Timing of integration into the chromosome is critical for the fitness of an integrative and conjugative element and its bacterial host

Saria A. McKeithen-Mead, Alan D. Grossman

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

* adg@mit.edu

Abstract

Integrative and conjugative elements (ICEs) are major contributors to genome plasticity in bacteria. ICEs reside integrated in the chromosome of a host bacterium and are passively propagated during chromosome replication and cell division. When activated, ICEs excise from the chromosome and may be transferred through the ICE-encoded conjugation machinery into a recipient cell. Integration into the chromosome of the new host generates a stable transconjugant. Although integration into the chromosome of a new host is critical for the stable acquisition of ICEs, few studies have directly investigated the molecular events that occur in recipient cells during generation of a stable transconjugant. We found that integration of ICEBs1, an ICE of Bacillus subtilis, occurred several generations after initial transfer to a new host. Premature integration in new hosts led to cell death and hence decreased fitness of the ICE and transconjugants. Host lethality due to premature integration was caused by rolling circle replication that initiated in the integrated ICEBs1 and extended into the host chromosome, resulting in catastrophic genome instability. Our results demonstrate that the timing of integration of an ICE is linked to cessation of autonomous replication of the ICE, and that perturbing this linkage leads to a decrease in ICE and host fitness due to a loss of viability of transconjugants. Linking integration to cessation of autonomous replication appears to be a conserved regulatory scheme for mobile genetic elements that both replicate and integrate into the chromosome of their host.

Author summary

Horizontal gene transfer contributes significantly to microbial evolution, enabling bacteria to rapidly acquire new genes and traits. Integrative and conjugative elements (ICEs) are mobile genetic elements that reside integrated in the chromosome of a host bacterium and can transfer to other cells via a contact-dependent mechanism called conjugation. Some ICEs contain genes that confer important traits to their bacterial hosts, including antibiotic resistances, metabolic capabilities, and pathogenicity. Central to the propagation of ICEs and other mobile genetic elements is the balance between dissemination to new hosts and maintenance within a host, while minimizing the fitness burden imposed
on their hosts. We describe an underlying regulatory mechanism that allows for an ICE that has entered into a nascent host to replicate and potentially spread to other hosts before integration into the chromosome. Integration occurs shortly after or concomitant with cessation of ICE replication. Disruption of this regulatory link results in premature integration and fitness defects for both the host and the ICE; lethality for the host and reduced spread of the ICE.

Introduction

Horizontal gene transfer (HGT) is a major driver of prokaryotic evolution, enabling the movement of genes from a donor organism to a recipient and subsequent maintenance of horizontally acquired genes within a population. Genes transferred horizontally can confer a wide range of traits, including antibiotic resistances, metabolic capacities, pathogenicity, and symbiosis (reviewed in [1]). Conjugation, a form of HGT, is the contact-dependent transfer of DNA from a donor to a recipient cell. Transfer is through a conjugation machine (a type 4 secretion system, T4SS) that is typically encoded by a conjugative element in a donor cell [2–4].

There are two broad classes of conjugative elements. Integrative and conjugative elements (ICEs, also known as conjugative transposons) normally reside integrated in a host genome and can become activated to excise and express the conjugation machinery [1,5]. Conjugative plasmids are extra-chromosomal and also contain genes needed for their transfer to recipient cells (1–4,6). Conjugative transfer of ICEs and plasmids originates at the origin of transfer (oriT) in the element and is initiated by the element-encoded relaxase protein that nicks and becomes covalently attached to the 5'-end of the nicked strand. The nicked DNA is unwound and linear single-stranded DNA (ssDNA) with the attached relaxase can be transferred from the donor to a recipient through the conjugation machinery [6]. Once inside the recipient, the ssDNA circularizes, and undergoes second-strand synthesis to generate circular dsDNA. ICE genes can now be expressed and the double-stranded circular form of the ICE is a substrate for integration into the host chromosome [1,5].

