Highly variable individual donor cell fates characterize robust horizontal gene transfer of an integrative and conjugative element

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Horizontal gene transfer is an important evolutionary mechanism for bacterial adaptation. However, given the typical low transfer frequencies in a bacterial population, little is known about the fate and interplay of donor cells and the mobilized DNA during transfer. Here we study transfer of an integrative and conjugative element (ICE) among individual live bacterial cells. ICEs are widely distributed mobile DNA elements that are different than plasmids because they reside silent in the host chromosome and are maintained through vertical descent. Occasionally, ICEs become active, excise, and transmit their DNA to a new recipient, where it is reintegrated. We develop a fluorescent tool to differentiate excision, transfer, and reintegration of a model ICE named ICE_{dc} (for carrying the *dc* genes for chlorocatechol metabolism) among single *Pseudomonas* cells by using time-lapse microscopy. We find that ICE_{dc} activation is initiated in stationary phase cells, but excision and transfer predominantly occur only when such cells have been presented with new nutrients. Donors with activated ICE develop a number of different states, characterized by reduced cell division rates or growth arrest, persistence, or lysis, concomitant with ICE excision, and likely, ICE loss or replication. The donor cell state transitions can be described by using a stochastic model, which predicts that ICE fitness is optimal at low initiation rates in stationary phase. Despite highly variable donor cell fates, ICE transfer is remarkably robust overall, with 75% success after excision. Our results help to better understand ICE behavior and shed a new light on bacterial cellular differentiation during horizontal gene transfer.

bacterial evolution | *Pseudomonas* | conjugation | modeling | time-lapse microscopy

Bacterial genomes represent dynamic constellations of core and accessory genomic regions (1–3). The latter are dominated by mobile elements; DNA that can be transferred horizontally from a donor to a recipient bacterium, even of a different species. Integrative and conjugative elements (ICEs) are frequently detected mobile genome inhabitants. They can contribute to host adaptation by dispersing dozens to hundreds of genes in a single transfer event (4–6). It has been estimated that conjugative systems of ICEs are more abundant among bacteria than those of plasmids (7), yet we know far less about ICE behavior because they are difficult to follow and isolate. ICEs have attracted interest because they can transmit antibiotic resistance genes (8) and suspected virulence factors (9–11), but also genes for toxic compound degradation (12, 13) and heavy metal resistance (14). Several evolutionary distinct families of ICEs have been described, which have a mechanistically similar lifestyle (4, 6, 15). In contrast to conjugative plasmids, ICEs invade cells through conjugation but subsequently insert at one or more specific sites in the genome, from where they need to excise for a next round of transfer (4–6). Similar to a prophage, the ICE is coreplicated with the bacterial chromosome in its integrated form, ensuring stable vertical transmission. Conjugation frequencies of ICEs vary widely from approximately 1×10^{-2} to 1×10^{-7} per donor cell, which is considered to be the outcome of an infrequent bistable switch triggering activation of the ICE (16,

17). Environmental cues eliciting the “SOS response” (18) or growth on specific carbon substrates (19) can enhance ICE transfer rates, likely by influencing the frequency of the bistable switch.

Horizontal transmission of ICEs starts by their excision from the host chromosome through site-specific recombination and subsequent processing of the excised circularized DNA for conjugation, similar to conjugative plasmids (20, 21) (Fig. 1A). In the receiving cell, the ICE must ensure effective integration to avoid being lost upon cell division because of its limited self-replication capacity (22, 23) (Fig. 1B). We showed for the model ICE_{dc} (named for carrying the *dc* genes for chlorocatechol metabolism) in the bacterium *Pseudomonas knackmussii* B13 (13, 24) that conjugative transfer necessitates development of the host bacterial cell into a “transfer competence” state (25). Development of transfer competence is orchestrated by ICE_{dc} and results in a bistable differentiation of transfer competent (tc) cells and non-tc cells (in which ICE_{dc} remains silent; Fig. 1C) (25). Initiation of transfer competence occurs in stationary phase, when the bacteria in culture run out of nutrients and stop dividing, but affects only 3–5% of the cells in a clonal population harboring WT ICE_{dc} (17, 19, 26). This proportion can increase to 50% or more in strains with a deletion of the ICE_{dc} gene *mfsR* (major facilitator superfamily regulator) (27). Initiation is assumed to trigger a cascade of events (17, 27), leading to the synthesis of the ICE_{dc} conjugation proteins (28, 29) and, finally, to activation of the P_{int} promoter driving *intB13* integrase expression and causing ICE_{dc} excision (Fig. 1A) (17, 27, 30). Intriguingly, tc cells do not only specifically commit to ICE transfer, but their further cell division becomes compromised by

Significance

DNA conjugation is a fascinating process that bestows the prokaryotic world with the ability to exchange hundreds of genes in a single event. What has long been puzzling is the low rate of horizontal gene transfer at the population level. Here, we develop a fluorescent tool to study the dynamic fate of a chromosomally located conjugative DNA. We find that donor cells becoming transfer proficient undergo highly variable cell fates, likely as a result of the conjugative element wreaking havoc in the cell. Modeling of the partnership between the cell and integrative and conjugative element (ICE) suggests that ICE fitness is optimal at low activation rates, explaining why gene transfer in bacterial populations occurs at low frequencies despite its ecological importance for adaptation.

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Fig. 1. Life cycle of the ICEclc element in *Pseudomonas*. (A) ICEclc is normally integrated in the host's chromosome at the 3' end of a gene for tRNA^{Gly} (white-green box). Excision occurs through site-specific recombination between two 18-bp sequences (purple boxes) (39). Note how the stronger P_{circ} promoter faces outward from ICEclc in the integrated form but is placed upstream of the weaker P_{int} promoter in front of the *intB13* integrase gene in the excised form (30). The developed ICEclc reporter has an *egfp* gene located in the same transcriptional unit as *intB13*. (B) Upon transfer to a recipient cell, ICEclc integrates through site-specific recombination in the reverse process of A. Shown here is the constructed recipient with the conditional trap, consisting of a promoterless *mCherry* gene downstream of the integration site. Upon integration, *mCherry* will be strongly transcribed from the P_{circ} promoter. (C) ICEclc activation is a bistable process arising in tc cells. Tc cells are characterized by activation of the P_{int} promoter, which can be visualized through EGFP fluorescence. Cells with excised ICEclc are expected to have stronger EGFP expression as a result of the P_{circ} promoter. Incoming ICEclc-*intB13-egfp* in the recipient may lead to temporary green fluorescence for as long as the ICE is in its excised form. When it has been integrated into the trap, the cell will appear orange because of mCherry expression (plus the remaining green). (D) Transfer competence is initiated in stationary phase (stat) cultures but only in a small proportion of cells. Some individuals transferred from flask to nutrient surface for time-lapse microscopy ($t = 0$) will be tc starters; others will be non-tc cells. Upon

factors encoded on ICEclc (25). This limited cell division leads to the formation of small groups of tc donor cells, which are thought to benefit the overall transfer success of ICEclc by increasing the chance to contact recipients (31).

