (Figure 1A). Expression of \textit{intB13} in the integrated form is under control of the promoter \(P_{\text{int}}\) which by single cell reporter gene analysis was shown to become active only in 3-5% of a bacterial population during stationary phase [26]. Direct single cell visualization further confirmed that only cells which express reporter gene fused to \(P_{\text{int}}\) above a threshold are capable of transferring ICE\textit{clc} to new recipients, a bistable state which we recently named “transfer competence” (tc) [30]. Irrespective of the success of ICE\textit{clc} transfer, tc cells can only divide a few times once they re-enter exponential phase before they arrest growth. We recently showed that this is due to the expression of the ICE\textit{clc} genes \(\text{shi}\) and \(\text{parA}\) [30]. Expression of \textit{intB13} is dependent on a variety of factors, most notably a gene named \textit{inrR} (Figure 1A), which itself is also bistably expressed [26]. Both \textit{inrR} and \textit{intB13} expression are dependent on the abundance of the stationary phase sigma factor RpoS, with cells having highest RpoS levels being more likely to activate \(P_{\text{int}}\) and \(P_{\text{parA}}\) [29]. RpoS and InrR are important for activating ICE\textit{clc} excision and transfer, but are not sufficient. Therefore, we hypothesized that additional factors are necessary for the tc state to develop [29].

In this study, we report a locus of three consecutive regulatory genes on ICE\textit{clc}, which is essential for controlling its transfer. The locus was uncovered by random transposon mutagenesis, and further studied by creation of deletion mutants and complementation. The effect of mutations was studied at the level of ICE\textit{clc} expression through microarray hybridizations, RT-PCR and reporter gene-based single cell fluorescence microscopy, and further in ICE\textit{clc} transfer assays. Fitness of mutants compared to wild-type was examined in growth assays and individual cell fates were followed by microscopy. Bioinformatics was used to analyze the configuration of the ICE\textit{clc} regulatory locus within this ICE family, and to possibly reconstruct the steps that may have led to selection of the specific regulatory control mechanism of ICE\textit{clc}. The results of our study help to explain why a careful balance has to be maintained between ICE transfer frequency and fitness loss.

**Results**

Discovery of an ICE\textit{clc} transfer control locus by transposon mutagenesis

In order to discover ICE\textit{clc}-located factors involved in its self-transfer, a library of \textit{P. knackmussii} B13 mutants was generated by using random Tn5 mutagenesis [31]. Next, we recovered ICE\textit{clc}...
elements with Km-insertions by conjugating the pool of B13 mutants en masse to Pseudomonas putida UWC1 and selecting for Km-resistant P. putida (Figure S1). We hypothesized that mutant ICEclc with insertions in genes implicated in self-transfer could still be transferred to UWC1, when the second copy of ICEclc in the same B13 donor cell is intact and complements transfer of the mutant copy. A total of 1920 Km-resistant P. putida transconjugants was recovered and subsequently conjugated each individually with a second P. putida recipient, resistant to nalidixic acid (Figure S1). For those conjugations in which no Km- and nalidixic acid-resistant transconjugant growth was detected, the corresponding P. putida donor was recovered and the location of the KmR-gene insertion on ICEclc was mapped. A total of 18 clones was recovered, which had insertions in an ICEclc open reading frame numbered of18502, that we renamed mfsR (Figure 1B). Surprisingly, apart from one donor with an insertion in mfsR, no other mutants with impaired ICEclc transfer were found in this screening. The KmR-gene had been inserted in four different positions in mfsR, at ICEclc nucleotide positions 19033, 18758, 18730 and 18618 (Figure 1B; accession number: AJ617440.2). This suggests that transposon insertions in strain B13 were sufficiently frequent to cover all genes, but that the selection procedure was biased for the recovery of the mfsR insertion, which may have been due to the function of mfsR as regulator in ICEclc transfer (see below). Alternatively, it is possible that insertions in ICEclc genes needed for transfer might not be efficiently complemented by the second ICEclc copy and would thus be underrepresented in the P. putida library. Frequencies of ICEclc transfer of the strains P. putida UWC1-2961 (KmR-gene insertion at 19033) and UWC1-2962 (insertion at 18618) in a filter-based conjugation assay were 103-fold and 102-fold lower than of a P. putida with one integrated wild-type ICEclc copy, respectively (Figure 2A).

### mfsR is part of an operon formed by three consecutive transcriptional regulators

Closer inspection indicated mfsR to be the first open reading frame in a series of three consecutive transcriptional regulators, previously designated as of18502, of17984 and of17162 (Figure 1B). mfsR encodes a TetR-like regulator harboring helix-turn-helix motifs TetR-N and TetR-C,7 (pfam00400 and pfam14246, respectively; see Figure 1D). The of17984 gene overlaps with the end of the mfsR open reading frame by 4 bp and encodes a putative regulator of the MarR family (smart00347 HTH_MARR motif). The last gene of this cluster starts 24 bp downstream of the stop codon of of17984 and is predicted to code for a LysR-type transcriptional regulator, harboring an N-terminal HTH_1 motif (pfam00126) and a C-terminal substrate-binding domain (PBP2_LTTR_aromatics_like; cd08414). The gene of17162 was renamed tciR (transfer competence inducer regulator) in anticipation of the results described further below. Reverse transcription of P. putida UWC1 (ICEclc) RNA isolated from exponential phase-grown cells, followed by specific PCR amplification confirmed that the three genes are transcribed on the same mRNA, which ends downstream of tciR (Figure S2). This implies that mfsR-marR-tciR form a single polycistronic unit.

### Effects of tciR, marR, and mfsR deletions on ICEclc transfer

In order to more precisely investigate the role of the three regulators on ICEclc transfer, their open reading frames were each individually and partially deleted in separate strains, namely P. putida UWC1 (ICEclcΔmfsR, strain 4322), UWC1 (ICEclcΔmarR, strain 4372), UWC1 (ICEclcΔmfsRΔmarR, strain 3453) and P. putida UWC1 (ICEclcΔtciR, strain 4321) (Figure 1B, Table 1). ICEclc transfer frequencies in plate-mating assays with a gentamicin-resistant P. putida UWC1 as recipient were 2·10−fold lower for UWC1 donors with ICEclc having an internal deletion in tciR compared to intact ICEclc (Figure 2B). Complementation of the ICEclc-tciR mutation with a single copy mini-Tn7 transposed fragment containing the tciR gene under the PmarR-promoter (strain 4649, Figure 1C) restored transfer, even to much higher levels than wild-type ICEclc (Figure 2B).

ICEclc transfer frequencies were 27-fold lower for UWC1 donors with ICEclc having an internal deletion in marR compared...
downstream-located is rather due to polar disturbance of the expression of the
*P. putida* transfer frequencies by 104-fold, also here much stronger than
a single copy mini-Tn5 element (from nucleotide position 18395 to 19166).
Complementation of the
mfsR gene under its own promoter, KmR,
mfsR-tciR genes, plus the intact
mfsR, mcherry, KmR.
complementation, KmR 
3432 Derivative of strain 3482 having a gfp gene inserted downstream of intB13.
This study
P. putida UWC1 (ICEclc) + mini-Tn7-mfsR, mfsR, gfp 4612 Derivative of 3422 carrying a single copy mini-Tn7 insertion of the
mfsR gene under its own promoter.
This study
P. putida UWC1 (ICEclc) + mini-Tn7-mfsR, mini-Tn5-mfsR, mfsR, gfp 4302 Derivative of strain 3482 but with a mini-Tn7 insertion containing the
intact mfsR gene expressed from its own promoter, KmR, GmR.
This study
P. putida UWC1 (ICEclc), mini-Tn5-Pint-mcherry 3482 Single copy insertion of a mini-Tn5 mfsR promoter-mcherry fusion, KmR
This study
P. putida UWC1 (ICEclc) + mini-Tn5-Pint-mcherry 3497 Derivative of 2737, single copy insertion of a mini-Tn5 mfsR promoter-mcherry
fusion, KmR
This study
P. putida UWC1 (ICEclc), mini-Tn5-Pint-mcherry 3606 Derivative of 3453, single copy insertion of a mini-Tn5 mfsR promoter-mcherry
fusion, KmR
This study
P. putida UWC1 (ICEclc), mini-Tn7-mfsR, mini-Tn5-Pint-mcherry 4282 Derivative of 3606, but with a mini-Tn7 insertion containing the intact mfsR
gene expressed from its own promoter, KmR, GmR.
This study
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### Table 1. Strains used in this study and their specifications.

