Activation of the integrative and conjugative element Tn916 causes growth arrest and death of host bacteria

Emily L. Bean, Lisa K. McLellan, Alan D. Grossman*

Department of Biology Massachusetts, Institute of Technology Cambridge, Massachusetts, United States America

*adg@mit.edu

Abstract

Integrative and conjugative elements (ICEs) serve as major drivers of bacterial evolution. These elements often confer some benefit to host cells, including antibiotic resistance, metabolic capabilities, or pathogenic determinants. ICEs can also have negative effects on host cells. Here, we investigated the effects of the ICE (conjugative transposon) Tn916 on host cells. Because Tn916 is active in a relatively small subpopulation of host cells, we developed a fluorescent reporter system for monitoring activation of Tn916 in single cells. Using this reporter, we found that cell division was arrested in cells of Bacillus subtilis and Enterococcus faecalis (a natural host for Tn916) that contained an activated (excised) Tn916. Furthermore, most of the cells with the activated Tn916 subsequently died. We also observed these phenotypes on the population level in B. subtilis utilizing a modified version of Tn916 that can be activated in the majority of cells. We identified two genes (orf17 and orf16) in Tn916 that were sufficient to cause growth defects in B. subtilis and identified a single gene, yqaR, that is in a defective phage (skin) in the B. subtilis chromosome that was required for this phenotype. These three genes were only partially responsible for the growth defect caused by Tn916, indicating that Tn916 possesses multiple mechanisms to affect growth and viability of host cells. These results highlight the complex relationships that conjugative elements have with their host cells and the interplay between mobile genetic elements.
Tn916 causes growth arrest and cell death in two bacterial hosts: *Bacillus subtilis*, which has been used for many studies of the element; and *Enterococcus faecalis*, a natural host of the element. We demonstrate that these deleterious effects in *B. subtilis* are due, at least in part, to interactions between components of the Tn916 conjugation machinery and a co-resident prophage-like element. Our findings highlight the complex nature of interactions that can occur between an integrative and conjugative element, its host, and other horizontally acquired elements.

Introduction

Integrative and conjugative elements (ICEs), also called conjugative transposons, are mobile genetic elements that contribute to bacterial evolution. Typically, an ICE resides integrated in the chromosome of a bacterial host. Either stochastically or in response to a signal, an ICE can excise to form an extrachromosomal circle. ICE-encoded conjugation machinery (a type IV secretion system, T4SS) can transfer the ICE into a recipient cell in a contact-dependent manner [1–5].

Conjugative elements often carry genes that confer phenotypes to host cells, including antibiotic resistances, pathogenic or symbiotic abilities, and various metabolic capabilities. Conjugative elements were frequently identified based on the phenotypes that they confer to bacterial hosts [reviewed in: [3]]. Advantageous phenotypes conferred by ICEs likely mitigate potential costs of maintaining these elements.

Conjugative elements can also have more complex relationships with their host cells. Some elements encode functions that manipulate host development, growth, and viability (for examples see [6–9]). Excessive mating events can be detrimental to host viability [10,11]. Additionally, interactions between conjugative elements and other horizontally-acquired elements, including phages, can impact a host cell. For instance, pili that are part of some conjugation systems can be targeted by male-specific phages [12–14]. Some phages can prevent conjugation events from occurring [15–17], and at least one ICE can prevent growth of a specific phage via an abortive infection mechanism [18].

Here, we present evidence that the ICE Tn916 possesses a previously unknown ability to cause a growth arrest and kill its host cell. Tn916 was the first described ICE and was identified based on its ability to spread tetracycline resistance between two strains of *Enterococcus faecalis* [19,20]. Tn916 and its relatives have since been found in other Gram-positive bacteria including *Streptococcus*, *Staphylococcus*, and *Clostridium* species [4,21–26], and it is functional in *Bacillus subtilis* [27–32]. Tn916 is regulated, at least in part, by a transcriptional attenuation mechanism that is relieved in the presence of tetracycline or other antibiotics that inhibit translation [4,33–35]. These drugs stimulate excision and transfer of Tn916 [32,33,36,37]. However, Tn916 only activates and excises in ~0.1–3% of cells in a population [32,33,38–40]. Therefore, any effects Tn916 activation has on the host cell would be masked in population-level analyses.

To study the effects of Tn916 gene activation on the population level in *B. subtilis* host cells, we used a hybrid conjugative element that contains the regulatory and recombination genes from a heterologous element and the DNA processing and conjugation genes from Tn916 [41]. Using this hybrid element, we identified two Tn916 genes that are sufficient to cause *B. subtilis* host cells to stop growing. We also identified a gene in the defective phage skin that was required for the growth defects caused by the two Tn916 genes.
We also analyzed the effects of Tn916 on cell growth in single cells using a fluorescent reporter to monitor activation of Tn916. We found that cell growth and division was inhibited in cells with an activated (excised) Tn916. Furthermore, most of these cells died. When activated in its natural host, E. faecalis, Tn916 also caused growth arrest and cell death. We suggest that these growth defects may be a common feature across other bacterial hosts of Tn916 and Tn916-like elements. Our results also indicate that the growth arrest likely functions to limit the spread of the element.

**Results**

Increased activation of Tn916 genes causes defects in cell growth and viability

Tn916, like many conjugative elements, only becomes active and excises from the genome in a small portion (~0.1–3%) of the cells in a population [32,33,38–40]. In previous work, we created hybrid ICEs that contained the DNA processing and conjugation functions of Tn916 (Fig 1A) and the efficient regulatory and recombination (integration and excision) systems encoded by ICEBs1 (Fig 1B) [41]. We refer to this hybrid element as (ICEBs1-Tn916)-H1, or ICE-H1 for short (Fig 1C). ICEBs1 and ICE-H1 can be activated in ~25–90% of the cells in a population by overproduction of the ICEBs1-encoded activator protein RapI. The presence of active RapI in the cell stimulates cleavage of the ICEBs1 repressor ImmR by the anti-repressor and protease ImmA. This causes derepression of transcription from the major promoter in ICEBs1, Pxis, which drives transcription of most of the genes in the element, including those needed for excision, autonomous replication, and conjugation [41–44]. In the hybrid ICE-H1, Pxis drives transcription of the genes from Tn916 that were inserted in place of the ICEBs1 genes and are needed for autonomous replication and conjugation (Fig 1). The ability to activate Tn916 genes needed for DNA processing and conjugation in a large proportion of cells enables population-level analyses of effects of these genes on host cells.

We monitored the growth and viability of host cells that were growing in defined minimal medium under activating and non-activating conditions: xylose was added to induce expression of rapI (from Pxyl-rapI, located at the non-essential amyE locus) and activation of either ICE-H1 (ELC1214) or ICEBs1 (MMB970); tetracycline (2.5 μg/ml) was added to stimulate Tn916 activation. ICE excision events were monitored via qPCR by quantifying the amounts of empty ICE attachment sites in the chromosome relative to a nearby chromosomal locus (Materials and Methods). By two hours after induction, excision had occurred in ~69%, ~95%, and 0.07% of cells containing ICE-H1, ICEBs1, and Tn916, respectively.

Approximately one hour after induction of ICE-H1, growth of the culture stopped as measured by optical density (Fig 2A). The optical density of the culture then declined (Fig 2A), indicating that cell lysis was likely occurring. Indeed, after three hours, there was an approximately 100-fold drop in viable cells in the culture in which ICE-H1 was activated relative to the uninduced culture, as measured by colony forming units (CFUs) (Fig 2B). The combination of the drop in optical density and the drop in CFUs indicated that the majority of cells in the culture were lysing.

In contrast, cells in which ICEBs1 had been induced continued to grow, plateaued at a relatively normal optical density (Fig 2A), and there was no evidence of a large drop in cell viability (Fig 2B). Cultures of Tn916-containing cells also grew normally (Fig 2A) and there was no apparent drop in cell viability (Fig 2B). Of course, even if all of the cells in which Tn916 had become activated (~0.1%) had lost viability, we would not detect this on a population level with the assays used (Fig 2). Based on the effects of ICE-H1 and ICEBs1 on cell growth and viability, we infer that the defects caused by ICE-H1 were either due to increased expression of
Additional experiments demonstrated that the growth arrest and cell death caused by induction of ICE-H1 were not due to loss of some putative protective gene(s) in ICE Bs1. We used a mutant of ICE Bs1 that contained only the ICE Bs1 genes present in ICE-H1. That is, the mutant {ICE Bs1(ΔhelP-ΔcwlT, ΔyddJ-ΔyddM); strain ELC1226} was missing all the ICE Bs1 genes that were also missing in ICE-H1, and also did not contain any genes from Tn916. Activation of this element did not cause a growth arrest or drop in viability (Fig 3A and 3B).
Fig 2. Activation of ICE-H1 causes growth arrest and cell death. Strains containing ICEBs1 (MMB970), Tn916 (CMJ253) or ICE-H1 (ELC1214) were grown in defined minimal medium with arabinose to early exponential phase. Cultures were split into two at an OD of ~0.2 (indicated as time = 0 hours) and the appropriate inducer was added (1% xylose to stimulate rapl expression, or +2.5 μg/ml tetracycline to stimulate Tn916 activation) to one part and the second part was left without induction. Data from four or more experiments (except for the growth curves for ICEBs1 and ICE-H1, time points 0.5, 1.5, and 2.5 h which were from two independent experiments) are presented as averages (A) or individual data points (B), and error bars represent standard error of the mean. A) Growth was monitored by OD$_{600}$ for three hours. Gray lines indicate growth of uninduced cultures. Black lines indicate growth of the induced cultures; ICEBs1 (filled circles); Tn916 (open squares); ICE-H1 (filled squares). Error bars could not always be depicted due to the size of each data point. B) The relative colony forming units per ml (CFUs/ml) of cultures after three hours of element activation was calculated as the number of CFUs formed by the induced culture, divided by that from the uninduced culture (a value of "1" indicates there is no change in CFUs with induction). Significant differences based on P < 0.05 in unpaired two-tailed T-tests include comparisons between ICE-H1 and each of the other elements.