Unlike that for conjugative plasmids, stable acquisition of an ICE requires integration into the host chromosome where the ICE is then passively propagated as the host replicates and segregates its DNA to daughter cells. Many ICEs are known to undergo limited rounds of autonomous replication and both replication and integration are often essential for stable acquisition of the element [1,7–16]. The requirement for integration for stable acquisition of an ICE is a clear delineation between conjugative plasmids and ICEs and introduces an area for differential selection and evolution of regulatory schemes.

Little is known about the regulatory factors that drive integration and stable acquisition of an ICE after transfer to a new host. Analysis of events in transconjugants has typically relied on end-point readouts of overall mating efficiency to discern the importance of ICE functions, thus obscuring the context in which a particular function is important. A primary challenge for these types of studies is that transfer events are relatively infrequent and, for most ICEs, only a subpopulation of cells participate.

We used ICEBs1 (Fig 1) from Bacillus subtilis to investigate the timing of successful integration and acquisition in transconjugants. ICEBs1 is well-suited for investigating conserved ICE dynamics as it has been well-studied, is easily manipulated, and can be activated in a relatively large fraction (~20%–90%) of cells in the population [17–19] ICEBs1 integrates via site-specific recombination into one site in the B. subtilis chromosome: in trnS-leu2 which encodes a
Fig 1. ICEBs1 integrated versus excised state per regulation with qPCR-assay schematic. A) Map of genes and some of the sites in ICEBs1. Black rectangles at the end of ICEBs1 represent the flanking 60 bp repeats attL and attR, the junctions between the chromosome and ICEBs1 on the left and right, respectively. Genes are represented by horizontal block arrows, not precisely to scale, indicating the direction of transcription. Genes are colored according to function (black = regulation, white = cargo/unknown function, blue = conjugation, grey = replication). Black lines above ICEBs1 indicate oriT, the origin of transfer, and ssoL, one of the single stranded origins of replication. Vertical right-angle arrows indicate promoters. Below the ICEBs1 gene map is a schematic of mutations. Gaps represent deletions (all strains have deletions of rapI-phrI). Insertions (Pxis-cre and Ppen-mNeongreen) are indicated by promoter arrows and horizontal block arrows.

B) General schematic for detection of products of ICEBs1 excision and integration. PCR primers are indicated by arrows with letter a, b, c, d, e, or f. Different combinations of primers can detect different forms of ICEBs1, as indicated. Black rectangles indicate ICEBs1 attachment sites: attP on ICEBs1; attB on B. subtilis chromosome; attL and attR for the integrated element and the junctions with the chromosome. i) Excised ICE in circular form with empty attB in the chromosome. ICEBs1 is represented by a black circle. The B. subtilis chromosome is represented by a blue line. ii) Integrated ICEBs1 in recipient/transconjugants (top) and original donors (bottom). The different flanking sequences in the experimental set-up are shown (blue for recipients/transconjugants; red for donors). PCR primers a and b are specific for the native locus in recipients and transconjugants, and primers e and f are specific for the ectopic locus in (amyE:attB) in donors. For example, the combination of primers c and b would detect integration of ICEBs1 (attR) at its native site attR. C) ICEBs1 regulation. The ICEBs1 gene products RapI (activator), ImmA (anti-repressor and protease), ImmR (repressor) all regulate ICEBs1 gene expression from the major promoter Pxis. Pxis is repressed by ImmR. When RapI is present and active, it causes the protease ImmA to cleave ImmR, thereby causing de-repression of transcription from Pxis [21,22]. ImmR both activates and represses transcription from its own promoter (PimmR) by binding with different affinities to operator sites. Transcription from PimmR is activated at low and repressed at high concentrations of ImmR [18].

https://doi.org/10.1371/journal.pgen.1010524.g001
leucyl-tRNA [17,19]. The specific integration site (attB; attachment site in the bacterium) allows for precise measurements of integration (stable acquisition).

Using a combination of population-based and single-cell assays, we found that integration in transconjugants occurs several generations after transfer of the element. Deletion of xis (encoding the recombination directionality factor that is needed for element excision from the chromosome) caused premature integration in transconjugants and was lethal for most transconjugants. Lethality was caused by integration of an active element that was undergoing autonomous rolling circle replication. Our results highlight the importance of ICE regulation in transconjugants and reveal the important underlying linkage between the cessation of autonomous replication (a plasmid-like function) and integration.