| Strain name | Strain collection number | Relevant characteristics | Reference or source |
|-------------|--------------------------|--------------------------|---------------------|
| Escherichia coli DH5α | [36] | | |
| E. coli DH5α λpir | | | |
| E. coli BW20767/pRL27 | 1853 | tra+, pRL27 containing hyperactive mini-Tn5 element (oriV, KmR). | [31] |
| *Pseudomonas knackmussii* B13 | 78 | Original host of ICEclc (2 identical copies). | [43] |
| *Pseudomonas putida* UWC1 | 1291 | plasmid-free derivative of *P. putida* KT2440, RifR | [44] |
| *P. putida* UWC1 (Nal) | | Spontaneous NaCl mutant of UWC1. | This study |
| *P. putida* UWC1 | 2744 | Single copy mini-Tn5-Pint-echerry insertion, GmR | [33] |
| *P. putida* UWC1 | 2756 | Single copy mini-Tn5-jimX-gfp insertion, KmR | [45] |
| *P. putida* UWC1 (ICEclc) | 2737 | Derivative of strain 1291 with one ICEclc copy integrated into tRNA55-5. | [45] |
| *P. putida* UWC1 (ICEclc) | 2738 | As 2737, but integrated into tRNA55-6. | [45] |
| *P. putida* UWC1 (ICEclc-KmR<sub>19033</sub>) | 2961 | Transposon mutant of strain 2737 with a KmR-gene inserted at nucleotide position 19033 in ICEclc. | This study |
| *P. putida* UWC1 (ICEclc-KmR<sub>18131</sub>) | 2962 | Transposon mutant of strain 2737 with a KmR-gene inserted at nucleotide position 18131 in ICEclc. | This study |
| *P. putida* UWC1 (ICEclc-ΔmfsR-ΔmarR) | 3453 | Derivative of strain 2737 with mfsR and part of marR deleted (from nucleotide position 18395 to 19166). | This study |
| *P. putida* UWC1 (ICEclc) + mini-Tn7-Pint-echerry | 3531, 3532, 3533 | Derivatives of strain 2737 with single copy random insertion of a mini-Tn-Pint-gfp/Pint-echerry, KmR | This study |
| *P. putida* UWC1 (ICEclc-ΔtciR) | 4321 | Derivative of strain 2737 with an internal deletion in tciR (from nucleotide position 17164 to 17985). | This study |
| *P. putida* UWC1 (ICEclc-ΔmfsR) | 4322 | Derivative of strain 2737 with an internal deletion in mfsR (from nucleotide position 18581 to 19143). | This study |
| *P. putida* UWC1 (ICEclc-ΔmarR) | 4372 | Derivative of strain 2737 with an internal deletion in marR (from nucleotide position 18018 to 18468). | This study |
| *P. putida* UWC1 (ICEclc-ΔmarR) + mini-Tn7-mfsR | 4469, 4470, 4471 | Derivatives of strain 4322 with single copy random insertion of a mini-Tn-Pint-gfp/Pint-echerry, KmR | This study |
| *P. putida* UWC1 (ICEclc-ΔmarR) + mini-Tn5-Pint-echerry | 4475, 4476, 4477 | Derivatives of strain 4372 with single copy random insertion of a mini-Tn-Pint-gfp/Pint-echerry, KmR | This study |
| *P. putida* UWC1 (ICEclc-ΔtciR) + mini-Tn5-Pint-echerry | 4479, 4480, 4481 | Derivatives of strain 4321 with single copy random insertion of a mini-Tn-Pint-gfp/Pint-echerry, KmR | This study |
| *P. putida* UWC1 (ICEclc-ΔmfsR, gfp) | 4612 | Derivative of 3422 having a gfp gene inserted downstream of intB13. | This study |
| *P. putida* UWC1 (ICEclc, mini-Tn7-mfsR, mini-Tn7-mfsR, mini-Tn5-mfsR) | 4646 | Derivative of 4322 carrying a single copy mini-Tn7 insertion of the mfsR gene under its own promoter. | This study |
| *P. putida* UWC1 (ICEclc-ΔtciR, mini-Tn7-mfsR, mini-Tn5-mfsR, tciR) | 4649 | Derivative of 4321 carrying a single copy mini-Tn7 insertion of the (frameshifted) mfsR and marR genes, plus the intact tciR gene under the mfsR promoter. | This study |
| *P. putida* UWC1 (ICEclc-ΔmarR, mini-Tn7-mfsR, mini-Tn5-mfsR, tciR) | 4804 | Derivative of 4372 carrying a single copy mini-Tn7 insertion of the (frameshifted) mfsR and tciR genes, plus the intact marR gene under the mfsR promoter. | This study |
| *P. putida* UWC1 mini-Tn5-Pint-mcherry | 3482 | Single copy insertion of a mini-Tn5 mfsR promoter-mcherry fusion, KmR | This study |
| *P. putida* UWC1 mini-Tn7-mfsR, mini-Tn5-Pint-mcherry | 4302 | Derivative of strain 3482 but with a mini-Tn7 insertion containing the intact mfsR gene expressed from its own promoter, KmR, GmR | This study |
| *P. putida* UWC1 (ICEclc), mini-Tn5-Pint-mcherry | 3497 | Derivative of 2737, single copy insertion of a mini-Tn5 mfsR promoter-mcherry fusion, KmR | This study |
| *P. putida* UWC1 (ICEclc-ΔmfsR), mini-Tn5-Pint-mcherry | 3606 | Derivative of 3453, single copy insertion of a mini-Tn5 mfsR promoter-mcherry fusion, KmR | This study |
| *P. putida* UWC1 (ICEclc, mini-Tn7-mfsR, mini-Tn5-Pint-mcherry) | 4282 | Derivative of 3606, but with a mini-Tn7 insertion containing the intact mfsR gene expressed from its own promoter, KmR, GmR | This study |

to intact ICEclc (Figure 2B). Complementation of the ICEclc-ΔmarR mutation with a similar single copy mini-Tn7-marR insertion did not change transfer rates (strain 4804, Figure 2B). This suggests that the effect of the marR deletion on ICEclc transfer is rather due to polar disturbance of the expression of the downstream-located tciR.

In contrast, ICEclc elements with mfsR deletions [i.e., *P. putida* UWC1 (ICEclc-ΔmfsR) and UWC1 (ICEclc-ΔmfsR-ΔmarR)] transferred with 25- and 15-fold higher frequencies than wild type ICEclc, respectively (Figure 2A, B). Complementation of the ICEclc-ΔmfsR mutation with a single copy mini-transposed mfsR gene under control of its own promoter reduced ICEclc-ΔmfsR transfer frequencies by 10<sup>3</sup>-fold, also here much stronger than predicted from wild-type ICEclc itself (Figure 2B). These results suggested that tciR is the actual regulator of ICEclc transfer, and further that mfsR is regulating expression of the mfsR-marR-tciR
operon. Since MfsR is expected to be a repressor, its deletion would lead to higher expression of the downstream genes marR and tciR, which results in increased ICEclc transfer. The effect of the transposon insertions in mfsR (i.e., lower ICEclc transfer rates, Figure 2A) seems therefore due to a polar effect on marR-tciR expression.

tciR encodes a global activator of the genes in the ICEclc core region

Next, we examined the effect of regulatory gene deletions on gene expression of ICEclc as a whole, using semi-tiling microarray analyses (Figure 3). When *P. putida* UWC1 (ICEclc) wild-type cells are growing exponentially on 3-chlorobenzoate (3CBA), expression from the genes in the ICEclc core region (roughly the second half of ICEclc) plus the integrase *intB13* is silent, whereas they are highly transcribed when cells are in stationary phase (Figure 3A). Among others, the core region encodes genes implicated in ICEclc conjugal transfer [32,33]. *P. putida* with mutant ICEclc lacking either *marR* or *tciR* strongly diminished expression in the core region and of the integrase gene in stationary phase when compared to wild type (Figure 3C, Figure S3). Lower core and integrase gene expression explains the lower ICEclc transfer rates from these mutants (Figure 2B). In contrast, *mfsR* deletion resulted in much higher expression from the ICEclc core genes in exponentially growing cells (Figure 3B), and even slightly higher expression in stationary phase than in wild-type ICEclc (Figure S4), which explains the 10- to 100-fold ICEclc higher transfer rates (Figure 2B). Expression of the *mfsR-marR-tciR* cluster itself was the same in the *tciR* and *marR* deletion mutants, and no different to the wild-type (Figure 4A, C, E). In contrast, expression of the *mfsR-marR-tciR* cluster was higher in the *mfsR* deletion mutants than in wild-type, both in exponential and stationary phase cells (Figure 4B, D). Since gene expression from ICEclc is similar in mutants lacking *mfsR* alone or *mfsR* plus the first 117 bp of *marR* (Figure S4), we conclude that it is the LysR-type regulator encoded by *tciR*, which is the main activator for ICEclc core gene expression.