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Fig 3. orf16 and orf17 are involved in the growth arrest caused by ICE-H1. Strains containing ICEBs1-ΔattR (Rep- due to ΔnicK) (closed circles, ELC1095), ICEBs1 (ΔhelP-cwtT, ΔyddJ-yddM) (open circles, ELC1226), or ICE-H1-ΔattR (Rep- due to Δorf20) (closed squares, ELC1076) with the indicated deletion(s) are indicated in the figure. Deletions are indicated by gene name or number and include: Δorf23-22 (open squares, ELC1945), Δorf21 (open hexagons, ELC1916), Δorf19 (open hexagons, ELC1915), ΔardA (stars, ELC1707), Δorf17 (closed downward triangle, ELC1419), Δorf16 (closed upward triangle, ELC1418).
triangles, ELC1420), Δorf17-16 (closed diamonds, ELC1942), Δorf17-orf16 (with lacA::Pxyl-orf17-orf16; plus signs, ELC1550), orf16 (K477E) (asterisks, ELC1899), Δorf15 (open downward triangles ELC1418), Δorf14 (open upward triangles, ELC1708), and Δorf13 (open diamonds, ELC1705). Strains were grown in minimal arabinose medium to early exponential phase. At time = 0 hours, when cultures were at an OD

indicating that the growth arrest was not due to the absence of one or more postulated protective gene from ICEBs1. Together, our results indicate that, when expressed, one or more genes from Tn916 that are present in ICE-H1 cause growth arrest and cell death.

Neither excision nor replication were required for the growth arrest and cell death caused by ICE-H1

We wished to determine which of the genes from Tn916 that are present in ICE-H1 caused cell death. To simplify the analysis, we used an ICE-H1 mutant that was unable to excise from the host chromosome, and unable to replicate, even after activation of element gene expression. We made an excision-defective mutant of ICE-H1 by deleting the “right” attachment site (attR) (Fig 1) that is necessary for element excision. The gene encoding the relaxase (nicK from ICEBs1, orf20 from Tn916 in ICE-H1) was also removed to prevent the relaxase from nicking the origin of transfer (oriT) in the element and initiating rolling circle DNA replication with the element unable to excise from the chromosome. Rolling circle replication from oriT of ICEBs1 that is unable to excise causes a dramatic drop in host cell viability [44,45] and we wished to prevent this contribution to cell death. For simplicity, we refer to this hybrid element as ICE-H1-ΔattR (Rep-) (Materials and Methods), noting that although usually not indicated, it is also missing orf20, the gene that encodes the relaxase.

Similar to the effects of ICE-H1, activation of ICE-H1-ΔattR (Rep-) led to a decrease in cell growth (Fig 3A) and viability (Fig 3B and 3C). This decrease was apparent within one to two hours after activation of ICE-H1-ΔattR (by inducing expression of Pxyl-rapI) (Fig 3A, 3C). By three hours after induction, the relative viability of cells with ICE-H1-ΔattR (Rep-) was ~3000-fold lower than that of cells with the uninduced element (Fig 3B and 3C). The drop in viability caused by ICE-H1-ΔattR (Rep-) was ~30-fold more severe than that caused by ICE-H1 that was capable of excision and replication (Fig 2B). The less severe phenotype caused by ICE-H1 (capable of excision and replication) was likely due, at least in part, to the loss of the extrachromosomal form of this element following excision and then the selective advantage of cells that had lost the element.

Based on these results, we conclude that neither excision, nor nicking by the relaxase, nor rolling circle replication (initiated by the relaxase) are required for growth arrest or cell death. These phenotypes must be caused by other genes in ICE-H1.
Multiple genes in Tn916, including orf16 and orf17, contribute to the growth arrest and killing caused by activation of the ICEBs1-Tn916 hybrid ICE-H1

Using a series of deletions in ICE-H1-ΔattR (Rep-), which cannot excise from the chromosome or initiate autonomous replication, we found that several genes contributed to the growth arrest and cell death phenotypes caused by activation of the element. We monitored the growth of host strains containing ICE-H1-ΔattR (ELC1076), and ICE-H1-ΔattR (Rep-) with deletions of: Δorf23-22 (ELC1945), Δorf21 (ELC1916), Δorf19 (ELC1915), ΔardA (ELC1707), Δorf17 (ELC1419), Δorf16 (ELC1420), Δorf17-16 (ELC1942), Δorf15 (ELC1418), Δorf14 (ELC1708), and Δorf13 (ELC1705). As above, each element could be activated by expression of Pxyl-rapI.

We found that loss of both orf16 (virB4-like; homolog of conE in ICEBs1) and orf17 (virB3-like; homolog of conD in ICEBs1) (Δorf17-16) almost completely suppressed the growth arrest, at least out to four hours after activation of the element, as determined by monitoring the OD of induced cultures (Fig 3A). Loss of orf16 alone had a partial effect and loss of orf17 alone had little or no effect (Fig 3A). These results could indicate that orf16 and orf17 are partly redundant for causing growth arrest. However, because the Orf17 homolog (ConD) from ICEBs1 affects the subcellular location of the Orf16 homolog (ConE) [46], we suspect that these two proteins work together.

No other deletions caused improved growth following element activation (Fig 3A). However, a few genes contributed to cell death, including orf17 and orf16. Strains missing orf17-16, orf21 (encoding the coupling protein virD4-like; conQ in ICEBs1), orf19 (essential for conjugation; same predicted topology as the conC gene product in ICEBs1), or orf15 (virB6-like; conG in ICEBs1) all had an increase in viable cells three hours after activation, relative to that of the parent strain containing ICE-H1-ΔattR (Rep-) (Fig 3B). Notably, no single gene deletion fully restored viability of cells containing ICE-H1-ΔattR (Rep-), indicating that multiple Tn916 genes contribute to death of host cells. We decided to focus on orf17 and orf16 due to their requirement for the growth arrest caused by activation of ICE-H1-ΔattR (Rep-) and their effect on cell viability.

In contrast to the almost complete restoration of cell growth in the Δorf17-16 mutant, there was still a large drop in cell viability (Fig 3B). Deletion of both orf16 and orf17 caused a delay in the drop in viability as measured by CFUs, relative to that caused by ICE-H1-ΔattR (Rep-) (Fig 3C). One hour after activation of the element, the number of viable cells was ~100-fold greater in cells with ICE-H1-ΔattR (Rep-) Δorf17-16 compared to those with ICE-H1-ΔattR (Rep-) (Fig 3C). However, by three hours, the effect of Δorf17-16 was much less pronounced and the drop in CFUs was similar to that caused by ICE-H1-ΔattR (Rep-) (Fig 3B and 3C). The improvement in viability caused by Δorf17-16 was not due to polar effects on downstream genes. When Δorf17-16 was complemented with orf17-16 at an ectopic site (lacA::Pxis orf17-16, ELC1550), the growth defects were completely restored to the levels exhibited by ICE-H1-ΔattR (Rep-) (Fig 3B).

We hypothesized that the effect Orf16 was having (in combination with Orf17) on the host cell might be due to its activity as a VirB4-like ATPase. This was not the case. We monitored the impact of activation of an element containing a mutation affecting the predicted Walker A motif of Orf16 (K477E) that should eliminate ATPase activity. Although the orf16Δ(K477E) mutation abolished conjugative transfer of Tn916 and ICE-H1, the phenotypes caused by the point mutation with respect to cell growth and viability were indistinguishable from those caused by wild type orf16 (Fig 3B).

Our results indicated that together, Orf16 and Orf17 are largely responsible for the arrest in cell growth, and partially responsible for the cell death caused by expression of the conjugation
genes from Tn916 that are present in ICE-H1. Based on these phenotypes, we decided to further analyze the effects of orf16 and orf17 on bacterial cells.

**orf17 and orf16 together are sufficient to cause growth arrest and a drop in viability**

We found that expression of orf16 and orf17 together was sufficient to cause growth arrest in the absence of other Tn916 genes. In strains devoid of any ICEs, we placed orf17, orf16, or orf17-16 together under the regulatory control of Pxis from ICEBs1 at an ectopic site, lacA (strains ELC1494, ELC1491, and ELC1496, respectively). A strain containing the vector with no genes inserted (ELC1495) was used as a control. These strains all contained the genes required for regulation of Pxis (immR, immA, and Pxyl-rapl). We monitored effects of expression of orf17 and-or orf16 on cell growth and viability.

We found that by one hour after expression of orf17 and orf16 together, cell growth had decreased relative to that of no expression or expression of each gene separately (Fig 4A). The arrest in cell growth was similar to that caused by activation of ICE-H1-ΔattR (Rep-), although the decrease in OD was more severe following activation of ICE-H1-ΔattR (Rep-) (Fig 4A). In contrast, expression of orf17 or orf16 individually had little or no effect on cell growth (Fig 4A).

Expression of orf17 and orf16 together caused an approximately 50-fold drop in cell viability three hours after induction of expression (Fig 4B). This decrease in viability was less severe than that caused by ICE-H1-ΔattR (Rep-) (~2,000-fold, Fig 3B), indicating that orf17 and orf16 contribute to the drop in viability following induction of Tn916 genes in ICE-H1, but that other Tn916 genes are also required for the nearly 2000-fold drop in CFUs observed following expression of Tn916 genes in ICE-H1-ΔattR (Rep-).

In contrast to the effects of orf17 and orf16 together, expression of each alone had relatively little effect on cell viability. There was an ~2–3 fold drop in cell viability after three hours of expression, but this occurred in the control that had no inserts (Fig 4B), indicating that this drop in viability likely resulted from the gene regulatory system (Pxyl-rapl, immR, immR, and Pxis).

Together, our results indicate that expression of orf17 and orf16 together, in the absence of any other Tn916 genes, is sufficient to cause growth arrest and cell death of B. subtilis. We suspect that orf17 is needed for the proper expression of orf16. This is by analogy to the homologous genes conD (orf17) and conE (orf16) in ICEBs1 where ectopic expression of conE (orf16) is improved in the presence of the upstream gene conD (orf17), likely due to translational coupling [47]. Alternatively, or in addition, both proteins may be required to interact with one or more host components thereby leading to growth arrest and cell death. In the context of ICEBs1, ConD assists in localizing ConE, a cytoplasmic protein, to the membrane [46]. We suspect that a similar interaction is occurring in the context of Tn916, although we do not know if this interaction is needed for the observed cellular phenotypes.