With few exceptions (25, 32), most of the current knowledge on ICE behavior derives from population-level genetic studies. Very little is known about the cellular events during transfer, which, given the low frequencies of ICE conjugation, are challenging to study. Of particular interest is what ICE transfer provokes in a donor cell and how or whether ICE can maximize successful transfer. Our starting hypothesis based on previous results (25) was that tc donor cells, when they have been initiated, would efficiently deliver the ICEclc at a high rate of successful transfer. Single-cell studies have been previously used to follow plasmid conjugation in which case donor cell fates have not been particularly addressed (33–35), but because ICEs behave very differently, those results are not immediately adaptable. Hence, to better study and understand donor cell fates, we develop a fluorescence reporter that distinguishes the four key steps of ICE transmission: activation, excision, actual transfer, and integration into new recipients. The reporter is calibrated on donor populations alone and in real-time transfer experiments between donors and recipients, and compared with quantitative PCR (qPCR) measurements of ICEclc excision. In contrast to our expectations, we find that donor cells display highly variable phenotypes characterized by dynamic ICEclc behavior, which can be explained by stochastic modeling of different cell state transitions. Despite the variability in cell fates, horizontal transmission is successful in 75% of donor cells in which ICEclc excises. Further work will be needed to understand the molecular events underlying the emergence of the different cell fates.

Results

Development of a Tool to Visualize ICEclc Excision and Transfer from Single Donor Cells. To better understand the dynamic fate of ICEclc during transfer, we developed a tool to follow experimentally single donor cell fates in absence or presence of recipients. The tool consists of an ICEclc reporter variant in which *egfp* is placed directly downstream of the *intB13* integrase gene in the same bicistronic transcription unit (Fig. 1A, Table S1, and Fig. S1). Initiation of transcription of the integrase gene is a key feature of donor cells in which ICE becomes active (25) and would be visible as single cells producing EGFP above background levels (Fig. S2) (36). However, upon excision and ICEclc-DNA circularization, a stronger constitutively transcribed promoter (P_{circ}) is placed upstream of *intB13* (30), which we expected would be reflected in higher EGFP production (Fig. 1A). *Pseudomonas putida* carrying a single integrated copy of such ICEclc-*intB13-egfp* (strain 4611) expressed EGFP in $5.9 \pm 0.4\%$ of individual cells after 72 h in stationary phase (Fig. S3A). This is consistent with previously measured proportions of initiated tc cells in stationary-phase cultures of *P. putida* ICEclc and *P. knackmussii* B13, estimated from EGFP expression of single-copy P_{int} -*egfp* insertions located outside ICEclc (17, 37). In *P. putida* carrying the same ICEclc-*intB13-egfp* bicistronic fusion but with an additional deletion of the *mfsR* global negative regulator [strain 4612 (27)], $45 \pm 5\%$ of individual cells expressed EGFP after 72 h in stationary phase (Fig. S3A). The ICEclc

renewed cell growth (expo), non-tc cells divide normally to microcolonies, and, in stationary phase (stat), a proportion of cells again starts the tc development (tc formers). tc starter cells divide abnormally and cease growth earlier than non-tc cells. (E) Starting condition of a tc starter cell (tcs) and non-tc cells of *P. putida* ICEclc-*intB13-egfp, ΔmfsR* 1 h after inoculation on nutrient surface (EXPO), and the same area with tc formers (tcf) in stationary-phase (STAT) microcolonies after 28 h.

Fig. 2. ICEclc excision and transfer from tc starter cells. (A) EGFP fluorescence development in individual tc starters and daughters (tcs; gray), tc formers (tcf; blue) and non-tc cells (red; Right) of *P. putida* ICEclc-intB13-egfp, $\Delta mfsR$, P_{inR} -mcherry (strain 4673). (Inset) How the slope of EGFP fluorescence increases during at least 150 min (dashed line) is operationally used to define tc starters with ICEclc excision (>1.65 EGFP U·min⁻¹; example yellow trace) or without (example magenta trace). Traces in tc formers are aligned to the point of last cell division ($t = 0$). Further individual tc starter traces are shown in Fig. S5. (B) Box-plot distributions of EGFP production rates among non-tc cells, tc former, and tc starter cells of *P. putida* ICEclc-intB13-egfp (WT, strain 4611), *P. putida* ICEclc-intB13-egfp, $\Delta mfsR$, P_{inR} -mcherry (strain 4673), and *P. putida* ICEclc-intB13-egfp, $\Delta mfsR$, $\Delta attL$ (strain 4755) in absence of recipient. TRA⁺ and TRA⁻ tc starters of *P. putida* ICEclc-intB13-egfp, $\Delta mfsR$ (strain 4612) in the presence of recipient, with confirmed ICEclc transfer or without transfer, respectively. Green asterisk indicates tc formers in presence of recipient with confirmed ICEclc transfer. Dashed line indicates the threshold above which cells are considered to have an excised ICEclc. Letters above box plots indicate statistically significant similarity among groups by multiple-comparison one-way ANOVA, Tukey test. N, number of cells analyzed. Whiskers represent the minimal and maximal values. (C) ICEclc transfer from tcs donors (d) of *P. putida* ICEclc-intB13-egfp, $\Delta mfsR$ (strain 4612) to *P. putida* P_{tac} -mcherry, resulting in two transconjugants (t) receiving ICEclc. Overlay image combined from individual images recording EGFP (green) and mCherry (red). Schematic shows expected color transitions. (D) Single tc donor double transconjugant event. (E) Single tc donor triple transconjugant event. (F) Single-donor ICEclc transfer and integration into *P. putida* with the conditional *attR*-mcherry trap (*r*). PhC, phase-contrast. (G) Stimulation of ICEclc excision in tc starters by addition of 0.1 mM 3-CBA on silica-gel patches (+3-CBA) or without (-). $P = 0.0015$, two-tailed *t* test, equal variance.

variant with the *mfsR* deletion is useful for studying single-cell transfer, as it causes a higher transfer frequency and increases the likelihood of observing such events in live-cell time-lapse microscopy. Strains carrying an additional randomly inserted *mcherry* reporter construct controlled by the P_{inR} promoter (strain 4673), which is also exclusively transcribed in tc cells (17, 26), coexpressed EGFP and mCherry in the same cell (Fig. S3B), confirming that those cells initiate the ICEclc transfer competence process.