Microarray analysis also helped to understand the behaviour of the *mfsR* Km-insertion mutant (Figure 3D). As for the *tciR* deletion mutant, expression of the ICEclc core region and of the integrase was dramatically lower than wild-type in stationary phase cells (Figure 3D, Figure S4). On the other hand, both *mfsR* deletion and *mfsR* Km-insertion mutants showed increased expression of a group of genes on ICEclc coding for a putative efflux system.

![Figure 3. Differential expression of the ICEclc gene region from micro-array data in selected mutant ICEclc versus wild type in *P. putida* UWC1.](image-url)
Detailed inspection of mfsR operon expression in the Km-insertion mutant revealed that the first 160 bp of mfsR, upstream of the KmR-gene insertion were higher expressed than in wild-type cells (Figure 4F). In contrast, the downstream genes marR and tciR were lower expressed compared to wild-type and to the mfsR deletion mutant (Figure 4B, D, F). This confirmed, therefore, that insertion of the KmR-gene had caused a polar effect on expression of marR and tciR genes, which explains the strongly diminished expression of the ICEclc core genes in stationary phase in the mfsR Km-insertion mutant, and decreased ICEclc transfer.

Inserting the presumed mfsR promoter region upstream of a promoterless mcherry gene in single copy on the chromosome of P. putida UWC1 without ICEclc produced strong and homogenous mCherry expression among all cells (Figure 5A, strain 3482). Inserting into this strain a single copy mfsR gene expressed from its own promoter abolished mCherry expression (Figure 5A, strain 4302). Expression of mCherry from PmfsR in P. putida ICEclc was very low, whereas disruption of mfsR on ICEclc again resulted in high mCherry expression (Figure 5A, strain 3606). Complementation of this strain by a single copy mfsR gene under its own promoter caused repression of mCherry expression (Figure 5A). All these data are consistent with the hypothesis that MfsR is repressing expression of itself and the downstream located marR and tciR genes.

Figure 4. Detailed view on the differential expression of the mfsR operon in P. putida ICEclc wild-type or mutants. (A) MarR deletion mutant versus wild-type. (B) mfsR deletion mutant versus wild type. (C) tciR deletion mutant versus wild-type. (D) mfsR-marR deletion mutant versus wild-type. (E) marR versus tciR deletions. (F) mfsR-transposon insertion mutant versus wild-type. Panels show 2log-fold change of expression level per microarray probe in this region of ICEclc for exponential (dark dots) and stationary phase cells (white dots). Genetic map of the region drawn at the bottom of each section for clarity. Arrows represent genes, deleted regions are indicated by stippled bars and corresponding probes are within brackets.

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mfsR deletion leads to an increase in the number of cells activating ICEclc

We then tested whether changed ICEclc transfer rates and core gene expression were in fact due to changes in the proportion of cells activating ICEclc. Here, a double promoter-reporter construct, carrying Pint-<em>gfp</em> and PinR<-mcherry was inserted in single copy on the chromosomes of <em>P. putida</em> UWC1 (ICEclc wild-type), ΔmfsR, ΔtciR or ΔmarR deletions, equipped with a single copy mini-transposon containing the Pint-<em>gfp</em> and PinR<-mcherry fusions. Panels show expression of both markers at different growth phases, as indicated, with colors representing genotypes with independent mini-Tn5 insertions. Note as example the subpopulation of wild-type cells (dotted ellips) expressing both reporters, compared to the majority of cells in the mfsR deletion mutant but a complete absence of such subpopulation in the tciR and marR deletion mutants. 

Figure 5. Effect of mutations in the <em>mfsR</em> region on the expression of the P<em>mfsR</em>-, P<em>int</em>- and P<em>mar</em>-promoters of ICEclc in <em>P. putida</em> UWC1. (A) mCherry expression from the <em>mfsR</em> promoter added in single copy to the chromosome of the indicated <em>P. putida</em> UWC1 strains (relevant genotypes and strain numbers specified below the graph). mCherry expression measured on individual cells (n = 1000) by epifluorescence microscopy in late exponential phase of cultures grown on 10 mM succinate and expressed as box plots (AU, arbitrary units at 20 ms exposure time). (B) Scatter plot of GFP and mcherry fluorescence in single cells of <em>P. putida</em> UWC1 (ICEclc) wild-type, ΔmfsR, ΔtciR or ΔmarR deletions, equipped with a single copy mini-transposon containing the P<em>mfsR</em>-<em>gfp</em> and P<em>mar</em>-mcherry fusions. Panels show expression of both markers at different growth phases, as indicated, with colors representing genotypes with independent mini-Tn5 insertions. Note as example the subpopulation of wild-type cells (dotted ellips) expressing both reporters, compared to the majority of cells in the mfsR deletion mutant but a complete absence of such subpopulation in the tciR and marR deletion mutants.

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Mutants with <em>mfsR</em> deletion in ICEclc face a strong fitness cost

Given that <em>P. putida</em> UWC1 carrying ICEclc-ΔmfsR transferred at a much higher rate than wild-type ICEclc, and also expressed both P<em>int</em>- and P<em>mar</em>-promoters (Figure 5B), Expression of both promoters in <em>P. putida</em> UWC1 (ICEclc-ΔmfsR) occurred in early stationary phase whereas in wild-type cells their expression is maximal in late stationary phase (Figure 5B). Conversely, <em>P. putida</em> UWC1 (ICEclc-ΔtciR) and <em>P. putida</em> UWC1 (ICEclc-ΔmarR) did not produce any detectable <em>PmfsR</em>- or <em>Pmar</em>-expressing cells, neither in exponential nor in stationary phase (Figure 5B). Considering a detection limit by microscopy of ~1 fluorescent cell among 1000-10,000 non fluorescent cells, the absence of detectable <em>PmfsR</em>- or <em>Pmar</em>-expressing cells in those mutants would be in accordance with absence of ICEclc core gene activation on microarrays (Figure 3D) and lower transfer frequencies (Figure 2B).
such mutants did not become selected spontaneously. Both \textit{P. putida} UWC1 \textit{(ICEclc)} wild-type and \textit{(ICEclc-ΔmfsR)} displayed statistically indistinguishable generation times during exponential growth on minimal medium with either 3CBA or succinate as carbon source (Table 2), although \textit{P. putida} UWC1 \textit{(ICEclc-ΔmfsR)} went through a longer lag phase (Figure S5A). In contrast, the proportion of colony forming units (CFU) in samples taken from stationary phase cultures both on 3CBA and succinate was lower than that for UWC1 \textit{(ICEclc)} (Figure 6). Moreover, 8 of 10 tested colonies of UWC1 \textit{(ICEclc-ΔmfsR)} grown on MM plates with succinate did no longer amplify the \textit{clc} gene of \textit{ICEclc} (not shown), the remaining two still being able to grow on 3CBA. Furthermore, half or more of microcolonies formed from UWC1 \textit{(ICEclc-ΔmfsR)} with a single copy \textit{P_{mfsR}egfp} insertion showed incidence of malformations and cell lysis, similar to what was reported previously for nutrient-reactivated tc cells \cite{30} (Figure 6B), but cells in the other microcolonies divided with generation times even slightly faster (1.49±0.15 h) than those in microcolonies of \textit{P. putida} UWC1 \textit{(ICEclc, 1.79±0.09 h)}. This indicates that the \textit{mfsR} deletion in \textit{ICEclc} imposes a strong fitness cost on \textit{P. putida} UWC1. Survival of UWC1 was restored to wild-type level when the \textit{ICEclc-ΔmfsR} was complemented by the mini-Tn\textsubscript{7} inserted \textit{mfsR} gene (Figure S6). In contrast, neither \textit{P. putida} UWC1 \textit{(ICEclc)} with \textit{marR} or \textit{tciR} deletion, nor the mini-Tn\textsubscript{7} complemented strains of \textit{P. putida} UWC1 \textit{(ICEclc-ΔtciR)} and \textit{(ICEclc-Δclc)} displayed standard deviation from the average. (B) Phase-contrast micrographs at 1000-fold magnification of microcolonies of \textit{P. putida} UWC1 (ICEclc) and \textit{P. putida} UWC1 \textit{(ICEclc-ΔmfsR)} \textit{P_{mfsR}egfp}, 4612) growing on agarose surface supplemented with 0.1 mM 3CBA. Shown are a regular stationary phase microcolony of \textit{P. putida} with wild-type \textit{ICEclc} and a transfer competent microcolony (tcm), occurring at 1-3% frequency as reported previously \cite{30}.