In contrast to the deleterious effects of Orf17 and Orf16 encoded by Tn916 on cell growth and viability, the homologs encoded by ICEBs1, ConD (19% identity, 35% similarity to Orf17) and ConE (23% identity, 43% similarity to Orf16) do not cause similar phenotypes. From this and previous works, we know that activation of ICEBs1 does not cause growth arrest and death (Fig 3). Furthermore, previously published ectopic expression constructs of ConD and ConE did not cause such effects [47]. Although these homologs almost certainly perform similar functions during conjugative transfer of the two elements, the differences in the sequences enable the Tn916 products to have dramatic effects on host cell physiology. We do not know which parts of the sequence of either protein from Tn916 contribute to growth arrest or cell death.
Fig 4. Orf17-16 are sufficient to cause arrest of cell growth. Strains containing overexpression alleles (indicated in the figure with an upwards arrow) of orf17 (downward triangle, ELC1494), orf16 (upward triangle, ELC1491), orf17-16 (diamonds, ELC1496), or an empty vector (open circles, ELC1495) and a strain containing ICE-H1-ΔattR (Rep-) (squares, ELC1076) were grown in minimal medium with arabinose to early exponential phase. At an OD$_{600}$ of ~0.2 (time = 0), cultures were split into inducing (+1% xylose to stimulate rapI expression) and non-inducing conditions. Data from three or more experiments are presented as averages (A) or individual data points (B), and error bars represent standard error of the mean. A) Growth was monitored by OD$_{600}$ for three hours. Black lines indicate growth of the induced cultures; gray lines (difficult to see as they are clustered in the set of strains at the top of the graph) indicate growth of uninduced cultures. The growth curve of ELC1076 (containing ICE-H1-ΔattR (Rep-)) from Fig 3A is included as reference. Error bars could not always be depicted due to the size of each data point. B) The relative CFUs/ml of cultures after three hours of element induction was calculated as the number of CFUs formed by the induced culture, divided by that from the uninduced culture (a value of “1” indicates there is no change in CFUs with induction). Results from the overexpression of orf17-orf16 were significantly different from the empty vector control based on P < 0.05 in unpaired two-tailed T-tests.

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Host-encoded yqaR is necessary for orf17-16-caused growth arrest and cell death

We set out to identify host genes that are required for the cell death caused by expression of orf16 and orf17 from Tn916. Because expression of orf17-16 causes cell death, we simply isolated suppressor mutations that enable cell survival. We expected to get mutations that prevent expression of functional orf17-16 from Pxis. These could include mutations in orf17-16 themselves, or in the regulatory genes (rapI and immA) needed for inactivation of the repressor ImmR and derepression of Pxis. To reduce the frequency of mutations in these genes, we enriched for survivors in a strain that contained two copies each of orf17-16, rapI, and immA (ELC1760). In addition, we included a Pxis-lacZ fusion that would be derepressed similarly to Pxis-orf17-16. In this way, we could monitor production of β-galactosidase to eliminate mutants in which Pxis could not be expressed.

To ensure that we isolated independent mutants, we grew eighteen separate cultures of ELC1760 and isolated one candidate from each culture. Cells were grown in defined minimal medium (with 1% arabinose) and expression of Pxis-orf17-16 was induced with 1% xylose and grown overnight (approximately 18 hours). Cultures were diluted and this process was repeated 1–2 times to enrich for suppressor mutants (Materials and Methods). Cells were then plated onto LB agar plates under non-inducing conditions and candidate mutants were colony-purified and checked for presence of all antibiotic resistance markers. Additionally, we confirmed these isolates properly activated Pxis-lacZ when streaked on LB plates containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 1% xylose, indicating that the RapI-driven induction of Pxis was functional (and likely orf17 and orf16 were still being expressed). We isolated 18 independent suppressor mutants, one from each of the separate cultures. Genomic DNA from each of these 18 mutants was used for whole genome sequencing to locate chromosomal mutations.

DNA sequencing indicated that 15 of the 18 mutants were cured of skin (sigK intervening), a genetic element that interrupts sigK. sigK encodes the mother-cell specific sigma factor (σK) that is required for sporulation [48,49]. The remaining three mutants each contained a frameshift mutation (either (A)₈₋₇ at nucleotide 50 of 465, or (T)₂₋₆ at nucleotide 450) in yqaR, a gene in skin. Skin is a remnant of a prophage [50] and contains several homologs of genes in PBSX, a co-resident defective prophage in B. subtilis [51]. Although yqaR is encoded between homologs of a PBSX transcription factor (yqaQ) and phage terminase proteins (yqaST), there are no homologs of yqaR in PBSX [51]. Little has been reported about YqaR, although it was identified as a membrane protein found in B. subtilis spores [52]. It is expressed in a variety of growth conditions [53], indicating that normal expression of this gene does not cause growth arrest or cell death.

We reconstructed strains to verify that loss of yqaR or skin suppressed the phenotypes caused by overexpression of orf17-16. These strains contained the xylose-inducible orf17-16 (Pxyl-orf17-16) and a deletion of either skin (ELC1891) or yqaR (AyqaR::cat) (ELC1892). Growth and viability were monitored before and after expression of orf17-16, essentially as described above. The growth and viability of these strains under inducing conditions was indistinguishable from non-inducing conditions (Fig 5A and 5B), indicating that deletion of yqaR or skin suppressed growth arrest and cell killing caused by expression of orf17-16. In other words, yqaR of the skin element was needed for orf17-16-mediated growth arrest and cell death.

We found that yqaR was the only gene in skin needed for the killing caused by expression of orf17-16. Introduction of a copy of yqaR, expressed from its predicted promoter, PyqaR, at an ectopic site (yhdGH) in the absence of skin completely restored the growth defect and cell
Fig 5. Effects of skin-encoded yqaR on growth arrest caused by orf17-16 and ICE-H1. Indicated strains were grown in defined minimal arabinose medium to early exponential phase. At time = 0 hours, when cultures were at an OD_{600} ~0.2, cultures were split into inducing (+1% xylose to stimulate rapI expression and de-repression of orf17-16 or of the indicated ICE hybrid) and non-inducing (no xylose) conditions. Data from three or more experiments are presented as averages (A, C) or individual data points (B, D), and error bars represent standard error of the mean.

A,C) Growth was monitored by OD_{600} for three hours. Black lines indicate growth of the induced cultures; gray lines (some are difficult to see as they are clustered in the set of strains at the top of the graph) indicate growth of uninduced cultures. Error bars could not always be depicted due to the size of each data point.

B,D) The relative CFUs/ml of cultures after three hours of induction of orf17-16 or the indicated element was calculated as the number of CFUs formed by the induced culture, divided by that from the uninduced culture (a value of “1” indicates there is no change in CFUs with induction). In panel B: Significant differences based on P < 0.05 in unpaired two-tailed T-tests include the overexpression of orf17-16 compared to: Δskin, ΔyqaR, and ΔyqaR yhdGH::empty. The small differences apparent in panel D are not statistically significant.

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death caused by expression of orf17-16 (Fig 5A and 5B). This construct also complemented the ΔyqaR mutation (Fig 5A and 5B). Together, these results indicate that yqaR is necessary for effects of orf17-16 on cell growth and viability, and that of all the genes in skin, it is sufficient for these effects.

Deleting yqaR partially relieves growth defects caused by activation of ICE-H1

We wished to determine if yqaR (or skin) contributed to the growth arrest and cell death caused by ICE-H1. If the other Tn916 genes present in ICE-H1 functioned similarly to orf17-16 in affecting cell viability, then loss of yqaR should similarly suppress those effects. However, if yqaR function was limited to orf17-16, then loss of yqaR would only partly suppress the cell death caused by activation of ICE-H1.

We found that deletion of yqaR (or skin) in strains containing the excision- and replication-deficient ICE-H1-ΔattR (Rep-) partially, but not fully, relieved the growth and viability defects caused by activation of the hybrid ICE. We monitored the growth and viability of strains containing ICE-H1-ΔattR (Rep-) with either Δskin or ΔyqaR-cat (ELC1908 and ELC1856, respectively) following element activation. Cell growth was largely restored, although there was a consistent and small decrease in OD relative to that of the uninduced cultures (Fig 5C). The introduction of PyqaR-yqaR into both Δskin or ΔyqaR-cat strains (ELC1911 and ELC1909, respectively) was sufficient to restore the growth defects indicating that the suppressive phenotype was caused by loss of yqaR and that yqaR is the only skin-encoded gene necessary for growth arrest and the decrease in optical density.

In contrast to the effect on cell growth, loss of yqaR or skin only had a minor effect on cell viability following activation of ICE-H1-ΔattR (Rep-) (Fig 5D). The ΔyqaR or Δskin mutants consistently had slightly more colonies (~2-fold) post-induction than the wild type host strain. The strains in which PyqaR-yqaR was introduced to complement the deletions behaved indistinguishably from the wild type host. These results indicate that yqaR does not greatly contribute to the killing effects caused by other Tn916 genes in the context of ICE-H1-ΔattR (Rep-), despite its involvement in growth arrest and killing mediated by orf17-16.

Construction of a fluorescent reporter system to visualize Tn916-activated cells

These heterologous expression systems revealed that activation of Tn916 genes negatively impacts population growth and cell viability. However, these are artificial systems in which Tn916 genes in ICE-H1 are regulated quite differently from that in their normal context in Tn916. Therefore, we wished to evaluate the effects of normal, endogenous activation of Tn916 on host cells. Because Tn916 is only activated in a small fraction of cells, we could not do these analyses on a population level. Instead, we analyzed behavior of single cells in which Tn916 had been activated.

To identify individual cells in which Tn916 had been activated and to monitor potential effects of Tn916 in its native context on cell growth and viability, we generated a fluorescent reporter to track element activation in single cells. We took advantage of the fact that the DNA processing and conjugation genes in Tn916 are not expressed until the element excises from the chromosome and forms a circular intermediate [33]. Circularization allows promoters on the “right” side of the element to be joined with those genes encoded on the “left” side of the element (as drawn in Fig 1A). By inserting gfpmut2 upstream of orf24 in Tn916 (Tn916-gfp), we generated a reporter system in which cells only fluoresce green when the element has been activated and excised (Fig 6A).
Insertion of gfp near the left end of Tn916-gfp did not abolish excision. We found that after 3 hours of growth in the presence of tetracycline (to stimulate excision), Tn916-gfp had excised in ~0.1% of cells, as measured by qPCR and by counting cells that produced GFP. These results indicated that this reporter is a reliable method to monitor Tn916 activation in single cells.

Growth defects caused by Tn916 activation in B. subtilis are observable on the single-cell level

By using this fluorescent reporter system, we found that B. subtilis cells in which Tn916 was activated under its endogenous regulatory system underwent limited to no cell divisions and frequently lysed. Cells containing Tn916-gfp (ELC1458) were grown in a minimal medium to early exponential phase, then treated with tetracycline to stimulate activation. Three hours later, we visualized cells microscopically, tracking cells that had activated Tn916-gfp (green, Tn916-gfp on) and comparing them to cells that had not activated Tn916-gfp using time-lapse microscopy over the course of three hours.