To distinguish potentially different states of ICEclc in donor cells (i.e., initiation of *intB13* expression in the integrated state and ICEclc excision), we followed EGFP production over time

by live-cell time-lapse microscopy. Individual cells of *P. putida* carrying WT ICEclc-intB13-egfp or ICEclc-intB13-egfp with the $\Delta mfsR$ mutation were placed on solid agarose surfaces with nutrients in closed microscope chambers (38) and imaged during exponential growth and subsequent stationary phase. Importantly, because cells inoculated on the surface come from liquid precultures that were grown to stationary phase, some of them already initiated the ICEclc transfer competence process (hereafter named tc starters; Fig. 1D). Individual cells visible on the nutrient surface at the start of the experiment can therefore be a tc starter or a non-tc cell, which can be distinguished on the basis

of their EGFP fluorescence level (Fig. 1 *D* and *E*, *EXPO*, and Fig. S2). Dividing non-tc cells reach new stationary phase on the surface after 9–10 h, during which expression of EGFP from the *intB13-egfp* fusion increases again in some cells, indicating initiation of the transfer competence process (Fig. 1 *D* and *E*, *STAT*). In the following, we will name such cells tc forming cells (or tc formers) to distinguish them from tc starters and their daughters (Fig. 1*D*). tc starters divide not at all (Fig. 1*E*) or only a few times, even in the presence of new nutrients (as discussed later).

EGFP production rates in individual cells of *P. putida* ICEcl_c-*intB13-egfp*, Δ *mfsR* (strain 4673), measured from the slope of EGFP fluorescence increase over time for at least 150 min in individual cells (Fig. 2*A*, *Inset*), were statistically significantly higher in tc starter cells and their daughters than in tc formers or non-tc cells ($P < 0.0001$, multiple-comparison one-way ANOVA, Tukey test; Fig. 2*B*). Also, EGFP fluorescence maxima observed during the lifetime of individual cells were statistically significantly higher in tc starters and daughters than in tc formers ($P < 0.0001$; Fig. S4). Visibly, EGFP production rates were also more variable in tc starter cells than in tc formers (Fig. 2*A* and Fig. S5*A*). No significant differences in EGFP production rates could be detected in tc starter cells of *P. putida* with the Δ *mfsR* mutation compared with the WT ICEcl_c-*intB13-egfp* fusion (strain 4611; $P = 0.9898$; Fig. 2*B*). This indicates that, whereas the *mfsR* deletion strongly increases the proportion of tc cells in a population, it does not change the level of ICEcl_c activation in individual cells compared with WT ICEcl_c. The difference in EGFP production rates between non-tc, tc starter, and tc former cells was thus in agreement with our hypothesis that the reporter could detect different ICEcl_c states in donor cells.

ICEcl_c Excision Occurs in Donor Cells Only After Renewed Nutrient Addition. To verify whether higher EGFP expression in tc starters was indeed a consequence of ICEcl_c excision, we compared with a *P. putida* strain with ICEcl_c in which the recombination sequence *attL* (Fig. 1*A*) was deleted (strain 4799). A Δ *attL* mutant cannot recombine between *attR* and *attL* and, therefore, ICEcl_c excision and transfer are strongly impaired (25) (Table 1). As a consequence, *IntB13* and EGFP cannot be expressed from P_{circ} but only from P_{int} (Fig. 1*A*). EGFP production rates and fluorescence maxima from tc starters of the Δ *attL* mutant were indeed significantly lower than those of *P. putida* tc starters with intact *attL* ($P < 0.0001$, multiple-comparison one-way ANOVA, Tukey test; Fig. 2*B* and Fig. S4).

To further confirm that ICEcl_c excision is more prevalent among tc starters than among tc formers, we measured by qPCR the ratio of covalently closed excised ICEcl_c DNA compared with the ICEcl_c-located *clcB* gene in stationary-phase cultures of *P. putida* ICEcl_c-*intB13-egfp*, Δ *mfsR* and in freshly diluted cultures after 4 h growth with 0.1 mM 3-chlorobenzoate [3-CBA; a selective carbon substrate for growth of cells carrying ICEcl_c (39)]. If the hypothesis from live-cell microscopy observations is correct, tc cells in stationary-phase cultures should have proportionally low ratios of excised ICEcl_c DNA (i.e., tc formers), but addition of new nutrients would result in stimulation of excision of ICEcl_c DNA (i.e., tc starters). Indeed, the measured ratio of amplified ICEcl_c circular junction compared with amplified *clcB* fragment was, on average, 1.49 times higher (SD = 0.35; triplicate independent cultures) in exponential than in stationary-phase cultures. This difference seems small but is in agreement with a predicted calculated ratio when considering the difference in growth rate between tc starters and non-tc cells and the average measured number of generations before tc starters stop dividing (Table S2 and Fig. S6). As expected, we were unable to detect by PCR any circular form of ICEcl_c in stationary phase or in exponential cultures of *P. putida* carrying ICEcl_c with the Δ *attL* mutation.