**Table 2. Effects of the \textit{mfsR} deletion on the growth characteristics of \textit{P. putida} UWC1 carrying \textit{ICEclc}.**

| Strain                      | Generation time (min) | Survival rate (%) |
|-----------------------------|-----------------------|-------------------|
|                             | MM Succ | MM 3CBA | MM Succ | MM 3CBA |
| \textit{P. putida} UWC1 (ICEclc) | 74.4±6.8\textsuperscript{a} | 186±11 | 100±12 | 113±8.5 |
| \textit{P. putida} UWC1 (ICEclc-ΔmfsR) | 68.2±8.1 | 184±3 | 0.3±0.3 | 5.6±2.5 |

\textsuperscript{a}Generation time was calculated as ln2/\mu, whereby \mu (min\textsuperscript{-1}) is the slope of the regression line on a plot of the log\textsubscript{culture turbidity} versus time from at least 5 points during exponential phase. Coefficients of determination (R\textsuperscript{2}) were >0.96 for each growth curve.

\textsuperscript{b}Survival rate in stationary phase of cultures on the indicated media was calculated as the ratio of the number of CFU/ml counted on MM+3CBA and the number of CFU/ml on MM+succinate plates. Succ, succinate; 3CBA, 3-chlorobenzoate.

\textsuperscript{c}Calculated standard deviation from triplicate measurements.

\textsuperscript{d}Calculated p-value in a two-tailed Student’s t-Test using equal variance.

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**Figure 6. Fitness loss of \textit{P. putida} UWC1 \textit{(ICEclc)} caused by the \textit{mfsR} deletion.** (A) Survival of \textit{P. putida} UWC1 \textit{(ICEclc, 2737)} and \textit{P. putida} UWC1 \textit{(ICEclc-ΔmfsR, 4322)} pregrown in suspended culture to stationary phase on 3-chlorobenzoate (3CBA) or succinate (succ), and plated from there on 3CBA or succinate agar. (E.g., 3CBA-3CBA, suspended culture on 3CBA, plated on 3CBA agar). Survival expressed as colony forming units (CFU) on the agar plate per ml of stationary phase culture. Data bars indicate the average from independent biological triplicates. Error bars indicate the calculated standard deviation from the average. (B) Phase-contrast micrographs at 1000-fold magnification of microcolonies of \textit{P. putida} UWC1 \textit{(ICEclc)} and \textit{P. putida} UWC1 \textit{(ICEclc-ΔmfsR) P_{mfsR}egfp}, 4612) growing on agarose surface supplemented with 0.1 mM 3CBA. Shown are a regular stationary phase microcolony of \textit{P. putida} with wild-type \textit{ICEclc} and a transfer competent microcolony (tcm), occurring at 1-3% frequency as reported previously \cite{30}. For comparison, massive lysis (white arrows) and cellular malformations formed in many microcolonies of \textit{P. putida} UWC1 with the \textit{mfsR} deletion.

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Figure 7. Conservation of tciR analogues in putative ICEclc-like regions in a variety of other bacterial genomes. Illustration represents tciR analogues (identified on the basis of a BLASTN E-value lower than 1·10^-15), and surrounding relevant gene regions in the indicated bacterial genomes (species name, accession numbers) compared to ICEclc. Genes are indicated as in the respective genome accession. Rectangles show annotated genes and their orientation (top, orientation towards the left; bottom, gene orientation towards to right); common colors indicate similar predicted functions. Stippled rectangles indicate common gene regions inferred from Artemis comparison, but not present in the respective annotation. ICE were inferred from (i) more than 75% nucleotide identities across the complete core region of ICEclc, and within a 1-100 kb window from the tciR position, and (ii) the presence of an integrase gene (in brown) within a 5-20 kb window from the tciR-analogue. Note how some genomes carry multiple different ICE from the same family (e.g., Achromobacter xylosoxidans, Acidovorax sp. strain JS42), and further how pair-wise identical ICE regions (shaded in grey) occur between different genomes. Finally note how the tciR-analogues often co-occur with a xer-type regulatory gene on the other strand (light green), and a further lysR gene member (yellow), but in none of the cases shown here with an mfsR counterpart (in red).

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Regulation of ICEclc Transfer

Figure 8. Model for regulation of ICEclc transfer competence. MfsR autoregulates itself and that of the TciR activator, without which ICEclc transfer decreases by 2-103-fold. TciR may activate specifically one or more promoters on ICEclc, such as the RpoS-dependent Ppar4-promoter [29], or a promoter upstream of the parA-like gene [32]. Expression of the Ppar4-promoter occurs preferentially in cells having highest RpoS levels, and only 1-3% of cells in a population in stationary phase visibly express reporter gene from Ppar4 [29]. InrR transmits bistable activation through an unknown process to the intB13 promoter [26], and possibly simultaneously to other promoters for the genes for the conjugative system in the ICEclc core region [32]. The exact mechanism of arousal of bistability is unclear as yet. For gene locations on ICEclc, see Figure 1A. doi:10.1371/journal.pgen.1004441.g008

Discussion

ICEclc has two distinctive modes of existence: the integrated form, which is transmitted vertically, and the circular form, which can be horizontally transferred. Previous work in our laboratory has shown that the transition between these two states occurs in only a few percent of cells in a population under stationary phase conditions [26,30,34]. We have recently suggested to name cells in which the molecular decision occurs to activate the ICEclc horizontal transfer mode transfer competent (tc) cells [30]. Single cell time-lapse experiments indicated that ICEclc transfer - at least insofar as detectable by microscopy, only occurs from tc cells, which can be distinguished through simultaneous expression of fluorescent proteins from single copy transcriptional fusions to the Pint and Pmar promoters of ICEclc [30]. Activation of those two promoters is the likely outcome of a multi-step regulatory cascade that orchestrates expression of some fifty genes [32], but the key factors that determine the onset of this cascade and control the extent of bistability are still obscure. Previous work provided evidence for the role of the stationary phase sigma factor RpoS in activation of ICEclc promoters, and we could show that tc cells on average have higher levels of RpoS [34]. In the present study, we report the discovery of a cluster of three regulatory genes, two of which globally control ICEclc activation and transfer, and additionally maintain a cap on fitness loss induced by the ICE (Figure 8).

The three regulatory genes occur in a unique configuration of a TciR-type repressor (encoded by mfsR), followed by a MarR-type (mfsR) and a LysR-type regulator (tciR). Transcript and microarray analysis of the locus in wild-type and mutant ICEclc, plus analysis of reporter gene expression from the mfsR promoter in a variety of host backgrounds, showed that the three genes are expressed as a polycistronic unit and are under autoregulatory control by MfsR (Figure 5A).