We tracked 34 cells in which Tn916-gfp had excised (GFP on) (Fig 6B, S1 and S2 Videos). Of these 34 cells, 31 (91%) did not undergo any further cell divisions and 3 (9%) divided once.
For comparison, we tracked 76 cells in which Tn916-gfp had not excised (GFP off). Of these 76 cells, only 2 (3%) did not divide and 74 (97%) underwent one or more cell divisions.

We also found that cells containing an activated Tn916 often lysed (S1 and S2 Videos). We used propidium iodide (PI) to monitor the viability of these cells [54]. Propidium iodide only penetrates cells with damaged membranes and is widely used as an indicator of cell death. Of the 34 cells that had activated Tn916-gfp, 91% stained with PI during the course of the experiment (Table 1). In contrast, only 3% of the 76 cells that had not activated Tn916-gfp were PI-positive or had a PI-positive daughter cell by the end of the time lapse. The division frequencies and cell lysis were similar for cells in which Tn916 was already activated (GFP on) prior to placement on the agarose pads compared to those cells in which Tn916 appeared to become activated (GFP-off to GFP-on) while on the agarose pad. Results from either case are combined in Fig 6C and Table 1.

We were concerned that the growth defect and loss of viability that we observed might be due to treatment with tetracycline. This was not the case. Neither growth arrest nor cell death were dependent on the presence of tetracycline. Tetracycline modestly enhances, but is not necessary for Tn916 activation [32,33,35–37]. To confirm that the presence of the antibiotic was not impacting the growth of activated cells, we monitored the growth and viability of Tn916 activated cells without the addition of tetracycline in conditions otherwise identical to those described above. We identified 20 cells that had activated Tn916-gfp (in the absence of tetracycline). Of these 20 cells, 19 (95%) did not divide, and all 20 (100%) lysed during the course of the experiment (Table 1). These results are indistinguishable from those observed with tetracycline added. Because tetracycline modestly enhances Tn916 activation, consequently increasing the number of activated cells to track, it was included for experiments described below.

We found that the growth and viability defects caused by excision of Tn916-gfp from the insertion site between yufK and yufL were not unique to the particular Tn916 genomic insertion site. Tn916 can insert into many sites in AT-rich regions in the bacterial chromosome [40,55–57]. Therefore, we monitored the growth of cells in which Tn916-gfp was located at two different locations on the chromosome, one inserted between ykuC and ykyB (LKM20) and one between nupQ and maeN (LKM18). We observed similar results of limited divisions.

Table 1. Growth defects caused by an active Tn916 in various host strains.

| B. subtilis Tn916-gfp integration site | Tn916 activation state; mutations | # cells | Cells that divided | PI-stained cells |
|--------------------------------------|----------------------------------|---------|-------------------|-----------------|
| yufK-yufL                            | Active                           | 34      | 9%                | 91%             |
|                                      | Non-active                       | 76      | 97%               | 3%              |
|                                      | Active; no tetracycline          | 20      | 5%                | 100%            |
|                                      | Active; Δorf17-16                | 36      | 3%                | 83%             |
|                                      | Active; ΔyqaR                    | 23      | 13%               | 65%             |
| ykuC-ykuB                            | Active                           | 20      | < 5%              | 95%             |
| nupQ-maeN                            | Active                           | 28      | 7%                | 79%             |

*Strains were grown in defined minimal glucose medium to late exponential phase with 2.5 μg/ml tetracycline to stimulate Tn916 excision (unless otherwise indicated). At time = 0 h, cells were spotted on minimal glucose agarose pads containing 2.5 μg/ml tetracycline, 0.1 μM propidium iodide, and 0.035 μg/ml DAPI. Cells were monitored by phase contrast and fluorescence microscopy for three hours. Cells that contained an active (GFP-positive) or non-active (GFP-negative) Tn916-gfp cells were monitored and the number of cell divisions was counted. Cells were counted as dead if either they or any daughter cells turned PI-positive during the 3-hour time lapse.

*Host strains examined: Tn916-gfp was located in 3 different chromosomal locations: between yufK-yufL (ELC1458), ykuC-ykyB (LKM20), and nupQ-maeN (LKM18), and two mutant strains with Tn916-gfp integrated between yufK-yufL with the indicated deletions: Δorf17-16 (ELC1512) and ΔyqaR (ELC1857).

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and frequent lysis for these two insertion sites compared to the insertion between yufK and yufL (Table 1), indicating these results were not dependent on the integration site of Tn916. Together, these results indicate that cells in which Tn916 became activated were unable to divide and lost viability. These results are consistent with those we observed on the population-level using the ICEBs1-Tn916 hybrid (ICE-H1), that activates Tn916 genes in the majority of cells in a population.

Host cells lacking orf17-16 or yqaR have growth defects following element activation

Because yqaR had relatively little effect on viability of cells with an activated ICE-H1 (Fig 5D), we anticipated that it would have little effect on cell death caused by Tn916. Similarly, we were interested in the effects of orf17-16 in the context of Tn916. Under conditions identical to those described above, we monitored the number of divisions and viability of ∆orf17-16 and ∆yqaR Tn916-gfp host cells (ELC1512, ELC1857, respectively). Loss of orf17-16 or yqaR did not drastically increase the number of cell divisions of the activated host cells (Table 1), nor was there a dramatic change in cell viability (PI staining). Overall, these results indicated that these mutations did not enable restored growth or viability of activated cells, although the data from the microscopy assays lack the resolution necessary to detect small changes. These results further support the conclusion that Tn916 has multiple mechanisms to manipulate host cell growth and viability beyond the relationship between Orf17-16 and YqaR.

Deleting yqaR in Tn916 host cells increases conjugation efficiency

The phenotypic interactions between orf17-16 and yqaR indicated that yqaR (and skin) might have some effect on the function of Tn916. For example, yqaR might limit the ability of Tn916 to efficiently move from cell-to-cell. Alternatively, Tn916 might be manipulating the host through yqaR, perhaps enabling the formation of "mating bodies" [9], or the release of DNA [58] that would better enable spread of Tn916.

We found that yqaR limits transfer of Tn916. The mating efficiency of Tn916 from donor cells with a null mutation in yqaR (ELC1851) was approximately 10-fold greater than that of their yqaR+ (CMJ253) counterparts (Fig 7A). However, deleting yqaR in recipient cells (ELC1854) did not cause detectable differences in mating efficiencies compared to yqaR+ recipients (ELC301), indicating that the effects of yqaR are specific to donors. In parallel experiments, we confirmed that no other genes in skin affected the mating efficiency of Tn916. Δskin donor cells (ELC1846) had an increase in mating efficiency similar to that of ΔyqaR donor cells (Fig 7A). Additionally, complementing yqaR under its native promoter at an ectopic locus (yhdGH) restored Tn916 mating efficiencies to WT levels (Fig 7A). These results indicate that the presence of yqaR negatively affects the transfer efficiency of Tn916.

We found that the effects of yqaR on Tn916 conjugation are downstream of the activation step of the element’s lifecycle. Deleting yqaR did not alter the activation frequency of Tn916. Tn916 had excised (and was therefore activated) in 0.79 ± 0.02% of ΔyqaR donors compared to 0.83 ± 0.02% of WT Tn916 donors immediately prior to the start of the mating.

Deleting yqaR had less of an impact on the transfer efficiency of the hybrid conjugative element ICE-H1 than on that of Tn916. ICE-H1 ΔyqaR donors (ELC1843) mated only ~2-fold more efficiently than yqaR+ donors (ELC1213) (Fig 7B). We suspect that the increased activation frequency of ICE-H1 relative to that of Tn916, and consequently increased transfer efficiencies (and perhaps increased transcription of element genes) masks possible effects of yqaR on mating efficiencies.
Fig 7. Effects of yqaR and skin on mating efficiency of Tn916. The indicated strains were grown to early exponential phase in LB medium. Activation of Tn916 (A) was stimulated by adding 2.5 μg/ml tetracycline for one hour prior to mixing with the indicated recipients. ICEBs1 and ICE-H1 (B, C) were activated by addition of 1 mM IPTG for one hour prior to mixing with recipients. Data are shown from three or more independent experiments. Error bars represent standard error of the mean. Typical conjugation efficiencies in these experiments were: Tn916 ~0.0005%, ICE-H1 ~1%, ICE-ΔΔ (ELC1922), Δskin (ELC1846), ΔyqaR PyqaR-Δ916 (ELC1923) were activated by addition of 1 mM IPTG for one hour prior to mixing with recipients. Δskin PyqaR-yqaR donors were mixed with recipients yqaR+ (ELC301) or ΔyqaR (ELC1854). Conjugation efficiencies (the number of transconjugants produced divided by the number of donors applied to mating) were normalized to those calculated for WT Tn916 donors mated into yqaR+ recipients, which were completed in parallel for each experimental replicate. The mating efficiencies of Tn916 from wild-type, ΔyqaR PyqaR-ΔyqaR, and Δskin PyqaR-ΔyqaR donors were significantly different than those from ΔyqaR and Δskin donors based on P < 0.05 in ratio paired two-tailed T-tests. B, C) Indicated donors were mixed with yqaR+ (ELC301) recipients. B) donors were ICE-H1 (ELC1213) or ICE-H1 ΔyqaR (ELC1843). (C) donors were ICEBs1 (JMA168), or ICEBs1 ΔyqaR (ELC1844). Conjugation efficiencies of ΔyqaR donors were normalized to that of the yqaR+ donor in experiments conducted in parallel. The difference in mating efficiency of ICE-H1 (panel B) from the ΔyqaR mutant compared to wild type was consistently about 2-fold, although this was not statistically significant (P = 0.068) based on comparison in a ratio-pair two-tailed T-test. There was no significant difference comparing data from the two strains in panel C.

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Because ICEBs1 activation does not cause growth defects like those elicited by Tn916, we did not expect yqaR to influence ICEBs1 mating efficiencies. Indeed, ΔyqaR ICEBs1 donors (ELC1844) did not exhibit altered mating efficiencies compared to yqaR+ ICEBs1 donors (MMB766) (Fig 7C). This result confirms that yqaR is specifically interacting with Tn916-encoded genes, and not broadly impacting the transfer of conjugative elements.

Together, these results indicate that the YqaR-dependent growth defects caused by Tn916 genes are not beneficial for efficient element transfer. Instead, it appears that B. subtilis encodes a mechanism to limit the spread of Tn916 through a population of cells. However, these results do not elucidate any effects that other Tn916-mediated growth defects may have on transfer efficiency.