Finally, we followed ICEcl_c transfer from individual *P. putida* donors mixed with ICEcl_c-free recipient cells, expecting that transfer would occur only from donors in which the ICE excises. Transfer can be followed at the single-cell level, because the *egfp* gene is inserted downstream of *intB13* on ICEcl_c, and, upon its entry into a new recipient (temporary), *egfp* expression may occur (as illustrated in Fig. 1*C*). As recipients, we used derivatives of *P. putida* UWC1 constitutively expressing *mcherry* (29) to distinguish them from donor cells or equipped with a conditionally fluorescent “trap” (lighting up after ICEcl_c insertion) (40). Recipient cells producing EGFP were observed exclusively when they had been in direct contact with tc starters or their daughters (57 transfers among 328 observed donor–recipient contacts with *P. putida* ICEcl_c-*intB13-egfp*, Δ *mfsR* in triplicate independent experiments; Table 1), but never with non-tc cells ($n = 202$ observed contacts with recipient cells). Three recipients with increased EGFP fluorescence occurred next to tc formers ($n = 316$ contacts; Table 1). In contrast, no EGFP-producing recipient cells were detected in matings with *P. putida* donors carrying the ICEcl_c Δ *attL* mutant ($n = 90$ tc starter contacts; Table 1). The EGFP production rates of tc starters with confirmed ICEcl_c transfer (Fig. 2*B*, TRA⁺) were statistically indistinguishable from those of tc starters in donor populations alone, but

Table 1. ICEcl_c transfer between *P. putida* donor and recipients

Donor*	Transfer rate [†]	Dividing tcs						Nondividing tcs						tcf	
		With contact to recipient						With contact to recipient							
				No. tcm (cells)		No. tcm (cells)				No. tcm					
		No. at start	No. of contacts [‡]	with transfer	without transfer	No. traco	without contact	No. at start	No. of contacts [‡]	with transfer	No. w/o transfer	No. traco	Without contact	No. of contacts [‡]	No. traco
<i>P. putida</i> (4612)	$12.8 \pm 4.6 \times 10^{-2}$	99	294	28 (93)	19 (73)	43	52	75	34	10	34	14	31	316	3
<i>P. putida</i> (4755)	$2 \pm 2 \times 10^{-4}$	44	39	0	11 (30)	0	33	41	51	0	14	0	27	28	0

Data pooled from independent triplicate live-cell microscopy transfer experiments, each following three to five randomly located areas of observation on the surface. tcf, transfer competent forming cells; tcm, microcolonies formed from tcs; tcs, transfer competent starters; traco, transconjugants (i.e., ICEcl_c in recipient). Number of cases with one transconjugant from one donor, 52 (87%); two transconjugants from a single donor, 2–4 (7%); and three transconjugants from a single donor, 1 (5%).

**P. putida* (4612) is ICEcl_c-*intB13-egfp*, Δ *mfsR*; *P. putida* (4755) is ICEcl_c-*intB13-egfp*, Δ *mfsR*, Δ *attL*. Recipient is *P. putida* UWC1 (2744).

[†]Number of colonies on plates with 3-CBA as sole carbon source plus gentamicin divided by the number of colonies on plates with 3-CBA only.

[‡]Multiple contacts per donor allowed.

higher than of tc starters without observed transfer ($P = 5.6 \times 10^{-5}$, multiple-comparison one-way ANOVA, Tukey test; Fig. 2B, TRA⁺). Also, the rate of EGFP production of the three tc formers with confirmed ICE $_{\text{Ecl}}$ transfer fell within the distribution of TRA⁺-tc starters (Fig. 2B, green asterisk). This confirmed that tc starters with the highest EGFP expression rates are the ones in which ICE $_{\text{Ecl}}$ excises.

Collectively, these results indicate that, although ICE $_{\text{Ecl}}$ transfer competence is initiated in cells in stationary phase (i.e., tc formers), the element predominantly excises when such cells receive fresh nutrients again (i.e., tc starters on the agarose surface or in diluted liquid batch cultures with new 3-CBA). To corroborate this more specifically, we repeated experiments with *P. putida* ICE $_{\text{Ecl}}$ -*intB13-egfp*, Δ *mfsR* cells inoculated on silica-gel surfaces in presence or absence of 0.1 mM 3-CBA. Silica gel, in contrast to agarose, contains no further traces of carbon substrate. Although cells divided more slowly on silica gel than on agarose surface, EGFP production rates among tc starters were significantly higher on silica gel with 3-CBA than without, suggesting that tc starter cells more frequently excise ICE $_{\text{Ecl}}$ in the presence of new nutrients (Fig. 2G).

ICE $_{\text{Ecl}}$ Transfer Competent Donors Display Variable Cell Fates. Given the high variability of EGFP production rates of tc starter cells of *P. putida* carrying WT ICE $_{\text{Ecl}}$ -*intB13-egfp* or its variant lacking Δ *mfsR* (Fig. 2A and B and Fig. S5A), we wondered whether all tc starters actually follow the same cell fate. From EGFP production rates in tc starters with confirmed ICE transfer (Fig. 2B, TRA⁺), we operationally defined a rate of 1.65 EGFP U·min⁻¹ during at least 150 min (on agarose surface, a rate higher than the 25th percentile of the tc starter population with confirmed ICE $_{\text{Ecl}}$ transfer; Fig. 2B, dotted line) to be considered as an individual cell with ICE $_{\text{Ecl}}$ excision. As the average EGFP production rate of tc starters in direct contact with recipient cells but for which no ICE $_{\text{Ecl}}$ transfer could be detected ($n = 27$) was statistically significantly lower than that from confirmed ICE $_{\text{Ecl}}$ transferring tc starters, this suggested that these cells did not excise the ICE. Averaged over all experimentally observed tc starters, the threshold of 1.65 EGFP U·min⁻¹ indicated an overall probability of 0.65 to excise ICE $_{\text{Ecl}}$, but a decreasing likelihood over time to excise for a given tc starter cell (Fig. S6G and H).

Time-lapse transfer experiments also showed further tc starter variability. Although, in most cases, one transconjugant was observed from contact to a tc starter with excised ICE $_{\text{Ecl}}$, in two to four cases, two transconjugant cells arose simultaneously from a single tc starter (Fig. 2D), and, in one case, three transconjugant cells arose (Table 1 and Fig. 2E). In some donor cells, EGFP continued to accumulate even after EGFP fluorescence in the transconjugants started to appear (Fig. S5B, asterisks). This suggests that, at least sometimes, ICE $_{\text{Ecl}}$ is replicated in the donor cell during transfer and that *intB13-egfp* continues to be transcribed. Many donor cells showed, at some point, an abrupt decrease in EGFP levels (Fig. S5), which, for 50% of the cases, corresponded to a loss of cell integrity in phase-contrast images and possible lysis (Fig. S7). In other cases, EGFP levels of donor cells decreased more smoothly (Fig. 2A and Fig. S5) and cells did not visibly lose integrity, suggesting that EGFP expression is turned off as a result of ICE $_{\text{Ecl}}$ reintegrating into the hosts' genome.