Precise gene deletions and complementations indicated that tciR is likely the main global regulator of ICEclc transfer activation. Deletion of tciR caused a 2-103-fold lower frequency of ICEclc transfer compared to wild type (Figure 2B), silenced expression of the ICEclc core region (Figure 3B) and reduced the proportion of cells expressing Pint and Pmar in stationary phase (Figure 5B). Deletion of the TciR deletion on ICEclc with a single copy mini-Tn7 inserted mfsR-marR-tciR fragment (Figure 1C) fused to the rpoS promoter region restored the expected phenotype (Figure 2B). TciR may act either directly as a regulator on a variety of individual ICEclc core promoters, or as a “master” regulator in a hierarchical activation cascade (Figure 8).

The role of marR is less clear and as yet unsolved. Deletion in marR resulted in essentially the same ICEclc transcriptome profile as deletion in tciR (Figure 3D). It also resulted in a lower transfer frequency than wild-type but not as low as the deletion in tciR (Figure 2B), and produced no detectable reporter gene expression from Pint or Pmar (Figure 5). In contrast, complementation of the marR deletion on ICEclc by a single copy marR gene through mini-Tn7 delivery (Figure 1G) did not restore ICEclc transfer

(ICEclc-DmarR) were impaired in survival compared to UWC1 (ICEclc) (Figure S6).

tciR is a very widespread ortholog among ICE closely related to ICEclc

Using bioinformatic queries, we retrieved orthologs to tciR and mfsR from sequenced bacterial genomes, and examined manually whether they occur in chromosomal regions qualifying as ICE (e.g., presence of an integrase gene nearby, see Materials and Methods). Interestingly, orthologs to the individual components of the mfsR operon are widespread but rarely occur in the same configuration (Figure 7). So far, the ICEclc mfsR-marR-tciR configuration is only found in the ICEclc variant of Burkholderia xenovorans LB400, whereas the Tn4371-element of Acidovorax sp. strain JS42 and Aerononas hydrophila SSU (accession number AGWR01000022.1) both carry an mfsR homolog and a MarR-type regulator immediately downstream, but not a tciR equivalent nearby. There is a tciR ortholog in Acidovorax sp. strain JS42, but not on the same chromosomal region as mfsR (Figure 7).

On the other hand, tciR seems much more widespread among ICEs, as homologs can be found in ICEclc-like elements G11 and G16 of Bordetella perta DSM12804, in PAGI-2 of P. aeruginosa strain C1, in diverse ICEs of X. campesiris pv. vesicatoria str. 85-106, suspected ICEs in Herminiimonas arsenicoxydans, Capnodiaceae metallidurans CH34, and Tolhuomanus amniss DSM 9187, among several dozens of others (Figure 7). Given that TciR is such a common regulator found in ICEs of the ICEclc family, it might be similarly implicated in their transfer control. This regulation is likely different in detail from ICEclc, given the absence of an mfsR and marR.
Furthermore, mutants with deletions in \textit{mfsR} or \textit{mfsR} plus the first 116 bp of \textit{marR} (Figure 1B), behaved quite similar in transfer frequency (Figure 2) and showed similar ICE\textsubscript{clc} transcription profiles (Figure S4). The \textit{marR} gene therefore seems to have no clear role in ICE\textsubscript{clc} core gene expression.

The most surprising effect of deletions in \textit{mfsR} was a complete deregulation of the ICE\textsubscript{clc} core gene expression. This became obvious from frequencies of ICE\textsubscript{clc} transfer being 10-100 fold higher than wild type, approximating 1 transfer per donor cell (Figure 2B). The deregulation was also obvious in microarray data showing the ICE\textsubscript{clc} core region in the \textit{mfsR} deletion mutants being already transcribed in exponential phase (Figure 3B, Figure S3).

Finally, 80-100\% of individual cells in stationary phase expressed the reporter genes from \textit{P}_{\text{at}} and \textit{P}_{\text{atk}} in the \textit{mfsR} deletion strain compared to 3-5\% in wild-type (Figure 5B). This can be explained by the fact that deletion of \textit{mfsR} would abolish autorepression, which would lead to constant high expression of \textit{marR} and \textit{tciR}. This overinitiates ICE\textsubscript{clc} core gene expression, leads to more cells entering the tc state and to higher transfer rates. The balance of \textit{mfsR} control appears to be extremely delicate, since even complementation with a single gene copy under control of the original promoter results in a stronger effect than the wild-type, both for \textit{tcR} and \textit{mfsR} (Figure 2B). The delicate balance became also obvious from the polar effects of insertion of the Km\textsubscript{R} resistance gene within \textit{mfsR}, leading to decreased transcription of \textit{marR} and \textit{tciR}, diminished core gene expression (Figure 3D) and reduced transfer rates (Figure 2A).

The finding that deletion and complementation of \textit{mfsR} or \textit{tcR} drastically changes the proportion of cells activating ICE\textsubscript{clc}, could imply that the bistability seen in wild-type situation (i.e., 3-5\% of cells in stationary phase becoming transfer competent) is a result of feedback at this locus. Cells activating ICE\textsubscript{clc} in the wild-type situation could arise as a consequence of “loopy” control by MfsR, incidentally causing a few cells to escape its control and transcribing \textit{marR} and \textit{tciR}. We think this is an unlikely scenario, because mCherry expression from the \textit{mfsR} promoter is homogeneous among cells (Figure 5A). Alternatively, there might be a chemical ligand that specifically binds to MfsR in a small subset of cells, upon which its repression is relieved in those cells. The resulting TciR would then be the necessary activator to trigger ICE\textsubscript{clc} core expression in cells with on average highest RpoS levels [29] (Figure 8). On the other hand, even though \textit{mfsR} may be the first level of control, bistability may also originate at later checkpoints in the regulatory cascade, which depend on the presence of sufficient TciR.

Quasi-global appearance of transfer competence across all cells in the \textit{mfsR} deletion mutant resulted in massive fitness loss (Figure 6), which became evident at two levels. First of all, time-lapse observations indicated lysis and aberrant cell growth in more than 50\% of microcolonies (Figure 6B). This lysis and growth arrest are similar to what we previously described as being a side consequence of becoming transfer competent in wild-type cells [30], and is caused by the \textit{parA-shi} gene products on ICE\textsubscript{clc} [30]. Secondly, there was a strong loss of the capacity to grow on 3CBA among cells sampled from stationary phase cultures of the \textit{mfsR} mutant compared to wild-type (Figure 6A, Figure S6), indicative for loss of ICE\textsubscript{clc} and counterselection against maintaining ICE\textsubscript{clc-}\textit{\Delta}mfs\textsubscript{R}. However, those cells that maintained ICE\textsubscript{clc-}\textit{\Delta}mfs\textsubscript{R} could still grow on 3CBA and showed indistinguishable exponential growth rate (Table 2). This paradox can be understood when modeling the number of tc cells in batch culture populations for ICE\textsubscript{clc} wild-type (probability of tc arrest, \textit{P}_{\text{at}} in stationary phase of 0.025) and for the ICE\textsubscript{clc-}\textit{\Delta}mfs\textsubscript{R} mutant (\textit{P}_{\text{at}} = 0.5). This model (Figure S5B) shows that whereas a large proportion of tc cells appear in ICE\textsubscript{clc-}\textit{\Delta}mfs\textsubscript{R} mutant cultures in stationary phase, these can only divide 2-3 times upon reinoculation into fresh medium before lysing. This causes an apparent prolongation of a lag phase visible as stagnant culture turbidity, but does not influence the overall predicted population exponential growth rate in batch culture (Figure S5B).

As expected from the postulated role of TciR, its complementation in trans also leads to increased ICE\textsubscript{clc} transfer, but interestingly, only the \textit{mfsR} deletion caused strongly decreased cell survival (Figure S6). We therefore hypothesize that ICE\textsubscript{clc} activation may follow two separate processes: transfer and tc cell growth arrest [30], that may both be initiated at the \textit{mfsR} locus. Deleting \textit{mfsR} would then deregulate both processes, whereas expressing \textit{tciR} in trans would only increase activation through the transfer branch (Figure 6).