Results

Timing of ICEBs1 transfer and integration in transconjugants

Integration of an ICE into the genome of a new host is essential for the stable acquisition of the element. Following the transfer of ICEBs1 from a donor to recipient, the element is capable of rapidly transferring through a chain of cells [20], indicating that the element is active for a period of time before it integrates into the chromosome. We sought to determine when ICEBs1 integrates into the chromosome of transconjugants.

Strains for distinguishing integration in donors and transconjugants. ICEBs1 has a unique integration site (attB) in the bacterial chromosome. When unoccupied, attB and the flanking sequences are identical in both donor and recipient cells. This makes it virtually impossible to physically distinguish, at the molecular level, integration or excision in transconjugants from that in the initial donor. In order to detect integration in transconjugants separately from the initial donors, we constructed a donor strain (SAM078) in which the site of ICEBs1 integration was in amyE (a nonessential gene often used for inserting cloned genes) rather than its normal position in trnS-leu2. We could then distinguish integrants in the transconjugant population (integration in the normal chromosomal site) from those in the initial donor (at amyE, the ectopic location) using PCR primer pairs that were specific for the sequences flanking attB in transconjugants (Fig 1B). We normalized the number of integrants in the transconjugant population relative to a genomic marker (ydds::mls) that was present in recipients (and transconjugants), but not in donors. yddS is located ~5 kb downstream of attB.

Timing of acquisition of ICEBs1. We measured the conjugation efficiency at various times after the start of mating (Methods). Transconjugants were determined by the number of colony forming units (CFUs) with indicative antibiotic resistances (Methods). Recipients (SAM103) lacked ICEBs1 (ICEBs10). ICEBs1 in donors (SAM078) was activated by overexpression of rapI from an inducible promoter at an ectopic locus (Methods). RapI causes the ICEBs1-encoded protease ImmA to cleave the element repressor ImmR, thereby causing derepression of ICEBs1 (Fig 1C) [17,18,21,22].

There were typically 5–10% transconjugants per initial donor after two hours and approximately 30% transconjugants per donor after three hours of mating (Table 1). The detection of transconjugants as CFUs at any time post-mating indicated that ICEBs1 had transferred from a donor to a recipient, but did not indicate when ICEBs1 had integrated into the chromosome of the transconjugant.

Timing of ICEBs1 integration in transconjugants. We measured the fraction of transconjugants in which ICEBs1 had integrated at two and three hours after the start of mating. Briefly, genomic DNA was isolated from cells that were recovered from mating filters and we
used qPCR to measure integration of ICEBs1. Because the location of the integration site was different in donors (SAM078) and recipients (SAM103), we could measure integration of ICEBs1 in the transconjugants (recipients) using a primer pair that would detect the junction between the right end of ICEBs1 and the host chromosome (attR) that is created only if ICEBs1 had integrated into the chromosome of a recipient. We also determined the total number of recipients in the population by qPCR of a sequence (mls) present in recipients (and transconjugants), but not in donors.

We found that ICEBs1 had integrated in approximately 5% of the transconjugants after two hours and approximately 70% after three hours of mating (Fig 2A). Based on these findings, we conclude that there was a delay between the time of ICEBs1 transfer and integration and that two hours post-mating, ICEBs1 had integrated into the chromosome of a relatively small fraction (~5%) of transconjugants. By three hours post-mating, ICEBs1 had integrated into the chromosome in the majority (~70%) of transconjugants.

Loss of xis caused premature integration in transconjugants

In many integrative elements, including temperate phages and ICEs, recombination into and out of the host chromosome is catalyzed by a recombinase, often named Int (integrase). For many elements, a recombination directionality factor, often called Xis or Rdf (excisionase or recombination directionality factor) is needed, along with Int, for recombination (excision) out of the genome. ICEBs1 encodes both Int and Xis; Int is needed for both integration and excision, and Xis is needed just for excision [19]. Regulation of ICEBs1 is such that int is expressed at a low level constitutively, and xis is expressed at a high level immediately upon de-repression of ICEBs1 gene expression (Fig 1A and 1C) [19].