As observed previously (25), approximately 40% (SD = 20% in triplicate experiments; Table 1) of tc starter cells did not divide at all (Fig. S5A), whereas the others divided irregularly but, on average, only 2.2 times (SD = 1.1, $n = 18$; Fig. S6A). Frequent lysis occurred among dividing tc starters, probably as a collateral consequence of the ICE $_{\text{Ecl}}$ activation (25) (Fig. S6D and Fig. S7). The average cell division rate of tc starter cells was also slower and more heterogeneous than that of non-tc cells [121 ± 55 min ($n = 115$ cells) vs. 96 ± 9 min ($n = 228$ cells), respectively; Fig. S6C).

Using average EGFP expression values validated here earlier as proxies for ICE $_{\text{Ecl}}$ behavior (e.g., EGFP production rates

greater than 1.65 U·min⁻¹ means ICE $_{\text{Ecl}}$ excision; Fig. 2A), we interpreted the fates of ICE $_{\text{Ecl}}$ in microcolonies formed from individual tc starters mixed with recipients (Fig. 3A and B and Fig. S8). In the example of the microcolony shown in Fig. 3A, ICE $_{\text{Ecl}}$ excised after the first cell division in one of the daughters (d1), but remained integrated in the other (d2). Subsequent division of the cell with excised ICE $_{\text{Ecl}}$ (d1) led to one daughter cell (d1.1) with rapidly increasing EGFP fluorescence levels and confirmed ICE transfer, perhaps even to two recipients (t1 and t2; Fig. 3A). The other daughter cell (d1.2) showed a rapid decrease in EGFP fluorescence (Fig. 3B), and lost contrast in phase-contrast images (Fig. 3A), which would be in agreement with that cell losing the ICE and possibly lysing. Daughter cell d2 divided once more, but ICE $_{\text{Ecl}}$ excised only after cell division in one of the daughters (d2.2), given the increase in EGFP fluorescence in that cell (Fig. 3B, green asterisk). Daughter d2.2 later transferred ICE $_{\text{Ecl}}$ successfully to a single recipient (t3; Fig. 3A). Daughter cell d2.1 displayed a gradual decline of EGFP fluorescence, suggesting arrest of P_{int} expression and degradation of EGFP (Fig. 3B). Analysis of a wider set of microcolonies from tc starters provided further evidence for replication and segregation of ICE $_{\text{Ecl}}$ in daughter cells (Fig. S8A), and cell division even after ICE $_{\text{Ecl}}$ transfer might take place (Fig. S8A). Missegregation of ICE in daughter cells also seemed to occur with occasionally ICE-free cells appearing (Fig. 3B and Fig. S8; note the rapid decrease in EGFP fluorescence in cells e13 and e31 in Fig. S8A). The dynamic fate of ICE $_{\text{Ecl}}$ and tc starters was similar in microcolonies of *P. putida* carrying WT ICE $_{\text{Ecl}}$ -*intB13-egfp*, indicating that the variable cell fates were not a result of the *mfsR* deletion (Fig. S8C).

Robust ICE $_{\text{Ecl}}$ Transfer Despite Variable tc Cell Fates. Despite this highly variable individual cell behavior, ICE $_{\text{Ecl}}$ transfer was quite robust. Among 174 tc starters, 91 contacted recipient cells (alone or one of their daughters after division), resulting in 57 transconjugants (Table 1). In total, 43 transconjugants among 57 (75%) originated from tc starters that had formed a small microcolony (3.0 ± 1.4 cells), whereas 14 (25%) transfer events came from single, nondividing tc starter cells (Table 1). Excision did not lead to ICE $_{\text{Ecl}}$ transfer in all cases, despite visible contact to recipients. Among 27 measured tc starters with contact to recipient cells but without detected transfer (Fig. 2B, TRA⁻), eight displayed EGFP production rates greater than the 25th percentile defined for tc starters with confirmed transfer, suggesting that these cells did excise ICE $_{\text{Ecl}}$ but somehow were unable to successfully deliver ICE $_{\text{Ecl}}$ to the recipient. The success of transfer upon donor-recipient contact in cells with excised ICE $_{\text{Ecl}}$ can thus be estimated as 75% (i.e., 28 TRA⁺ events among 28 + 8 cells with excision). As previously suggested (31), the formation of small tc donor microcolonies is indeed advantageous for ICE $_{\text{Ecl}}$ transfer, given that the 60% of tc starters that could divide contacted almost 10 times more recipients (294 contacts) than the nondividing tc starters (34 contacts), and provided 75% of all transfers (Table 1).

A Model for tc Cell Fate Transitions. To better understand the occurrence of the different cell fates accompanying ICE $_{\text{Ecl}}$ transfer, we developed a model that describes transitions between a limited number of donor cell states, which we defined and parameterized based on experimental observations (Fig. 4A and Fig. S6). The first transformation starts when cells with integrated ICE $_{\text{Ecl}}$ (i.e., non-tc cells) initiate ICE $_{\text{Ecl}}$ transfer competence (i.e., tc cells) during stationary phase when nutrients and carbon substrate become limiting for growth (Fig. 4B, stationary phase). This transformation rate, $t_{\text{non-tc}}$, has a low probability (0.04 for WT ICE $_{\text{Ecl}}$; Fig. S6F) and occurs, on average, after 4–5 h into stationary phase (Fig. S6H), leading to a proportion of approximately 4% tc cells over time (Fig. 4C, stationary phase). When tc cells are again provided with nutrients (Fig. 4B, growth

Fig. 3. Different cell fates in an ICEclc transferring tc starter cell microcolony. (A) Selected time-lapse microscopy images of cell fates in a tc starter microcolony of *P. putida* ICEclc-*intB13-egfp*, $\Delta mfsR$ transferring ICEclc to *P. putida* Ptac-mCherry. PhC, phase-contrast. Arrows point to acquisition of ICEclc by the recipient (transconjugants). Colored outlines on the left represent cell origin (green, tc starter; red, recipient; yellow, transconjugants; gray, other tc starter cell). d1, d1.1, etc., represent tc starter and daughters in B and C. EGFP panels all scaled to maximum intensity of 840, and mCherry panels are autoscaled for intensity. (B) EGFP fluorescence traces of individual cells in the tc starter microcolony. Stars indicate ICEclc excision deduced from the slope of EGFP fluorescence production rate ($>1.65 \text{ U} \cdot \text{min}^{-1}$). Note the rapid decline in EGFP fluorescence in cell d1.2 after 8 h, which occurs after ICEclc excision in the sister cell (d1.1), and thus might represent a case of missegregation and ICE loss. Further traces are shown in Fig. S5B. (C) Deduced genealogy of cell fates and ICEclc transfer. Fig. S8 provides more examples.