The configuration of the \textit{mfsR-marR-tciR} operon of ICE\textsubscript{clc} is unique, but \textit{tciR} alone is a very common part of ICE similar to ICE\textsubscript{clc} (Figure 7). We therefore speculate that \textit{mfsR-marR} are a more recent acquisition in ICE\textsubscript{clc}, which drastically changed the expression of the \textit{tciR} gene. Unfortunately, expression of \textit{tciR} analogs in other ICEs has not been studied and very little has been reported on the transferability of ICEs related to ICE\textsubscript{clc}. The exceptions are G3 of \textit{B. petrii} that transfers at extremely low frequencies ($\sim 10^{-7}$) [35], and the \textit{P. aeruginosa} PAG1-2 element for which transfer has not been detected at all [16]. In comparison, wild-type ICE\textsubscript{clc} transfers at rates of $10^{-2}$ to $10^{-3}$ per donor (Figure 2), suggesting that it was perhaps the acquisition of the \textit{mfsR} regulatory control that led to expression of transfer activity in a larger proportion of cells in the population. As we show here, the downside of increasing the proportion of ICE\textsubscript{clc} tc cells is an increase of the proportion of cells displaying growth arrest through the \textit{shi-parA} pathway [30]. Likely, the MfsR autoregulation evolved to a stage of permitting efficient transfer but avoiding too much fitness loss to the population. Even though the mechanistic details are different for ICE\textsubscript{clc}, double control layers are more common for various ICEs and typically involve a variety of regulators acting on each other and/or in response to specific chemical ligands [10,18,23-25]. It will be highly interesting to further study the mechanistic details of the control systems that maintain very low ICE transfer rates, and to understand whether and how such control can evolve to allow hyperefficient transfer.

**Materials and Methods**

**Strains and culture conditions**

Table 1 lists the strains used in this study. \textit{Escherichia coli} DH5\textsubscript{a} (Gibco Life Technologies, Gaithersburg, Md.), \textit{E. coli} DH5\textsubscript{a} Top10, \textit{E. coli} BW20767/pRL27 were cultured at 37°C on Luria-Bertani (LB) medium [36]. \textit{Pseudomonas} species were cultured at 30°C on LB or 21°C minimal medium (MM) [37] complemented with one of the following carbon sources: 0.5, 5, or 10 mM 3-chlorobenzoate (3CBA), 15 mM succinate or 10 mM fructose. Antibiotics were supplemented to the growth medium to select for maintenance of genetic constructions at the following concentrations: kanamycin (Km) 25 \mu g/mL, chloramphenicol (Cm) 20 \mu g/mL, rifampicin (Rif) 50 \mu g/mL, nalidixic acid (Nal) 50 \mu g/mL, gentamicin (Gm) 20 \mu g/mL, and ampicillin (Ap) 100 \mu g/mL.

**Strain constructions and DNA techniques**

DNA purification, PCR, restriction enzyme digests, DNA ligations and electro-transformations were performed according to standard procedures [36]. Deletions in ICE\textsubscript{clc} genes were created by double recombination techniques as described elsewhere.
Nucleotide positions are given according to AJ617740 (ICEclc). Primers used for strain constructions are listed in table S1.

For complementation of P. putida UWC1 (ICEclc-ΔmfsR, strain 4322) we first amplified the mfsR gene plus the 429 bp upstream region containing the mfsR promoter using PCR. This fragment was cloned into pGEM-T-easy and verified for correctness by DNA sequencing. The fragment containing the correct mfsR region was then recovered by restriction enzyme digestion with PstI and BamHI, and ligated into the mini-Tn7 vector pUC-miniTn7-Gm [39]. After transformation and verification in E. coli, the mini-Tn7 construct was introduced into P. putida UWC1 (ICEclc-ΔmfsR) by using the pUX-BF13 helper plasmid [40]. Clones resistant to Gm were selected and verified by PCR for correct insertion of the mfsR DNA in the attTn7 locus. To complement P. putida UWC1 (ICEclc-ΔmfsR, strain 4321) and (ICEclc-ΔmarR, strain 4372) we amplified the complete mfsR-marR-tecR locus including the 429-bp upstream region. This fragment was cloned into pGEM-T-easy and again verified for correctness by DNA sequencing. The fragment was recovered by digestion with BamHI and Stul, and ligated with the mini-Tn7 vector. A frameshift was then introduced in the mfsR coding region by digestion at the unique Ncol-site, filling in using Klenow and religation. This will cause premature ending of the mfsR gene product (mfsR'). A second frameshift was subsequently introduced to inactivate the marR gene product, using the unique BglII-site (marR'). After transformation and verification in E. coli, the construct was introduced in P. putida UWC1 (ICEclc-ΔmfsR) as outlined above. This procedure was repeated to create a fragment with frameshifts in mfsR and in tecR (using the unique KpnI site), but maintaining an intact marR. This construct was introduced into P. putida UWC1 (ICEclc-ΔmarR). Gm-resistant clones were verified by PCR for the correct insertion at the attTn7-site, and for the presence of ICEclc.

A 656-bp region upstream of mfsR was amplified by PCR and fused to a promoterless mcherry gene. This fragment was introduced in single copy on the chromosome of P. putida UWC1, P. putida UWC1 (ICEclc) or P. putida UWC1 (ICEclc-ΔmfsR) using mini-Tn5 delivery. Three independent Km-resistant colonies were verified by PCR for the correct insertion and stored individually. P. putida UWC1 mini-Tn5-PmfsR-Δmcherry and P. putida UWC1 (ICEclc-ΔmfsR) mini-Tn5-PmfsR-Δmcherry were then further used as recipient to introduce the mini-Tn7-mfsR construct.

Random mutagenesis and screening

Random mini-transposon insertions in P. knackmussii B13 were generated by mobilization of the pRL27 suicide plasmid from E. coli BW20767 in a biparental mating. Hereto both strains were each cultured overnight in 3 ml LB, pelleted down, resuspended in 50 μl sterile saline solution (0.9% NaCl), mixed in a 1:1 (v/v) ratio and incubated on the surface of an LB agar plate for 24 hours at 30°C. The mixture was then resuspended with 1 ml saline solution, which was inoculated in 100 ml MM with 0.5 mM 3CBA plus Km to select for the mini-transposon insertion and Cm to counterselect against E. coli, and incubated at 30°C for 16 h with orbital shaking (180 rpm). An aliquot of 3 ml of this pool of enriched KmR B13 mutants was used en masse as donor in a subsequent mating procedure. Hereto, cells from the 3 ml suspension were pelleted by centrifugation, washed with 3 ml sterile saline and mixed with 3 ml of suspension of P. putida UWC1 recipient, that had been grown for 16 h on LB, was pelleted by centrifugation and resuspended in sterile saline. The mating mixture was again centrifuged, the cell pellet was resuspended in 50 μl sterile saline solution and spotted on the surface of a MM agar plate containing 0.5 mM 3CBA. The mixture was incubated for 72 hours at 30°C, after which the cells were washed from the plate with 1 ml sterile saline, which was further serially diluted and plated on MM agar plates with 5 mM 3CBA plus Km and Rif to select for transconjugants carrying mutant ICEclc. Individual colonies were purified, recultured in organized 96-well format and stored at -80°C after addition of and mixing with glycerol to 15% (v/v). Libraries were replicated and regrown in 100 μl LB plus Rif for 16 h in 96-well microtiter plates, mixed with 100 μl P. putida UWC1 NalR recipient suspension, and incubated at 30°C for 48 h. Then 50 μl of each well was reinoculated into 170 μl of MM containing 5 mM 3CBA plus Km, Rif and Nal, and growth was measured by continuous OD-measurements in a multiplate reader (FluoStar Omega, BMG labtech). Absence of growth was taken as indication for absence of ICEclc transfer, in which case the donor culture was recovered for mapping of the transposon insertion.

Insertion mappings

DIG-labeled primers 070934 or 070935, annealing to one of the ends of the KmR insert but facing outward, were used (separately) in single-primer PCR with DNA from mutant UWC1 donors as templates. The reactions produced oligonucleotide probes with the 5'-DIG-label, the sequence of the end of the KmR gene and the adjacent sequence of the ICEclc insert position. Such products were used for rough localization of the insertion position by hybridizing to macroblot membranes (Eurogentec, UK), whose set of 55-mer oligonucleotides covers most of the ICEclc genes. Hybridization and detection of the DIG-marker were carried out according to the manufacturer’s instructions (Roche Diagnostics GmbH, Mannheim, Germany). Once the insertion was roughly mapped on ICEclc, PCR-based sequencing was used to determine exact position of the KmR-gene insertion.