Tn916 activation causes growth defects in Enterococcus faecalis

Whereas B. subtilis is a convenient host for analyzing Tn916 (e.g., [27–32]), it is not a natural host. Furthermore, homologs of YqaR are not found in any of its natural hosts (Enterococcus, Streptococcus, Staphylococcus, and Clostridium species). Therefore, we wondered if this killing elicited by activated Tn916 is specific to B. subtilis or might also occur in a natural host.

We found that Tn916 activation-related growth defects and death also occur in Enterococcus faecalis, a natural Tn916 host. We monitored the effects of Tn916 activation in Enterococcus faecalis, the first-discovered host species of Tn916 [19,20] using the same fluorescent reporter system described above. Tn916-gfp was mated into E. faecalis (ATCC 19433) and two independent transconjugants (strains ELC1531 and ELC1529) were isolated to evaluate the effects of activation (excision) of Tn916-gfp from different chromosomal integration sites (Materials and Methods). ELC1531 had a single copy and ELC1529 had two copies of Tn916-gfp integrated in the chromosome (see below). E. faecalis does not grow on the defined minimal medium used for B. subtilis microscopy; instead, we used a rich medium to grow and visualize E. faecalis during live cell imaging. Each E. faecalis strain containing Tn916-gfp (strains ELC1531 and ELC1529) was grown to early exponential phase (Materials and Methods), tetracycline (2.5 μg/ml) was added to stimulate excision of the element, and after an hour of growth with tetracycline, cells were visualized for two hours using time-lapse microscopy (Fig 8A and S3 Video).

Cells in which Tn916 had excised (green) had a decrease in cell divisions and were stained with PI more frequently than neighboring non-activated cells (Fig 8, S3 Video). We tracked 66 cells that were producing GFP (had an excised Tn916-gfp; in strain ELC1531): forty cells (61%) underwent no divisions, 26 cells (39%) underwent one division. Thirty-five cells (53%)
appeared to lose viability based on staining with PI during the time lapse. In contrast, of the 66 non-activated cells that were tracked, 100% underwent one or more divisions and only 2 (3%) became PI-positive or had progeny that became PI-positive.

We tracked 45 activated cells from the other \( E. \) \( faecalis \) isolate (strain ELC1529): 8 (18%) underwent one or more divisions and 98% appeared to lose viability based on staining with PI during the time lapse. Of the 45 non-activated cells that were tracked, 43 (96%) underwent one or more divisions and only 3 (7%) became PI-positive.

The growth defects were most pronounced in ELC1529, which has two copies of \( \text{Tn}916 \) in the chromosome (Materials and Methods). We suspect that having two copies of \( \text{Tn}916 \) exacerbated the growth phenotypes following \( \text{Tn}916 \) activation. It is likely that the activation and excision of one copy of \( \text{Tn}916 \) leads to activation and excision of the second copy \cite{36,59,60}. Thus, having two copies of \( \text{Tn}916 \) in a host cell likely leads to higher expression levels of
detrimental gene(s), which could be deleterious for the host. This result is interesting given that Tn916 does not possess any known mechanisms to prevent acquisition of multiple copies in a single host, such as exclusion systems that are used by conjugative plasmids and some ICEs [10,61,62].

Although we did not identify a homolog of yqaR in E. faecalis, we confirmed that the deleterious interactions between Tn916 and E. faecalis host cells were independent of orf17-orf16. We mated a Tn916-gfp (orf17-orf16) mutant from a B. subtilis donor into E. faecalis (ELC1696; the orf17-16 were provided ectopically in the donor; Materials and Methods). We monitored the growth of 23 activated cells. Twelve (52%) underwent one or more division and 11 (48%) appeared to lose viability based on PI staining. These results are similar to those observed for ELC1531, indicating that deleting orf17-orf16 from Tn916-gfp did not greatly improve growth or viability of host cells following activation. It is possible that these experiments do not possess the resolution necessary to detect growth improvements, as noted for B. subtilis microscopy experiments described above. However, these results highlight that Tn916 possesses mechanisms independent of orf17-orf16 to modulate growth and viability of multiple host species. We suspect that at least some of these mechanisms function similarly in different hosts.

**Discussion**

The experiments described here demonstrate that activation of Tn916 causes growth arrest and cell death, both in B. subtilis and a natural host E. faecalis. We suspect that these effects were previously undetected due to the low activation frequency of the element. We were able to detect the growth arrest and cell death caused by Tn916 by studying a hybrid ICE that can be activated in a large fraction of cells in a population, and by analyzing the low activation frequency Tn916 in single cells using a fluorescent reporter.

Two previous reports contained results indicating that when activated, Tn916 was deleterious to the host cell. We previously noted that the percentage of cells in a population that contained a circular (excised) Tn916 decreased over time, leading us to speculate that excision of Tn916 might cause some deleterious effect on cell growth [32]. Additionally, a previous report found that E. faecalis host cells containing a Tn916 mutant with increased excision frequencies (due to mutations in the regulatory region upstream of tetM) had decreased fitness relative to cells containing wild type Tn916 [63]. The authors hypothesized that the decreased fitness was due to increased production of TetM, possibly slowing protein production, and an additional cost of the hyper-conjugative phenotype of the mutant [63]. We suspect that in both these studies [32,63], the decreased fitness was due to the growth arrest and cell killing described here.

**Interactions between a conjugative element (Tn916) and a defective prophage-like element (skin)**

We found that multiple Tn916-encoded genes contribute to the growth arrest and cell death. Growth arrest and cell death caused by Orf17-16 was dependent on yqaR, a gene found within the defective phage-like element skin. Loss of yqaR (or skin) leads to an increase in Tn916 conjugation, indicating that one function of yqaR might be to limit activity and spread of this conjugative element. These interactions are reminiscent of abortive infection, in which bacterial cells possess a mechanism to kill themselves to prevent spread of an active phage [64]. An intriguing comparison is the abortive infection mechanism encoded by ICEBs1, in which spbK (from ICEBs1) protects host cells from predation by the co-resident prophage SP6 [18]. However, in the case of Tn916 and the defective prophage-like element skin, it is the prophage that appears to be limiting spread of the conjugative element. Following the abortive infection.
analogy, the ability of a host cell to limit spread of Tn916 might protect neighboring cells from killing by Tn916. Alternatively, host cells may limit the spread of Tn916 to limit the sharing of tetM and to possibly outcompete neighboring cells in the presence of tetracycline. Because Tn916 is only activated in a small fraction of cells in a population, this model could be feasible. The interactions (direct or indirect) between the yqaR and orf17-16 gene products represent a type of interaction and perhaps competition between mobile genetic elements that share a bacterial host.

Multiple mechanisms by which Tn916 causes growth arrest and cell death

Beyond the growth arrest and killing mediated by yqaR and orf17-16, Tn916 has other genes that cause host cell death. In the absence of yqaR, there is growth arrest and cell death when Tn916 is activated in B. subtilis. In addition, growth arrest and cell death also occur in E. faecalis, which has no recognizable homologs of yqaR. It is possible that there are functional analogs of YqaR, but we favor a model in which Tn916 influences cell growth and viability through other pathways, both in its natural host E. faecalis and in B. subtilis. We suspect that similar processes occur in other natural hosts, and that close relatives of Tn916 are likely to cause similar phenotypes.

Cell fate and the spread of integrative and conjugative elements

A major question arising from our findings centers around the fate of the transconjugant cells that acquire a copy of Tn916. During conjugation, a linear single-stranded copy of Tn916 is transferred from donor to recipient. Once in the recipient, the DNA re-circularizes and is replicated to form a dsDNA circle, which is the substrate for integration [reviewed in [3]]. The Tn916 genes are presumably expressed from the dsDNA; in particular, the integrase needs to be made. Based on our results, we expect that expression of Tn916 genes would be detrimental to the nascent transconjugants. However, it is clear that Tn916 is able to successfully transfer and produce viable transconjugants, indicating that at least some fraction of nascent transconjugants are able to survive. Perhaps Tn916 is able to integrate and thus halt expression of its detrimental genes in a short time scale that does not compromise the viability of its host cell. The initial acquisition of a conjugative element can be costly to host cell growth, and such a phenotype would not be unique to Tn916; previous reports have demonstrated the costs associated with conjugative element acquisition [e.g., [65–68]]. Future studies may explore the mechanisms of cell survival in transconjugants.

Similar questions apply to other ICEs. For example, ICEclc from Pseudomonas species is activated stochastically in 3–5% of cells in a population, and these cells differentiate into a “transfer competent” state that is characterized by slow growth, decreased viability, and the ability to transfer the element efficiently [9,69]. The genes required for the decreased cell growth and viability do not encode components of the conjugation machinery, but their loss causes a decrease in conjugation efficiency, indicating that the differentiated state is somehow important for efficient transfer of ICEclc [9]. This is in contrast to the situation with Tn916: at least some of the Tn916 genes that contribute to the growth arrest and cell death encode proteins that are part of the conjugation machinery.

More similarly to Tn916, an essential component of the conjugation machinery encoded by the ICE R391, originally isolated from Providencia rettgeri, permeabilizes the host cell membrane, causing death [70,71]. This killing is proposed to function as a back-up mechanism for spread of the element through a population [58,72]. However, the mechanisms by which Tn916 causes growth and viability defects appears to be different. First, the protein in R391 responsible for these phenotypes is not related to any of those encoded by Tn916. Second, for
Tn916, partial alleviation of the growth and viability defects leads to an increase in transfer. Thus, for R391 and ICEclc, the growth arrest and decreased viability stimulate transfer whereas for Tn916, they inhibit transfer. These differences highlight how various ICEs and their hosts have evolved multiple and contrasting mechanisms that impact growth and viability of host cells and spread of the element.

We suspect that other ICEs have similarly complex impacts on their host cells. However, since most ICEs are activated in only a relatively small fraction of cells in a population, these effects are difficult to observe. The ability to activate an ICE in a large fraction of cells and to visualize and analyze individual cells that contain an active ICE should reveal many of the complex interactions that occur between an ICE, a host cell, and other horizontally acquired elements.

Materials and methods

Media and growth conditions

*B. subtilis* cells were grown shaking at 37°C in either LB medium or MOPS (morpholinepropanesulfonic acid)-buffered 1X S7 defined minimal medium [73] containing 0.1% glutamate, required amino acids (40 μg/ml phenylalanine and 40 μg/ml tryptophan) and either glucose or arabinose (1% (w/v)) as a carbon source or on LB plates containing 1.5% agar.