We found that loss of xis caused early integration in transconjugants. We used donor cells that contained ICEBs1 without xis (ICEBs1 Δxis; SAM207). To enable excision, we provided xis in trans from an ectopic locus, essentially as described previously [19]. Thus, ICEBs1 Δxis could excise in this donor, but there would be no xis in transconjugants. At two hours post-mating, ICEBs1 Δxis had integrated in ~50% of transconjugants, an increase of ~10-fold
**Fig 2. Early integration of ICEBs1 causes cell death.**

**A**) Integration of ICEBs1 in transconjugants was measured by qPCR and normalized to the number of transconjugant CFUs. The identity of the donor and recipient strains is indicated below the x-axis. Donors: WT (SAM078); Δxis (SAM207). Recipients: WT (SAM103); +xis (xis expressed in recipients; SAM379). Mating pairs are shown below the graph with relevant genotypes indicated. Circles indicate the results from independent experiments. Error bars indicate standard error of the mean. Results are from the averages of at least three dilutions within the linear range with three technical replicates each for cycle threshold (CT) values from PCR, then normalized to CFUs.

**B**) Lysis of ICEBs1 Δxis transconjugants. Donors (ICEBs1 Δxis; SAM472; or ICEBs1 xis+; SAM288) harboring ICEBs1 with constitutively expressed mNeongreen were mated with recipients (SAM271) constitutively expressing mApple (and MLS resistant) for 4 hr. Samples were then spotted onto nutrient-rich agarose pads containing erythromycin and lincomycin (selecting for recipients and transconjugants, preventing growth of donors). Transconjugants were identified by co-expression of mNeongreen and mApple. Cell fate was tracked over the course of 3 hr. Each point represents the frequency of lysed transconjugant cells in an individual field of cells from one representative experiment. Top and bottom lines indicate the interquartile range and the middle line indicates the median. We tracked 732 and 635 transconjugants from Δxis and wild type (xis+), respectively. Of these 51 (~7%) of the Δxis and 1 (~0.2%) of the xis+ transconjugants lysed during the 4 hrs of observation.

**C**) Acquisition of ICEBs1 as measured by Cre-mediated recombination.
per recipient. Acquisition efficiency was measured by qPCR by amplifying across the inversion sequence (which would indicate Cre-mediated recombination) and normalizing to a unique sequence (mls) in recipients. Donors: WT (SAM830); Δxis donor (SAM892). Recipients: WT (SAM599). Circles indicate the results from independent experiments. Error bars indicate standard error of the mean. Results are from the averages of at least two dilutions within the linear range with three technical replicates each for CT values from PCR, then normalized to CFUs. D) Same experiment as in Fig 2C. qPCR from amplification across inversion sequence normalized to transconjungant CFUs. These are the data from Fig 2C divided by data from corresponding strains and conditions from S2 Fig. The frequency of inversions per viable transconjugant for Δxis decreased at 3 hr because the number of viable transconjugants increased. Circles indicate the results from independent experiments. Error bars indicate standard error of the mean. Results are from the averages of at least two dilutions within the linear range with three technical replicates each for CT values then normalized to CFUs. E) Experimental set-up as in B with the addition of DAPI to agarose pads. Chromosomal abnormalities were tracked over the course of 3 h. Results are from randomly sampling between 100–116 cells for each condition. Data were analyzed by two individuals who did not know the identity of the samples. Error bars indicate standard error of the mean. F) Representative micrograph images showing abnormal (Δxis) and normal (WT) chromosome segregation in transconjugants from the experiment in Fig 2E. Red arrow indicates cell with an elongated nucleoid. White arrow indicates a cell with asymmetrical chromosome segregation.

https://doi.org/10.1371/journal.pgen.1010524.g002

relative to that of wild-type ICEBs1 (xis+) (Fig 2A). Loss of xis also caused a decrease in the number of viable transconjugants per donor. At two hours post-mating, there were ~0.6% transconjugants per initial donor compared to ~10% for wild type (xis+) (Table 1).