ICEclc transfer assays

The frequency of ICEclc transfer was determined in experimental conditions described previously [33]. P. putida UWC1 ICEclc wild-type or mutant derivatives were used as donors, whereas P. putida UWC1G (constitutively fluorescent, KmR) or P. putida UWC1 KmR were used as the recipient (Table 1). Briefly, donors and recipient were each cultivated on 5 mM 3CBA MM and 10 mM fructose MM, respectively, and combined on 0.5 mM 3CBA agar plates as a single concentrated pellet. After 48 hours incubation at 30°C, mating mixes were resuspended, diluted and plated on 5 mM 3CBA MM agar (counting of donor CFU) or 5 mM 3CBA Gm or Km agar (counting of transconjugant CFU). Transconjugants were checked by PCR and frequencies were expressed as the number of transconjugant CFU per donor CFU. Donor survival was used for the data shown in Figure S6.

ICEclc transcriptome analysis by microarrays

The ICEclc transcriptomes of P. putida UWC1 (ICEclc), P. putida UWC1 (ICEclc), P. putida UWC1 (ICEclc-ΔmfsR, strain 19033), P. putida UWC1 (ICEclc-ΔmarR, strain 19034), P. putida UWC1 (ICEclc-ΔmarR, strain 19035), P. putida UWC1 (ICEclc-ΔtecR, strain 19036), P. putida UWC1 (ICEclc-ΔmfsR-ΔmarR, strain 19037) and P. putida UWC1 (ICEclc-ΔtecR-ΔmarR, strain 19038) were investigated by microarray analysis, as described previously [32]. Total RNA was extracted from cells grown on 10 mM 3CBA MM, and harvested at mid exponential phase (OD600 = 0.6) and 48 h after entrance in stationary phase. Reverse transcription using cyanine-dCTP among the dNTPs produced labeled cDNA that was further purified and hybridized on 8×15 K microarray slides (Agilent, Santa Clara, CA, USA). Slides were washed and scanned according to manufacturer’s instructions (Agilent). Data were recovered and analyzed using GeneSpring GX. Microarray data can be accessed from the GEO database (accession number: GSE51391).
Time-lapse microscopy

*P. putida* UWC1 strains were precultured for 16 h at 30°C in LB medium, after which 100 μl were added fresh MM 4 mM 3CBA medium in presence of the appropriate antibiotics. This culture was incubated for 96 hours at 30°C and 200 rpm shaking, after which the cells were 100-fold diluted in MM without C-source and inoculated on agarose surfaces (gel patches) for time-lapse microscopy [41]. Medium for gel patches consisted of 1% agarose dissolved by heating into MM with 0.1 mM 3CBA. Gel patches were created by pipetting 130 μl of the agarose-MM-3CBA solution kept at 55°C on the surface of a circular cover glass (42 mm ø and 0.17-mm thick), placed in an autoclaved perfusion chamber [POC; H. Saur, Reutlingen, Germany], separated with a 0.5 mm thick silicon spacer ring and covering them with a second cover glass. After solidification of the agarose, the upper cover slip was removed and 6 μl of the diluted cell suspension was placed onto the agarose gel patch. As soon as the drops were dried on the surface, the patches were turned upside down and placed bacteria-facing-down on a new round cover glass [41]. A second silicon spacer ring was added to allow air circulation within the closed chamber and the glass sandwich was fixed into the metal cast POC chamber with a metal ring. Up to four patches could be placed simultaneously within a single glass sandwich in a POC chamber.

Microcolony development was followed directly on a Nikon Inverted Microscope Eclipse Ti-E, equipped with a Perfect Focus System (PFS), eL-100 CoolLED and a Plan Apo λ 100x x1.45 Oil objective (Nikon), installed in a controlled temperature room (22°C). Ten random regions of every patch were imaged automatically during 48 hours with intervals of 1 h, in Phase Contrast mode (10 ns exposure), eGFP (500 ns) and eCherry (500 ms). Images were recorded using Micro-Manager 1.4 (http://www.micro-manager.org/) and fluorescence values were extracted using MetaMorph (Series 7.5, MDS, Analytical Technologies).

### P. putida UWC1 ICEclc and mutant fitness tests

Triplicates of strains UWC1 (ICEclc) and UWC1 (ICEclc-ΔmfsR) were grown for 16 h in LB medium at 30°C. Both strains were then 500-fold diluted (starting OD_{600} 0.001) in MM with 5 mM 3CBA or 10 mM succinate. Upon reaching early stationary phase, strains were again diluted into fresh MM (starting OD 0.001) with the same carbon source, and growth was followed by frequent turbidity measurements (OD_{600}). 24 h after reaching stationary phase, each replicate culture was serially diluted in MM and plated onto MM agar plates with 5 mM 3CBA or with 10 mM succinate. The number of CFU/ml was scored and the ratio was calculated between the number of CFU/ml on MM agar with 5 mM 3CBA and the number of CFU/ml on MM with 10 mM succinate.

Ten randomly chosen colonies of UWC1 (ICEclc-ΔmfsR) cultivated in MM with succinate and grown on MM-sucinate agar plates were restreaked for growth on MM agar with 5 mM 3CBA. The presence of ICEclc was determined by colony PCR on the same colonies by amplifying the *clcA* gene, which is carried by ICEclc and the gene product of which is essential for 3CBA metabolism.

**Bioinformatic screening for ICE related to ICEclc**

Homologues to *teiR* of ICEclc were detected by BLASTN to the nr/nt database at E-value <1·10^{-10}. The corresponding whole or draft genome sequences were retrieved and compared by aligning to ICEclc (Accession number AP8617740.2) using Megablast. Detected regions were manually recovered and searched for the *teiR* homologue and an intB13 homologue within a 1-100 kb window. If annotated, the presence of a gene for tRNA-Gly nearby the intB13 homologue was scored. Regions covering all criteria (i.e., homology to ICEclc core region, *teiR* homologue and presence of integrase gene) were retained as containing putative ICE. Selected regions were further individually pair-wise compared by using the Artemis Comparison Tool within the WebACT service [42].

### Supporting Information

**Figure S1** Outline of the random mutagenesis and subsequent selection procedure. Original ICEclc-host *P. knaasus* B13 is randomly mutagenized by miniTn5-mediated insertions of kanamycin resistance inserts (KmR). B13 mutant B13 are selected by culturing cells on minimal medium (MM) with Km and 3-chlorocatechol (3CBA) as sole carbon and energy source. The pool of B13 mutants is cultured in batch and mixed with recipient strain *P. putida* UWC1 (resistant to rifampicin, RifR). The mixture is incubated in mating conditions for 72 hours and plated on MM with 3CBA, Km and Rif, to select for transconjugants. Individual colonies of transconjugants were restricted and organized into a mutant library in 96-well plates. Each mutant is used as donor in a new 96-well mating with recipient *P. putida* UWC1 resistant to Rif and nalidixic acid (NalR). Individual mating mixtures are grown on MM agar with 3CBA, Km, Rif, Nal in order to select for transconjugants. In absence of transconjugant growth, the donor of that particular mating was recovered from the library and had its insertion position mapped.

(TIF)

**Figure S2** Reverse transcriptase polymerase chain reaction analysis of transcription in the mfsR-marR-tciR region. (A) Amplification of specific regions on reverse-transcribed (+) or not (-) mRNA purified from exponentially growing *P. putida* UWC1 (ICEclc) cultures on 3CBA, compared to amplification on purified DNA. (B) Schematic overview of the location of the used primers for the reverse transcription reaction (RT) and for the amplification of the gene regions.

(TIF)

**Figure S3** Pair-wise comparisons of expression in the ICEclc area by microarray analysis. (A) *P. putida* UWC1 (ICEclc-ΔmarR, strain 4372) compared to *P. putida* UWC1 (ICEclc, strain 2737). (B) *P. putida* UWC1 (ICEclc-ΔteiR, strain 4321) versus *P. putida* UWC1 (ICEclc, strain 2737). (C) *P. putida* UWC1 (ICEclc-ΔmfsR, strain 4372) versus *P. putida* UWC1 (ICEclc-ΔmarR, strain 4321). Panels indicate comparisons of exponentially growing or stationary phase cells, with hybridization signals on the plus- (open symbols) or minus-strand (closed symbols) of ICEclc. Dots indicate the log-fold change of hybridization signal per microarray probe in the comparison, plotted at their distance along the ICEclc sequence (X-axis; in kb). A scheme of ICEclc is redrawn at the bottom of each section, with regions of interest as grey boxes (+ or - indicate the DNA strand on which the region is encoded). Grey bars in the background indicate the two-fold cut-off level.