phase), their fates become more variable. Non-tc cells divide with a relatively coherent birth rate, $b_{\text{non-tc}}$, whereas tc cells divide more irregularly (b_{tc} ; Fig. S6 A–C). With a probability of 0.65, tc cells excise the ICE (e_{tc} ; Fig. 4A). They can again divide in excised state or lose the ICE and lyse (Fig. S6 D–I). Simulations of population development by stochastic sampling of experimentally derived parameters suggest that this leads to a temporary increase of the total number of tc starters and ICEclc excision, and also to a small increase in the population of cells without ICE (Fig. 4B, growth phase). Proportionally speaking, however, the number of tc starters rapidly decreases as a result of more consistent cell division of non-tc cells (Fig. 4C, growth phase), declining from 4% to an estimated 0.014% after 10 generations (mostly non-tc doublings). Non-tc and tc starter cell numbers counted on time-lapse imaging of *P. putida* ICEclc-*intB13-egfp* WT followed the simulated predictions quite nicely (Fig. 4D), although tc starters seem to persist slightly better than predicted (parameterization was performed on the basis of the $\Delta mfsR$ mutant data). Predictions from simulations using different non-tc to tc cell transformation rates (i.e., $t_{\text{non-tc}}$) suggest that ICEclc fitness markedly decreases if $t_{\text{non-tc}}$ increases from 4% (WT) to 20% (Fig. 4E). Fitness is counted here as the copy number of ICEclc in the donor population plus $0.75 \times$ the number of cells in which ICEclc excises (assuming that these would successfully transfer the ICE to a recipient). Similarly, ICEclc fitness would decrease if tc cells form already during growth phase instead of stationary phase (even at 4% WT transformation rate; Fig. 4E). These predictions would imply that the ICEclc system was selected for low tc cell activation in stationary phase, given the highest fitness.

Discussion

Horizontal gene transfer is a fascinating process in the prokaryotic world, through which large pieces of DNA can be

actively transmitted between cells. Until now, there has been only limited insight into and appreciation for the actual cell fates during transfer of mobile DNA (32, 34), and how these fates determine the ecological and evolutionary success of the types of mobile DNA becoming dominant in bacterial populations. ICEs are still remarkably poorly understood considering their wide distribution in bacterial genomes and importance among conjugative systems (7). The system we study here, ICEclc, is an example of an abundant ICE family in a wide variety of γ - and β -proteobacteria (14, 17), among which are several *P. aeruginosa* isolates (10, 11, 41). Our results enable us to draw a number of conclusions about the basic steps in the behavior of the ICEclc family. First, the process of ICEclc transfer competence development is initiated in a small proportion of stationary-phase cells from non-tc cells (i.e., tc formers). This differentiation to tc formers can be observed in time-lapse microscopy (Figs. 1E and 2A). When tc formers are presented with new carbon substrate, they develop different cell fates (as deduced here from cells we named tc starters). Although we did not experimentally show here that tc formers (appearing in stationary phase) are the same cells as tc starters (at the start of new growth phase), because they appear at different stages in the experimental setup (e.g., Figs. 1E and 2A), modeling suggests that this is the most likely explanation (Fig. 4C). In contrast, it is extremely unlikely that tc starters are senescent cells or persisters that maintain silently in the population and grow out when conditions have become more favorable [like in the case of antibiotic persisters (42, 43)]. We do acknowledge that tc starters can persist over long durations in growth phase (Figs. 1E and 4D), even though they frequently lyse and stop dividing or do not divide at all (Fig. S6 A and D and Fig. S7), and are very rapidly overgrown by dividing non-tc cells (Fig. 4D). However, as previous experiments have shown, elongation and division of such persisting tc cells cannot be induced a

Fig. 4. Stochastic modeling of tc cell and ICEclc fates. (A) Schematic cell fate transitions during growth and stationary phase, following experimental observations. Process parameters stochastically sampled from experimentally derived probability distributions (Fig. S6). (B) Mean predictions (solid lines) plus SD (lighter shading) of subpopulation development from 20 independent simulations in stationary and growth phase during 1,000 min each under starting conditions as indicated. (C) Same data as in B but displayed as the proportion of tc cells in the population over time. (D) Predicted and observed population development of tc and non-tc cells in *P. putida* WT ICEclc-*intB13-egfp* (strain 4611) during growth phase, at the indicated observed starting cell numbers. (E) ICEclc fitness (as ICE copy number in donor populations plus $0.75 \times$ the number of excised tc cells, assuming these can successfully transfer to recipient) after one or five cycles of alternating stationary-growth phases, and as a function of rate and timing of tc transformation ($t_{\text{non-tc}}$). Study cases (i to vii) are shown on the right. Letters indicate statistically significantly different groups (Kruskal-Wallis test, $df = 38$, $P < 0.001$). Data points indicate the mean abundance in 20 independent simulations, with whiskers indicating calculated SDs.

second time (that is, after passage through stationary phase as in Fig. 1D and E) by renewed addition of carbon source (25). The molecular details of this cell arrest are currently unknown.

Contrary to our expectations, in most instances, ICEclc does not excise or transfer from tc formers in stationary phase, but this predominantly (Fig. 2C–F) occurs when these cells receive fresh nutrients (i.e., tc starters; Fig. 1D). Our data strongly suggest that availability of carbon or some other nutrient is needed as a trigger for excision and subsequent transfer. Because at least 40% of tc starters can excise and transfer ICEclc without further cell division (Table 1), we conclude that it is not so much a renewed division cycle after stationary phase that acts as a trigger, but the actual presence of nutrients, as demonstrated by the qPCR data and silica-gel experiments (Fig. 2G). One could imagine that nutrients may be needed to provide sufficient energy to set in motion the complex DNA transfer machinery. As tc donor cells have already initiated the transfer competence cascade in stationary phase, it is a priori unclear how, mechanistically, nutrients can provide the trigger for ICE excision and transfer. Either somehow the tc cells in stationary phase only transcribe but do not translate the ICE mRNAs (which seems unlikely because EGFP is produced in such cells from the combined *intB13-egfp* mRNA), or all necessary transfer proteins and the integrase are produced in stationary-phase tc cells but some other unique factor only becomes available upon new nutrient stimulation that aids the integrase to excise the ICE DNA

and unleashes transfer. These and other questions should be studied in future experiments.