*E. faecalis* cells were grown shaking at 37°C either in an M9 medium, consisting of 1X M9 salts supplemented with 0.3% yeast extract, 1% casamino acids, 3.6% glucose, 0.012% MgSO₄, and 0.0011% CaCl₂ [74,75] or in BHI medium. *Escherichia coli* cells were grown shaking at 37°C in LB medium for routine strain constructions. As appropriate, antibiotics were used in standard concentrations [76]: 5 μg/ml kanamycin, 12.5 μg/ml tetracycline, and 100 μg/ml streptomycin for selection on solid media.

Strains, alleles, and plasmids

*E. coli* strain AG1111 (MC1061 F’ lacIq lacZM15 Tn10) was used for plasmid construction. *Bacillus subtilis* strains (Table 2), except BS49, were derived from JH642, contain the *trpC2 pheA1* alleles [77,78], and were made by natural transformation [76] or conjugation to introduce the indicated ICE. Key strains and newly reported alleles are summarized below.

Δ(rapl-pherI)342::kan was used to select for ICEBs1 during matings as described previously [42]. ΔattR::tet [44] was used to prevent element excision from the chromosome. ICEBs1 ΔnicK [84], and Tn916 Δorf20 [32] prevent nicking and subsequent DNA unwinding of the cognate ICE and were previously described.

*B. subtilis* strains cured of ICEBs1 (ICEBs1*) and the streptomycin resistance allele (str-84) were previously described [42]. ΔcomK::spc in ELC301 replaced most of the comK open reading frame from 47 bp upstream of comK to 19 bp upstream of its stop codon with the spectinomycin resistance cassette from pUS19 [85]. The spc marker was fused with up- and downstream homology regions via isothermal assembly [86] and used for transformation.

The construction of (ICEBs1-Tn916)-H1, a hybrid integrative and conjugative element, was previously described [41]. Essentially, we replaced the DNA processing and conjugation genes of ICEBs1 (helP-cwlT) with those of Tn916 (orf23-orf13) (Fig 1). In this hybrid element, the Tn916 genes are controlled by the promoter Pxis from ICEBs1 (regulated by ImmR, ImmA, and RapI). The integration and excision components (Int and Xis) are from ICEBs1. ICE-H1 is easily and efficiently activated by overproduction of the ICEBs1-encoded activator protein RapI [41].

ICEBs1, (ICEBs1-Tn916)-H1, and complementation constructs were under the regulatory control of Pxis (of ICEBs1) and were activated by overexpression of rapl using either a xylose-
| Strain       | Genotype* (reference[s])                                                                 |
|-------------|------------------------------------------------------------------------------------------|
| BS49        | metB5 hisA1 thr 5-att(yufK)[::]Tn916 att(ykuC-ykyB)[::]Tn916 ICEBs1* [27,79,80]         |
| CMJ253      | att(yufK)[::]Tn916 [81]                                                                   |
| JMA168      | ICEBs1 [Δ(rapl-pherl)342::kan] amyE::[(Pspank(by)-raplI) spc] [42]                       |
| JMA208      | ICEBs1 immR::cat (unstable and used to cure ICEBs1) [42,81]                              |
| JMA222      | ICEBs1-cured [42] (ICEBs1*)                                                              |
| KLM18       | att(nupQ-macN):Tn916-gfp (ICEBs1*)                                                       |
| KLM20       | att(ykuC-ykyB):Tn916-gfp (ICEBs1*)                                                       |
| MMB970      | ICEBs1 Δ(rapl-pherl)342::kan amyE::[(Pxyl-raphI) spc]                                   |
| MO1100      | Δskin [48,82] ICEBs1*                                                                   |
| ELC301      | comK::spc str-84 (ICEBs1*)                                                              |
| ELC1076     | ICE-H1-ΔattR Δorf20* (indicated as ICE-H1-ΔattR (Rep-)) amyE::[(Pxyl-raphI) spc]       |
| ELC1095     | ICEBs1[ΔwicK306 Δ(rapl-pherl)342::kan ΔattR::tet] amyE::[(Pxyl-raphI) spc]              |
| ELC1213     | (ICEBs1-Tn916)-H16* amyE::[(Pspank(by)-raphI) spc]                                     |
| ELC1214     | (ICEBs1-Tn916)-H16* amyE::[(Pxyl-raphI) spc]                                             |
| ELC1226     | ICEBs1[Δ(helP-cwT) Δ(ydd1-yddM) kan] amyE::[(Pxyl-raphI) spc]                           |
| ELC1418     | ICE-H1-ΔattR (Rep-)* Δorf15 amyE::[(Pxyl-raphI) spc]                                    |
| ELC1419     | ICE-H1-ΔattR (Rep-)* Δorf17 amyE::[(Pxyl-raphI) spc]                                    |
| ELC1420     | ICE-H1-ΔattR (Rep-)* Δorf16 amyE::[(Pxyl-raphI) spc]                                    |
| ELC1458     | att(yufK)[::]Tn916-gfpΔ (ICEBs1*)                                                        |
| ELC1491     | lacA::[(Pxis-orf16) mls]* cegD::[(PimmR-(immR-immA) kan] amyE::[(Pxyl-raphI) spc]      |
| ELC1494     | lacA::[(Pxis-orf17) mls]* cegD::[(PimmR-(immR-immA) kan] amyE::[(Pxyl-raphI) spc]      |
| ELC1495     | lacA::[(Pxis-empty) mls]* cegD::[(PimmR-(immR-immA) kan] amyE::[(Pxyl-raphI) spc]       |
| ELC1496     | lacA::[(Pxis-(orf16-16)] mls]* cegD::[(PimmR-(immR-immA) kan] amyE::[(Pxyl-raphI) spc] |
| ELC1512     | att(yufK)[::]Tn916-gfpΔorf17-16Δ (ICEBs1*)                                               |
| ELC1550     | ICE-H1-ΔattR (Rep-)* Δorf17-16 amyE::[(Pxyl-raphI) spc] lacA::[(Pxis-orf17-16) mls]*    |
| ELC1705     | ICE-H1-ΔattR (Rep-)* Δorf13 amyE::[(Pxyl-raphI) spc]                                    |
| ELC1707     | ICE-H1-ΔattR (Rep-)* ΔardA amyE::[(Pxyl-raphI) spc]                                    |
| ELC1708     | ICE-H1-ΔattR (Rep-)* Δorf14 amyE::[(Pxyl-raphI) spc]                                    |
| ELC1760     | lacA::[(Pxis-(orf17-16)] mls[(Pxyl-raphI) spc]* cegD::[(PimmR-(immR-immA) kan) amyE::[(Pxyl-raphI) cat (PimmR-(immR-immA)] (Pxis-(orf17-16)] yhdGH::[(Pxis-lac2) tetM] (ICEBs1*) |
| ELC1830     | Δskin ICEBs1-cured (ICEBs1*) (MO1100 cured of ICEBs1)                                   |
| ELC1843     | (ICEBs1-Tn916)-H16* amyE::[(Pspank(by)-raphI) spc] Δyqar::cat                           |
| ELC1844     | ICEBs1 Δ(rapl-pherl)342::kan amyE::[(Pspank(by)-raphI) spc] Δyqar::cat                   |
| ELC1846     | att(yufK)[::]Tn916 Δskin (ICEBs1*)                                                       |
| ELC1851     | att(yufK)[::]Tn916 Δyqar::cat (ICEBs1*)                                                   |
| ELC1854     | comK::spc str-84 Δyqar::cat (ICEBs1*)                                                    |
| ELC1856     | ICE-H1-ΔattR (Rep-)* amyE::[(Pxyl-raphI) spc] Δyqar::cat                                |
| ELC1857     | att(yufK)[::]Tn916-gfp Δyqar::cat (ICEBs1*)                                              |
| ELC1891     | amyE::[(Pxyl-raphI) spc (PimmR-(immR-immA) ] (Pxis-(orf17-16)] Δskin (ICEBs1*)         |
| ELC1892     | amyE::[(Pxyl-raphI) spc (PimmR-(immR-immA) ] (Pxis-(orf17-16)] Δyqar::cat (ICEBs1*)   |
| ELC1899     | ICE-H1-ΔattR (Rep-)* orf16[K477E] amyE::[(Pxyl-raphI) spc]                              |
| ELC1903     | amyE::[(Pxyl-raphI) spc (PimmR-(immR-immA) ] (Pxis-(orf17-16)] Δskin yhdGH::[(Pyqar-yqarR) kan (ICEBs1*) |
| ELC1904     | amyE::[(Pxyl-raphI) spc (PimmR-(immR-immA) ] (Pxis-(orf17-16)] Δyqar::cat yhdGH::[(Pyqar-yqarR) kan (ICEBs1*) |
| ELC1908     | ICE-H1-ΔattR (Rep-)* amyE::[(Pxyl-raphI) spc] Δskin                                     |
| ELC1909     | ICE-H1-ΔattR (Rep-)* amyE::[(Pxyl-raphI) spc] Δskin yhdGH::[(Pyqar-yqarR) kan]          |
| ELC1911     | ICE-H1-ΔattR (Rep-)* amyE::[(Pxyl-raphI) spc] Δyqar::cat yhdGH::[(Pyqar-yqarR) kan]     |

(Continued)
to be Met39). The misannotated start site lacked an obvious ribosome binding site; we found some (chloramphenicol resistant) by single crossover recombination. Transformants were predicted to overlap with the last 26 bp of orf19 between orf18 and orf20. Delion of Tn916 genes orf23-22 (Δorf23-22) extends from immediately after the orf24 stop codon through the orf22 stop codon. Δorf21 (encoding the coupling protein) removes the first 1272 bp of orf21 and leaves the remaining 114 bp (and oriT) intact. Δorf19 extends from 5 bp upstream of orf19 to 15 bp upstream of ardA (orf18), likely abolishing sos916 which is between orf19 and ardA [32]. ΔardA removes most of the open reading frame, leaving the final 26 bp intact (to leave a previously misannotated orf17 start codon intact).