(TIF)

**Figure S4** Pair-wise comparisons of expression in the ICEclc area of mfsR mutants by microarray analysis. (A) *P. putida* UWC1 (ICEclc-ΔmfsR, strain 4322) compared to *P. putida* UWC1 (ICEclc, strain 2737). (B) *P. putida* UWC1 (ICEclc-ΔmfsR-ΔmarR, strain 3433) versus *P. putida* UWC1 (ICEclc, strain 2737). (C) *P. putida* UWC1 (ICEclc-ΔmfsR:KmR, strain 2961) versus *P. putida* UWC1 (ICEclc, strain 2737). Panels indicate comparisons of exponentially growing or stationary phase cells, with hybridization signals on the plus- (open symbols) or minus-strand (closed symbols) of ICEclc.
Dots indicate the log-fold change of hybridization signal per microarray probe in the comparison, plotted at their distance along the ICEclc sequence (X-axis; in kb). A scheme of ICEclc is redrawn at the bottom of each section, with regions of interest as grey boxes (+ or - indicate the DNA strand on which the region is encoded). Grey bars in the background indicate the two-fold cut-off level.

**Figure S5** Observed and expected population growth in cultures of *P. putida* with wild-type ICEclc and the ΔmfsR mutant. (A) Measured turbidities in triplicate batch cultures growing on 3CBA as sole carbon and energy source. Note the increased lag time of the *P. putida* ΔmfsR mutant. (B) Modeled population growth of all cells (brown) and tc cells (red) in two subsequent batch cultures growing on 3CBA; the second being inoculated with 1:1000 volume from the first culture in stationary phase. Scenarios show predicted behavior for wild-type ICEclc (with a probability of 0.025 of tc cell appearance in stationary phase) and for ΔmfsR (with *P* = 0.5). Note how a tc population appears in stationary phase, which is transferred to a new culture, but rapidly dies as a result of activation (TIF).

**Figure S6** Population sizes of *P. putida* UWC1 carrying wild-type (2737, 2738) or mutant ICEclc, measured as colony forming units (CFU) on 5 mM 3-chlorobenzoate agar plates, per ml of resuspended culture spotted and incubated for 48 h on 0.5 mM 3-chlorobenzoate containing agar medium. (A) *P. putida* UWC1 strains: 4165, ICEclc with a deletion in the genes for the suspected efflux system (as unrelated control); 4321, tcR deletion; 4322, mfsR deletion; 4372, marR deletion; versus *P. putida* UWC1 (ICEclc), 2730. (B) *P. putida* UWC1 strains: 2737, ICEclc wild-type; 4321, tcR deletion; 4322, mfsR deletion; 4372, marR deletion; 4646, mfsR deletion but complemented in trans by a single copy mini-Tn7 inserted mfsR gene under its own promoter; 4649, tcR deletion but complemented in trans by a single copy mini-Tn7 inserted fragment with the (mfsR-)marR-iceR genes under the mfsR promoter. 4804, marR deletion but complemented in trans by a single copy mini-Tn7 inserted fragment with the (mfsR/marR)-iceR genes under the mfsR promoter. (A) and (B) Independently carried out experiments on different occasions and by different scientists. Letters indicate statistically indistinguishable groups identified from ANOVA tests on biological replicates, followed by Tukey’s post hoc testing. P-values indicate the significance for the overall group difference (α versus β), or in one specific case, between the samples connected by the line. (TIF)

**Table S1** Oligonucleotides used for amplification of ICEclc fragments. (DOCX)

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**Author Contributions**

Conceived and designed the experiments: NP SS JRvdM. Performed the experiments: NP SS FD II. RM JRvdM. Analyzed the data: NP SS FD RM JRvdM. Contributed reagents/materials/analysis tools: RM NP. Wrote the paper: NP JRvdM FD.
27. Gaillard M, Vallaeys T, Vorho¨lter FJ, Minoia M, Werlen C, et al. (2006) The clc element of Pseudomonas sp. strain B13, a genomic island with various catabolic properties. J Bacteriol 188: 1999-2013.

28. Miyazaki R, Minoia M, Pradervand N, Sentschilo V, Sulser S, et al. (2011) The clc element and related genomic islands in Proteobacteria. In: Roberts AP, Mullany P, editors. Bacterial integrative mobile genetic elements: Landes Bioscience.

29. Miyazaki R, Minoia M, Pradervand N, Sulser S, Reinhard F, et al. (2012) Cellular variability of RpoS expression underlies subpopulation activation of an integrative and conjugative element. PLoS Genet 8: e1002818.

30. Reinhard F, Miyazaki R, Pradervand N, van der Meer JR. (2013) Cell differentiation to “mating bodies” induced by an integrating and conjugative element in free-living bacteria. Curr Biol 23: 255-259.

31. Larsen RA, Wilson MM, Guss AM, Metcalf WW (2002) Genetic analysis of pigment biosynthesis in Xanthobacter autotrophicus Py2 using a new, highly efficient transposon mutagenesis system that is functional in a wide variety of bacteria. Arch Microbiol 178: 193-201.

32. Gaillard M, Pradervand N, Minoia M, Sentschilo V, Johnson DR, et al. (2010) Transcriptome analysis of the mobile genome ICEclc in Pseudomonas knackmussii B13. BMC Microbiol 10: 153.

33. Miyazaki R, van der Meer JR (2011) A dual functional origin of transfer in the ICEclc genomic island of Pseudomonas knackmussii B13. Mol Microbiol 79: 743-758.

34. Sentschilo VS, Ravatn C, Werlen C, Zehnder AJB, van der Meer JR (2003) Unusual integrase gene expression on the clc genomic island of Pseudomonas sp. strain B13. J Bacteriol 185: 4530-4538.

35. Lechner M, Schmidt K, Bauer S, Hot D, Hubans C, et al. (2009) Genomic island excisions in Burkholderia pseudomallei. BMC Microbiol 9: 141.

36. Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

37. Gerhardt P, Murray RG, Costilow RN, Nester EW, Wood WA, et al, editors (1981) Manual of methods for general bacteriology. Washington, D.C.: American Society for Microbiology.

38. Martinez-Garcia E, de Lorenzo V (2011) Engineering multiple genomic deletions in Gram-negative bacteria: analysis of the multi-resistant antibiotic profile of Pseudomonas putida KT2440. Environ Microbiol 13: 2702-2716.

39. Choi KH, Gaynor JR, White KG, Lopez C, Bosio CM, et al. (2005) A Tn7-based broad-range bacterial cloning and expression system. Nat Methods 2: 443-446.

40. Koch B, Jensen LE, Nybroe O (2001) A panel of Tn7-based vectors for insertion of the gfp marker gene or for delivery of cloned DNA into Gram-negative bacteria at a neutral chromosomal site. J Microbiol Methods 45: 187-195.

41. Reinhard F, van der Meer JR (2010) Microcolony growth assays In: Timmis KN, de Lorenzo V, McGinity T, van der Meer JR, editors. Handbook of Hydrocarbon and Lipid Microbiology: Springer Verlag. pp. 3562-3570.

42. Abbott JC, Aanensen DM, Bentley SD (2007) WebsACT: an online genome comparison suite. Methods Mol Biol 395: 57-74.

43. Dorn E, Hellweg M, Reineke W, Knackmuss H-J (1974) Isolation and characterization of a 3-chlorobenzoate degrading Pseudomonad. Arch Microbiol 99: 61-70.

44. 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.

45. Sentschilo V, Czechowska K, Pradervand N, Minoia M, Miyazaki R, et al. (2009) Intracellular excision and reintegration dynamics of the ICEclc genomic island of Pseudomonas knackmussii sp. strain B13. Mol Microbiol 72: 1295-1306.