As deduced from our *intB13-egfp* reporter tool, ICEclc behavior is highly variable among individual *P. putida* tc starters, and different cell (and ICE) fates can be inferred (Fig. 4A). Excision is not automatic after initiation of ICEclc transfer competence, but occurs at a probability of 0.65, whereas, in other cells, the ICE remains integrated. In some tc starters, the ICE replicates and correctly segregates copies among daughter cells (concluded from consistently high EGFP production rates in both daughter cells), whereas others seem to lose the ICE upon division (concluded from rapid decrease in EGFP production rate). An estimated 75% of cells with excised ICEclc successfully deliver the ICE upon cell contact to a new ICE-free recipient. Inferred ICEclc replication and correct segregation in some tc starters would be in agreement with accumulating evidence on temporary replication of other ICE after excision using qPCR methods (22, 44). It is tempting to suggest that the variance in EGFP production rates in tc starters with confirmed ICEclc transfer (Fig. 2B, TRA⁺) is a result of different ICE copy numbers in cells, but this cannot be firmly concluded from our reporter tool. Different excised ICEclc copy numbers would be a reasonable assumption to explain the small proportion of tc starters that transfer ICEclc twice or even three times almost simultaneously (Fig. 2D and E and Fig. S5). The mechanisms for multiple transfer from single donor cells is ill understood, but

can contribute to the overall transfer success. It may be the result of a TraI-dependent ICE $_{clc}$ replication after excision analogous to what has been described for ICE $_{SXT}$ from *Vibrio cholerae* (22) and ICE $_{Bs1}$ from *Bacillus subtilis* (44) and/or a consequence of its double origin of transfer (29), which might enable successive rounds of ICE $_{clc}$ DNA processing for conjugative transfer.

Our results demonstrate clearly that tc starter cells, when the ICE $_{clc}$ excision and transfer process has started, undergo highly variable fates, exemplified by limited or arrested cell division and cell lysis. Previous data indicated that this is, at least in part, the result of ICE $_{clc}$ -encoded factors (25). Further observations using cell staining for reactive oxidative species suggested that tc starters are more frequently damaged than non-tc cells (25), which would be in agreement with the idea that such cells are in poor shape. One could thus imagine that the various cell fates of tc starters are not just spontaneous stochastic events but a result of how the ICE tries to manipulate and transform the host cell into a successful transfer machine, with dire consequences for that cell. It is too early to conclude whether this behavior is general for horizontal gene transfer or specific for ICEs from the ICE $_{clc}$ family, as few other conjugative systems have been studied at the single-cell level. Studies on F and R751 plasmid transfer from *Escherichia coli* (33, 34) and ICE $_{Bs1}$ transfer in *B. subtilis* (32) did not particularly describe phenotypic variability among donor cells.

In conclusion, our results provide a unique, fascinating, and unprecedented view on dynamic cell fates during horizontal gene transfer. The robust transfer of ICE $_{clc}$ despite highly variable behavior at the level of individual donors can help to explain the success of distribution of ICE $_{clc}$ -like elements in a wide variety of γ - and β -proteobacteria (14, 17), but our results and modeling also help to understand why ICE fitness benefits from the element keeping a low transfer frequency.

Materials and Methods

Bacterial strains used in this study are listed in Table S1. *E. coli* strains used for plasmid cloning were routinely grown at 37 °C on Luria-Bertani (LB) medium. *P. putida* strains were cultured at 30 °C on LB or 21C minimal medium (MM) (45) complemented with 5 mM 3-CBA or 10 mM sodium succinate. If necessary, antibiotics were added at the following concentrations: ampicillin 100 $\mu\text{g}\cdot\text{mL}^{-1}$, kanamycin 25 $\mu\text{g}\cdot\text{mL}^{-1}$, and gentamicin 20 $\mu\text{g}\cdot\text{mL}^{-1}$.

Targeted chromosomal mutations in *P. putida* (insertion and deletions) were created by the I-SceI counterselection double recombination system as described elsewhere (26, 46). A 752-bp promoterless *egfp* gene was amplified from a derivative of plasmid pJAMA23 (47) and inserted 27 bp downstream of the stop codon of *intB13* (Fig. S1) in *P. putida* carrying WT ICE $_{clc}$ (strain 2737) or ICE $_{clc}$ - $\Delta mfsR$ [strain 4322 (27)], with the ICE inserted in the gene for tRNA-gly5 (40). The resulting strains *P. putida* 4611 and 4612, respectively) were further used to create an 18-bp deletion in *attL* of ICE $_{clc}$ by using the same double-recombination techniques (producing *P. putida* strains 4754 and 4755, respectively). A single copy of a P_{intR} -*echerry* reporter gene cassette was randomly inserted in *P. putida* ICE $_{clc}$ -*intB13*-*gfp* (strains 4669–4672) and *P. putida* ICE $_{clc}$ -*intB13*-*gfp*, $\Delta mfsR$ (strains 4673–4676) using mini-Tn5 delivery, as previously described (17). *P. putida* UWC1 strains containing an *attR*-*mcherry* conditional trap for ICE $_{clc}$ insertion (Fig. 1B) were constructed by cloning the *attR* fragment [formerly named ΔP_{int} -fragment (40)] in front of a promoterless *mcherry* on plasmid pJAMA39 (17). The NotI *attR*-*mcherry* fragment was then recovered and cloned into pCK218 (48), and the resulting plasmid was electroporated into *P. putida* UWC1 to deliver the mini-Tn5 cassette, as previously described (40), to produce strains 5001–5003.

Transfer frequencies of ICE $_{clc}$ from *P. putida* donors to *P. putida* recipients on plates were determined following experimental conditions described previously (27) and are further specified in SI Materials and Methods.