Early integration of ICEBs1 Δxis was due to the absence of xis in transconjugants and not loss of a site or an effect on a downstream gene. We expressed xis from its own promoter (Pxixs-xis, integrated as a single copy in the chromosome) in recipients. When these recipients (SAM379) were used in a mating with ICEBs1 Δxis donors (SAM207), the timing of integration was similar to that of wild-type ICEBs1 (xis+) (Fig 2A). Additionally, conjugation efficiency in the recipient expressing xis increased relative to that of ICEBs1 Δxis (Table 1). These results indicate that early integration of the Δxis mutant in transconjugants was due to loss of xis and not an unexpected secondary effect on downstream genes or loss of a site. We conclude that xis is needed in transconjugants for proper timing of integration.

Early integration caused cell death

Our data indicated that many of the initial transconjugants that acquired ICEBs1 Δxis were likely dying. For example, three hours post-mating, the integration efficiency per viable transconjugant of ICEBs1 Δxis was greater than 100% (Fig 2A). This indicated that ICEBs1 Δxis was integrating in the transconjugants, but that many of these transconjugants were unable to form colonies and that the resulting increase in the ratio of integrants per transconjugant CFU was likely due to the decrease in transconjugant CFUs. To further investigate this, we monitored the fate of individual transconjugants using fluorescence microscopy.

To distinguish donors, recipients, and transconjugants, the three cell types present in a mating mix, we constructed recipients that constitutively produced red fluorescent protein from mApple and donors that constitutively produced yellow-green fluorescent protein from mNeongreen that had been introduced into ICEBs1 (ICEBs1 mNeongreen). Transconjugants produce both mApple and mNeongreen and were easily distinguished from both donors and recipients that expressed only one fluorescent protein.

We found that a higher proportion of transconjugants that had acquired ICEBs1 Δxis lysed compared to those that had acquired ICEBs1 xis+. In these experiments, we mated donors (xis+, SAM288; Δxis, SAM472) with recipients (SAM271) for four hours and spotted the mating mixture onto agarose pads to observe individual cells by time-lapse microscopy for three hours (Methods). Transconjugants were identified as cells that had both red (mApple) and yellow (mNeongreen) fluorescence. During the three hours of observation, ~7% (51 of 732 cells observed) of Δxis transconjugants lysed, compared to less than 0.2% (1 of 635 cells observed) of wild-type (xis+) transconjugants (Fig 2B and S1 Movie). We suspect that Δxis transconjugants continued to lose viability after the three hours of observation.
Using the recombinase Cre to detect transfer of ICEBs1, irrespective of viability of transconjugants

To better measure integration-mediated death of transconjugants, separate from the ability of transconjugants to form colonies, we developed a reporter system that uses Cre-mediated recombination to demonstrate transfer of ICEBs1. We reasoned that if ICEBs1 Δxis had integrated prematurely, then ICEBs1 genes must have been expressed. If the element encoded a heterologous recombinase (e.g., Cre), then transconjugants could be detected by virtue of a Cre-catalyzed recombination event, even if the transconjugants lost viability. Briefly, we inserted cre driven by Pxis in place of rapI-phrI in ICEBs1 (xis+, SAM830; Δxis, SAM892). Recipients (SAM599) contained a spectinomycin-resistance cassette (spc) with a promoter in the wrong orientation {P(off)} between two lox sites. Transfer and expression of cre should cause inversion of the promoter-containing fragment such that the promoter would be driving expression of spc {P(on)} (S1 Fig). This Cre-mediated recombination is easily detectable by qPCR (S1 Fig and Methods) and does not require that the transconjugants be able to form colonies, although viable transconjugants should be spectinomycin resistant.