Of note, we found that orf17 actually begins 88 bp downstream of ardA (it was previously predicted to overlap with the last 26 bp of ardA; the actual start codon was previously predicted to be Met39). The misannotated start site lacked an obvious ribosome binding site; we found

### Table 2. (Continued)

| Strain   | Genotype* (reference[s])                                                                 |
|----------|------------------------------------------------------------------------------------------|
| ELC1915  | ICE-H1-ΔattR (Rep-) Δorf19 amyE::[(PxyI-rapI) spc]                                      |
| ELC1916  | ICE-H1-ΔattR (Rep-) Δorf21 amyE::[(PxyI-rapI) spc]                                      |
| ELC1918  | amyE::[(PxyI-rapI) spc (PimmR-(immR-immA) (Pxis-(orf17-16)))]ΔyqaR::cat yhdGH::kan (also indicated as ΔyhdGH::empty in contrast to ELC1922) (ICEBs1°) | |
| ELC1922  | att(yufK)::Tn916 ΔyqaR yhdGH::[(PxyI-rapI) kan] (ICEBs1°)                                |
| ELC1923  | att(yufK)::Tn916 Δkin yhdGH::[(PxyI-rapI) kan] (ICEBs1°)                                |
| ELC1942  | ICE-H1-ΔattR (Rep-) Δorf17-16 amyE::[(PxyI-rapI) spc]                                   |
| ELC1945  | ICE-H1-ΔattR (Rep-) Δorf23-22 amyE::[(PxyI-rapI) spc]                                   |

*B. subtilis strains, except BS49, are derived from JH642 (AG174) and contain the trpC2 pheA1 alleles (not written in the table) [77,78]. Genotypes indicate if a strain contains ICEBs1, Tn916, or an ICEBs1-Tn916 hybrid (ICE-H1). Many strains are cured of ICEBs1, indicated as ICEBs1°. Original Tn916 gene names (orf1-24) are used as appropriate.

°(ICEBs1-Tn916)-H1 expanded genotype: ICEBs1[ΔhelP-yddM::(Tn916(orf23-orf13) kan)] [41].

ICE-H1-ΔattR (Rep-) expanded genotype: ICEBs1[ΔhelP-yddM::(Tn916(orf23-orf21, orf19-orf13) kan) ΔattR::tet].

This is essentially (ICEBs1-Tn916)-H1, containing the indicated genes from Tn916, except that attR and orf20 are deleted. orf20 encodes the relaxase needed for nicking and replication and the element is indicated as (Rep-).

°Tn916-gfp contains gfpmut2 between attL and orf24.

°Pxis-driven alleles use the Pxis promoter from ICEBs1, which is repressed by ImmR and activated by the metalloprotease ImmA and the cell-signaling receptor RapI [42,43,83].

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inducible (amyE::[(PxyI-rapI) spc]) [47] or an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible (amyE::[(Pspank(ry)-rapI) spc]) [42] copy of rapI. When needed, ICE-cured strains contained cgeD::[(PimmR-(immR-immA) kan] [43] for regulation of Pxis used to drive expression of various genes.

Tn916 host strain CMJ253 contains a single copy of Tn916 between yufK and yufL [81] (at GAAAGGACT TTTTTATATG AAAAATACTT, where the underlined nucleotides indicate the Tn916-chromosome junction). It was generated by natural transformation of MJA222, a JH642-derived strain that was cured of ICEBs1 [42], with genomic DNA from BS49 [27,79,80], selecting for tetracycline resistance from Tn916, as previously described [32,81].

Unmarked deletions were generated for Tn916 genes orf23-orf13 in the context of the hybrid element ICE-H1. Briefly, flanking homology regions were amplified for each deletion and inserted by isothermal assembly into pCAL1422, a plasmid containing E. coli lacZ and cat in the backbone, cut with EcoRI and BamHI [87]. The resulting plasmids were used to transform an appropriate B. subtilis strain, selecting for integration of the plasmid into the chromosome (chloramphenicol resistant) by single crossover recombination. Transformants were screened for loss of lacZ and checked by PCR for the desired deletion. The deletion boundaries are described below.

Deletion of Tn916 genes orf23-22 (Δorf23-22) extends from immediately after the orf24 stop codon through the orf22 stop codon. Δorf21 (encoding the coupling protein) removes the first 1272 bp of orf21 and leaves the remaining 114 bp (and oriT) intact. Δorf19 extends from 5 bp upstream of orf19 to 15 bp upstream of ardA (orf18), likely abolishing sos916 which is between orf19 and ardA [32]. ΔardA removes most of the open reading frame, leaving the final 26 bp intact (to leave a previously misannotated orf17 start codon intact).
that an ectopic expression allele using the orf17 “downstream” start site was able to restore mating for a donor strain containing (ICEBs1-Tn916)-H1(Δorf17), which could not detectably mate.

Δorf17 extends from 78 bp upstream of the orf17 start codon and leaves the last 14 codons intact. orf16 removes codons 10–804 (of 815 total). Δorf15 removes codons 6–716 (of 754 total), based on the sequence for Tn916 in Enterococcus faecalis DS16 (GenBank U09422.1). However, orf15 in Tn916 from BS49 contains a cytosine insertion resulting in a 725-amino acid protein [79]. This frameshift was removed in the deletion, allowing codons 1–5 to be fused with the originally annotated codons 716–754. Δorf14 removes codons 32–325 (of 333 total).

Ectopic expression alleles controlled by Pxis to test the sufficiency of Tn916 genes to cause growth defects were cloned at lacA, as previously described [7]. Briefly, orf16, orf17, or orf17-16 together were fused to Pxis, an MLS-resistance cassette (with its own promoter), and up- and downstream homology arms were combined via isothermal assembly and transformed into B. subtilis, selecting for acquisition of MLS resistance. A lacA:[(Pxis-empty) mls] with no gene downstream of Pxis, was constructed similarly and was used as a control.

ELC1760 was used to screen for suppressors of the cell death caused by expression of orf17-16. The screen was done in a strain with two copies of orf17-16 and two copies of each of the genes required for regulation (immR, immA, and rapI). Having two copies of each gene virtually eliminated suppressor mutations related to orf17-16 expression because the frequency of mutations in both copies of a duplicated gene would be exceedingly low. Previous constructs were used to generate additional alleles. lacA:[(Pxis-orf17-16) mls (Pxyralp) spc] was constructed by inserting [(Pxyralp) spc] into the existing lacA:[(Pxis-orf17-16) mls] allele, selecting for spectinomycin resistance. amyE:[(Pxyralp) cat (PimmR-(immR-ImmA) (Pxis-orf17-16))] was constructed by inserting cat, PimmR-(immR-ImmA), and Pxis-(orf17-16) into amyE:[(Pxyralp) spc] and selecting for chloramphenicol resistance.

orf17-::cat is a deletion-insertion replacing yqaR from 18 bp upstream of the start of the yqaR open reading frame to 48 bp upstream of its stop codon with the chloramphenicol resistance cassette from pGEMcat [88]. Fragments were joined via isothermal assembly and used for transformation. A B. subtilis strain cured of the skin element (leaving behind intact sigK) was obtained from the Losick lab {askin allele described in [48,82]}. yhdGH:[(PyqaR-yqaR) kan] is an insertion of yqaR, from 275 bp upstream of the open reading frame through its stop codon and apparently containing its native promoter. yqaR and kan were cloned between yhdG (bcaP) and yhdH. The construct was inserted 19 bp downstream of yhdG and 98 bp upstream of the yhdH start codon such that yqaR and kan are in the opposite orientation of yhdG and yhdH. The transcriptional terminator from between serA and aroC (from 2 bp downstream of the serA stop codon to 6 bp downstream of the convergent aroC stop codon) was amplified from the B. subtilis chromosome and inserted upstream of yqaR to insulate it from possible transcription from upstream sequences.

Tn916-gfp was generated as a reporter to monitor Tn916 activation in single cells. It is an unmarked insertion of promoterless gfpmut2 29 bp upstream of orf24. gfpmut2 was cloned into pCAL1422 (described above) to generate pELC1329. This plasmid was used to generate B. subtilis strain ELC1458, which contains a copy of Tn916-gfp between yufL and yufK. In this context, gfpmut2 (along with the rest of the DNA processing and T4SS genes) will not be expressed until the element has excised from the chromosome and circularized [33]. To
confirm that the growth defects observed upon activation of Tn916-gfp in ELC1458 were not due to this particular integration site of Tn916, this element was subsequently mated into JMA222 (which lacks Tn916 and ICEBs1) to isolate strains LKM18 and LKM20, which contained Tn916-gfp at different chromosomal sites: between mutQ-maeN (TTAGTTTTTAACTTTAAAATATGGAATG) and between ykuC-ykyB (CAGGTTAAAAATGCGCTTTTTTTCTTAGAA), respectively. New integration sites were mapped by arbitrary PCR, as previously described [41,89,90].

Tn916-gfp was transferred via conjugation from B. subtilis donors into E. faecalis (ATCC 19433) recipients under standard mating conditions (described below). Briefly, B. subtilis Tn916-gfp donors were D-alanine auxotrophs (Δalr::cat) and the absence of D-alanine was used as a counter-selection against donors when selecting for E. faecalis Tn916-gfp transconjugants [41,89]. To transfer Tn916 (Δorf17-orf16) into E. faecalis, the Δorf17-16 deletion mutation was complemented with a copy of orf17-16 elsewhere in the donor genome.

Tn916 insertion sites were identified by arbitrary PCR or inverse PCR, similar to previously described methods [32,44]. Briefly, for inverse PCR, chromosomal DNA was digested with either PacI or AseI/NdeI restriction enzymes and then ligated to circularize the fragments. The following primer pairs were used to amplify and sequence the Tn916-chromosome junctions: oLM177 (5’- AACGCTTCGT TATGTACCCT CTG) and oLM178 (5’–ACCACTTCTGACAGCTAAGA CATG) for PacI digested DNA; oLW443 (5’–CTCTACGTCG TGAGAATCC) and oLW209 (5’–TTGACCTTGA TAAAGTGTGATAAGTCC) for AseI/NdeI digested DNA. Integration sites were mapped to the following chromosomal regions of the E. faecalis genome (Accession number: ASDA00000000.1).

We made two different E. faecalis strains containing Tn916-gfp and one that contained Tn916-gfp Δ(orf17-orf16) and determined the site of each insertion. The location of each insertion was based on the sequence annotation of E. faecalis KB1, accession number: CP015410.1. For genomic context, thirty bases of sequence are shown, and the underlined nucleotides indicate the predicted Tn916-chromosome junction.

ELC1529 has two copies of Tn916. One was between citG and a gene encoding a surface protein (SP), att(citG_SP)::Tn916-gfp: (AACCGCTGTC GCCTTTTTTT ATGAAATTTT). The second was between genes encoding a hypothetical protein (HP) and a cold shock protein (CSP), att(HP_CSP)::Tn916-gfp: (TTTCTTGTTCT TTTTTTAT AAAAAAAC).