Time-lapse microscopy was performed as described previously with minor changes (37). Briefly, all derivatives of UWC1 carrying ICE $_{clc}$ were pregrown overnight in LB medium supplemented with antibiotics to select for maintenance of their respective traits. An aliquot of 100 μL of this preculture was then transferred in 20 mL MM with 5 mM 3-CBA (without further antibiotics) and allowed to grow for 96 h, after which 1 mL of cells were 2- to 10-fold diluted in MM before being spotted on agarose patches containing 0.1 mM 3-CBA. Recipient cells were grown for 24 h in 20 mL MM with 10 mM sodium succinate before being mixed in a 2:1 (vol/vol) ratio with the 96-h-old donor

cell culture. Cells were not further diluted but were washed once in MM before being spotted onto the agarose patches. Agarose patches were prepared as previously described (27). Silica gel-based patches were prepared by mixing 0.5 mL of twofold concentrated MM with 0.42 mL of a silica-gel solution [composed of 10 g of silicate gel (no. 60738; no. 112926–00-8; Fluka) and 7 g of KOH dissolved in 100 mL Milli-Q water] with or without 3-CBA (at 0.1 mM final concentration). Immediately after addition of 83 μL phosphoric acid solution (fivefold dilution pure orthophosphoric acid), 130 μL was pipetted on a coverslip to create 1-mm-thick patches as described previously (27). Ten arbitrarily defined regions with cells were followed automatically for 50 h with image acquisition every 30 min. Image series were deconvoluted, allowing consecutive automated cell tracking using MetaMorph (series 7.5, MDS; Analytical Technologies) and the mean fluorescence intensities of single cells at all time points were extracted by using an in-house-written Matlab script. EGFP production rates in single cells were calculated by linear regression of periods of increasing EGFP fluorescence values, taking at least six consecutive time measurements into consideration (150 min). The 25th percentile value of EGFP production rates of confirmed ICE $_{clc}$ transferring tc starters (1.65 $\text{U}\cdot\text{min}^{-1}$; $n = 28$) was used as a cutoff, above which a tc cell was considered having excised ICE $_{clc}$. Cells with negative slopes of EGFP fluorescence over time, or for which fewer than six time points were available, and/or with $R^2 < 0.80$, were discarded for statistical analysis. Because the fluorescence intensities of non-tc cells remained constant and low, their slopes were calculated from two random time points by linear regression. *P. putida* P_{tac} -*mcherry* recipients were considered as having acquired ICE $_{clc}$ -*intB13*-*egfp* if EGFP values augmented for at least six consecutive measurement points (150 min) and were higher than the average EGFP of recipients not in contact to tc starters. Live-cell microscopy transfer data were pooled from triplicate independently started agarose patch growth experiments, either with donors alone or with donor-recipient mixtures, using tc starters present on three to five randomly selected image locations for each replicate.

For qPCR, three independent cultures of *P. putida* ICE $_{clc}$ -*intB13*-*egfp*, $\Delta mfsR$ (strain 4612) and *P. putida* ICE $_{clc}$ -*intB13*-*egfp*, $\Delta attL$, $\Delta mfsR$ (strain 4755) were grown for 96 h in 20 mL MM with 5 mM 3-CBA. At this time point, 1 mL of each culture was collected for DNA extraction (stationary phase), whereas another 1 mL was transferred into 20 mL MM containing 0.1 mM 3-CBA. Cells were allowed to grow for 4 h and then collected by centrifuging the 20-mL cultures (early exponential phase). DNA was extracted using the Wizard Genomic DNA purification kit (Promega) and used to quantify by real-time PCR the relative abundances of the *clcB* gene of ICE $_{clc}$ and of *attP* (formed exclusively when ICE $_{clc}$ excises) (40). The primer set for *clcB* amplified a 177-bp fragment (5'-GGTTCAGAGAGCGTGCTTC + 5'-GCGCTGAACCAT-CAAGGTC), whereas the *attP* primer set (5'-CTTCGCTGGCCACCTCGG + 5'-GTGGCGCTCGCTGGAATGA) amplified a 166-bp fragment. Primer set calibration curves were prepared by using 10-fold serial dilutions of DNA obtained from *P. putida* ICE $_{clc}$ -*intB13*-*egfp*, $\Delta mfsR$ grown for 96 h in MM with 5 mM 3-CBA. Real-time qPCR was performed on an ABI Prism Step One Plus Real-Time PCR system (Applied Biosystems) with 5 μL of SYBR Mix Select (Life technologies), 3.2 μL of water, 0.4 μL of each primer (5 μM), and 1 μL of DNA. Reactions were held for 2 min at 50 °C and 2 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 72 °C. Products were verified by melting curves. qPCR efficiencies for both primer sets were above 1.83 and with $R^2 > 0.996$. Samples were diluted such that *clcB* and *attP* threshold cycle-amplification values fell within both calibration curves. The abundance of the *attP* fragment was calculated relative to *clcB* and taking into account the qPCR efficiencies of both primer sets.

A stochastic model was built that predicts the fates of cells and ICE $_{clc}$ during a population growth phase (sufficient nutrients available for cell division) and during stationary phase (no net population growth). Four cell types were assumed (Fig. 4A): (i) cells that have initiated the tc pathway but have not excised the ICE yet (tc), (ii) tc but with excised ICE (excised tc), (iii) tc cells that have lost the ICE (non-ICE), and (iv) cells in which ICE $_{clc}$ remains silent (non-tc). During growth, the different cell types can divide or lyse. In addition, tc cells can excise their ICE and become excised tc cells, and excised tc cells can lose their ICE and become non-ICE cells, as illustrated schematically in Fig. 4A. Because of the need to carry ICE $_{clc}$ for metabolizing the substrate 3-CBA, non-ICE cells are not allowed to divide. Following experimental observations, none of the cells are allowed to divide during stationary phase, but non-tc cells can differentiate to tc cells, which can lyse (Fig. 4A), whereas the lysis rate for non-tc cells is set to zero. Transition rates are determined by stochastic sampling from probability distributions derived from experimental data (Fig. S6). Starting populations for simulations were composed based on experimental data. For example, populations enter stationary phase without any tc cells, and their appearance essentially

follows the differentiation rate from non-tc cells (Fig. 4B). Growth phase starts with an initial population of 1,000 cells with 4% tc cells as for the WT ICE clc situation. Both phases were allowed 1,000 time steps of simulation. We further simulated the effects of various tc differentiation rates and the timing of tc differentiation (stationary phase only or during growth and stationary phases) on ICE fitness after single or multiple growth/stationary phase cycles. Multiple cycles were simulated by proportionally diluting the final abundance of all cell types at the end of the stationary phase to a starting size of 1,000 cells. A new stationary phase started with the number of non-tc cells from the end of the previous growth phase and no tc cells, matching experimental

observations. ICE fitness was scored as the copy number in the donor population plus 0.75 times the number of cells with excised ICE (as these would be able to successfully transmit the ICE to a recipient) at the end of each cycle. The simulations were coded in Matlab R2014b. Statistical significance was inferred on averages from 20 independent simulations by using the Kruskal–Wallis test.

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