ELC1531 has a copy of Tn916 between genes encoding a predicted ABC transporter (ABC) and an acyl carrier protein (ACP), att(ABC_ACP)::Tn916-gfp: (TTTTTTACAT GTATGATTTT TTTTACAAAA).

ELC1696 contained Tn916 Δorf17-orf16 between genes encoding an alpha/beta hydrolase (ABH) and a hypothetical protein, att(ABH_HP)::Tn916-gfp Δ(orf17-orf16): (TCTTTTTTTTTTTACAAAAAATACAGAAATT).

**Growth and viability assays**

*B. subtilis* strains were grown in defined minimal medium with 1% arabinose as a carbon source to early exponential phase. At an OD$_{600}$ of 0.2, the cultures were split into activating or non-activating conditions: 1% xylose was added to stimulate activation of ICEBs1, (ICEBs1-Tn916)-H1, or ectopic expression constructs; 2.5 μg/ml tetracycline was added to stimulate activation of Tn916. The number of colony forming units (CFUs) was determined immediately prior to activation, and at one or more time points (typically three hours) after activation in induced and non-induced cultures. “Relative viability” was calculated as the number of CFUs present in the induced culture divided by the number of CFUs present in the non-induced culture. OD$_{600}$ was monitored every 30 minutes for four hours post-induction.
Excision assays

qPCR was used to monitor excision (and therefore activation) of Tn916, ICE-H1, and ICEBs1, as previously described [32,41]. Briefly, gDNA of ICE host strains was harvested using the Qiagen DNeasy kit with 40 mg/ml lysozyme. The primers described below were used to quantify the presence of the empty ICE attachment site, normalized to a nearby chromosomal locus that is present in every cell.

For Tn916 excision assays (integrated between yufK and yufL), we used previously described primers [32]: oLW542 (5’- GCAATGCGAT TAATACAACG ATAC) and oLW543 (5’- TCGAGGCATC CATCATACT AC) amplified the empty chromosomal attachment site (att1). oLW544 (5’- CCTGCTTGGG ATTCTCTTTA TC) and oLW545 (5’- GTCATCCTTG CACTTCTC T) amplified a region within the nearby gene mrgP.

For ICE-H1 and ICEBs1 (integrated at trnS-leu2), oMA198 (5’- GCCTACTAAA CCAGCCACCAAC) and oMA199 (5’- AAGGTGGTAA ACCCTTGG) amplified the empty chromosomal attachment site (attB). oMA200 (5’- GCAAAGCAGTC ACAAAAGGT T) and oMA201 (5’- AGCGTAAATC SCTGCAAAG) amplified a region within the nearby gene, yddN.

qPCR was performed using SsoAdvanced SYBR master mix and the CFX96 Touch Real-Time PCR system (Bio-Rad). Excision frequencies were calculated as the number of copies of the empty chromosomal attachment sites (as indicated by the Cp values measured through qPCR) divided by the number of copies of the nearby chromosomal locus. Standard curves for these qPCRs were generated using B. subtilis genomic DNA that contained empty ICE attachment sites and a copy of the nearby gene (yddN or mrgP). DNA for the standard curves was harvested when these strains were in late stationary phase and had an oriC/terC ratio of ~1, indicating that the copy numbers of these targets were in ~1:1 ratios.

Suppressor screen

Eighteen independent cultures of ELC1760, which contains two copies of orf17 and orf16 (described above, Table 2) were grown in defined minimal medium (with 1% arabinose). In early exponential phase, expression of orf17 and orf16 was induced with 1% xylose and cultures were grown overnight (approximately 18 hours) until they became dense. Cultures were back-diluted and this process was repeated once to enrich for suppressor mutants. Some cultures were back-diluted a second time to further enrich and increase the chances of isolating suppressors. Suppressors were colony-purified, and checked for presence of all antibiotic resistance markers. Additionally, we confirmed these isolates properly activated Pxis-lacZ when streaked on LB plates containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 1% xylose, indicating that the RapI-driven induction of Pxis was still working properly (and likely orf17 and orf16 were still being expressed).

Genome resequencing

Each suppressor mutant was grown in a minimal medium containing 1% glucose to an OD600 ~1.5–2. Cells were harvested, and DNA was extracted using a Qiagen 100 G tips purification kit. Sample preparation, including DNA shearing using a Covaris ultrasonicator, size selection (aiming for ~500 bp), adapter ligation, and paired-end read sequencing (300nt + 300nt) on an Illumina MiSeq were performed by the MIT BioMicro Center. Reads were mapped to the B. subtilis JH642 chromosome (Accession number: CP007800) [78], as previously described [91].

Mating assays

Mating assays were performed essentially as described previously [42]. Briefly, donor strains containing Tn916 (tetracycline-resistant), (ICEBs1-Tn916)-H1 (kanamycin-resistant), or
ICEBs1 (Δ(rapI-phrI)::kan), or derivatives were grown in LB medium to early exponential phase. At an OD$_{600}$ ~0.2, Tn916 activation was stimulated with 2.5 µg/ml tetracycline; ICE-H1 and ICEBs1 activation was stimulated with 1mM IPTG (via the Pspank(hy)-rapI allele). After one hour induction, donor strains were mixed in a 1:1 ratio with streptomycin resistant recipient cells (5 total ODs of cells) and filtered. Mating filters were placed on a 1X Spizizen’s salts [76] 1.5% agar plate at 37˚C for one hour. Cells were then harvested off the filter and plated on selective media to detect ICE transfer. The number of donor (tetracycline or kanamycin resistant), recipient (streptomycin resistant), and transconjugant (tetracycline/streptomycin resistant or kanamycin/streptomycin resistant) CFUs were determined both pre- and post-mating. Conjugation efficiency was calculated as the percentage of transconjugant CFUs per donor cell at the start of mating. Conjugation efficiencies were normalized to those of the wild type matings performed in parallel. Typically, conjugation efficiencies were as follows: Tn916 ~0.0005%, ICE-H1 ~0.5%, ICEBs1 ~1%.

Time-lapse microscopy and analysis

*B. subtilis* and *E. faecalis* cells were grown to early-exponential phase in the appropriate medium. When applicable, Tn916 activation was stimulated with 2.5 µg/ml tetracycline. After a 1–3 hour induction, cells were transferred to an agarose pad (1.5% UltraPure agarose; Invitrogen) containing growth medium, 0.1 µM Propidium iodide, DAPI (0.035 µg/ml for *B. subtilis*; 0.5 µg/ml for *E. faecalis*), and either 2.5 µg/ml tetracycline for Tn916 activation or 1% xylose for expression of orf17-orf16-gfp. The agarose pad was placed in an incubation chamber, which was made by stacking two Frame-Seal Slide Chambers (Bio-Rad) on a standard microscope slide (VWR). Cells were then grown at 37˚C for 2–4 hours while monitoring growth. Time-lapse images were captured on a Nikon Ti-E inverted microscope using a CoolSnap HQ camera (Photometrics) using a Nikon Intensilight mercury illuminator and appropriate sets of excitation and emission filters (Chroma; filter sets 49000, 49002, and 49008). ImageJ software was used for image processing.

Supporting information

**S1 Video. Growth arrest and death of *B. subtilis* cells with an activated Tn916.** Cells containing Tn916-gfp integrated in the *B. subtilis* chromosome between yufK-yufL (ELC1458) were grown in defined minimal glucose medium to late exponential phase with 2.5 µg/ml tetracycline to stimulate Tn916 excision. At time = 0 h, cells were spotted on minimal glucose agarose pads containing 2.5 µg/ml tetracycline, 0.1µM propidium iodide, and 0.035 µg/ml DAPI. Cells were monitored by phase contrast and fluorescence microscopy for three hours. A representative video from these experiments that highlights a single Tn916-activated cell undergoing lysis is shown here. GFP (green) was produced in cells in which Tn916 was activated and excised from the chromosome. Propidium iodide (red) indicates dead cells. (AVI)

**S2 Video. Growth arrest and death of *B. subtilis* cells with an activated Tn916.** As in S1 video, cells containing Tn916-gfp integrated in the *B. subtilis* chromosome between yufK-yufL (ELC1458) were grown in defined minimal glucose medium to late exponential phase with 2.5 µg/ml tetracycline to stimulate Tn916 excision. At time = 0 h, cells were spotted on minimal glucose agarose pads containing 2.5 µg/ml tetracycline, 0.1µM propidium iodide, and 0.035 µg/ml DAPI. Cells were monitored by phase contrast and fluorescence microscopy for three hours. A representative video from these experiments highlighting several Tn916-activated cells exhibiting growth defects and undergoing lysis is shown here. GFP (green) was
produced in cells in which Tn916 was activated and excised from the chromosome. Propidium iodide (red) indicates dead cells.

(AVI)

S3 Video. Growth arrest and death of *E. faecalis* cells with an activated Tn916. *E. faecalis* cells containing two copies of Tn916-<em>gfp</em> (ELC1529) were used to monitor effects of Tn916 activation. Cells were grown in a rich M9 medium (Methods) to late exponential phase with 2.5 μg/ml tetracycline to stimulate Tn916 excision. At time = 0 h, cells were spotted on rich M9 medium agarose pads containing 2.5 μg/ml tetracycline, 0.1 μM propidium iodide, and 0.5 μg/ml DAPI. Cells were monitored by phase contrast and fluorescence microscopy for two hours. A representative video monitoring ELC129 cells with activated Tn916-<em>gfp</em> (GFP-positive) is shown here. The black arrow in the final frame indicates a PI-stained, GFP-positive cell (appears red or reddish-yellow).

(AVI)

S1 Data. Underlying raw data for experiments presented in the figures. The excel spreadsheet contains the underlying data for the experiments presented in each of the figures.

(XLSX)

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Author Contributions

Conceptualization: Emily L. Bean, Alan D. Grossman.

Funding acquisition: Alan D. Grossman.

Investigation: Emily L. Bean, Lisa K. McLellan.

Methodology: Emily L. Bean, Lisa K. McLellan, Alan D. Grossman.

Project administration: Emily L. Bean, Lisa K. McLellan, Alan D. Grossman.

Supervision: Alan D. Grossman.

Validation: Emily L. Bean, Lisa K. McLellan.

Visualization: Emily L. Bean, Lisa K. McLellan.

Writing – original draft: Emily L. Bean.

Writing – review & editing: Emily L. Bean, Lisa K. McLellan, Alan D. Grossman.

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