Cre-mediated recombination enabled us to compare the frequency with which recipients acquired ICEBs1 Δxis to that for ICEBs1 xis+. We mated ICEBs1 Δxis cre (SAM892) with recipients that contained the Cre reporter (SAM599). The acquisition efficiency as measured by CFUs was ~0.01% transconjugants per recipient after two hours of mating (S2 Fig). In contrast, the acquisition efficiency as measured by Cre-mediated recombination (qPCR, Methods) was ~5% per recipient after two hours of mating (Fig 2C), indicating that using Cre-mediated recombination, we detected 500-fold more inversions than viable transconjugants (Fig 2D).

We found that the acquisition efficiency of wild-type ICEBs1 cre (xis+) (SAM830) as measured by Cre-mediated recombination was ~6% transconjugants per recipient after two hours of mating (Fig 2C). This is virtually indistinguishable from the results with ICEBs1 Δxis (~5% transconjugants per recipient by the Cre assay). Based on this comparison and the results presented above, we conclude that ICEBs1 Δxis was transferred to recipients, that the element integrated prematurely into the genome of these transconjugants, and that the vast majority of transconjugants were not viable. We suspect that loss of viability was likely due to DNA damage from the integration of an element undergoing autonomous rolling circle replication (see below).

Premature integration of ICEBs1 in transconjugants causes chromosome abnormalities

To test if integration of ICEBs1 Δxis in naive hosts caused effects on the host chromosome, we monitored chromosome segregation in transconjugants that had received ICEBs1 Δxis. We mated donors and recipients expressing different fluorescent proteins as described above (mApple in recipients; mNeongreen in donors and transconjugants) and then placed mating mixtures on agarose pads containing DAPI to visualize DNA (Methods). We found that ~60% of transconjugants that received ICEBs1 Δxis had abnormal chromosome segregation, defined as any division event that did not result in symmetrical segregation of the chromosome to daughter cells, or the appearance of either compacted or elongated nucleoids. In contrast to the ~60% for ICEBs1 Δxis, approximately 20% of ICEBs1 xis+ transconjugants had abnormal chromosome segregation (Fig 2E and 2F). These results indicate that premature integration of ICEBs1 in transconjugants caused an increase in chromosome abnormalities.

Detailed characterization of events in transconjugants is technically challenging due to the limited conjugation frequency and the need to distinguish transconjugants from the other two cell types (donors and recipients) present in the mating mix. Below, we describe experiments
that overcome these limitations and allow for more detailed characterization of cells in which
ICEBs1 is integrated and its genes are expressed.

Use of an ICEBs1 Δxis mutant host as a proxy for premature integration in
transconjugants

Previous work indicated that activation of ICEBs1 that was unable to excise, due to integration
in secondary sites, caused a host SOS response and cell death [23], similar to the phenotypes
caused by premature integration of ICEBs1 Δxis in transconjugants. We used a strain
(SAM249) containing ICEBs1 Δxis at its normal integration site (attB) as a proxy for prematu-
re integration of ICEBs1 Δxis in transconjugants. This is similar to the donor strain used in
conjugation experiments described above, except without providing a functional xis in trans
and with ICEBs1 Δxis integrated at its normal location in the chromosome (attB in trnS-leu2).
Without any xis in the cell, activation of ICEBs1 Δxis results in de-repression of ICEBs1 gene
expression and failure to excise from the chromosome, a situation similar to that caused by
premature integration in the ICEBs1 Δxis transconjugants.

We found that one hour after activation (addition of xylose to induce P
xyl-rapi) of ICEBs1
Δxis in cells growing in defined minimal medium (Methods), cell viability was ~2% of that
before activation (Fig 3). In contrast, the number of viable cells of ICEBs1 xis+ increased
2-fold under the same conditions.

Fig 3. Activation of ICEBs1 that is unable to excise from the chromosome causes loss of cell viability. Cells were
grown at 37°C in a defined minimal medium containing L-arabinose as a carbon source. Expression of rapI (P
xyl-rapi) was induced by the addition of D-xylose for 60 mins. Viability was determined by CFUs and normalized to the
number of CFUs at the time of induction (addition of xylose). ICEBs1 WT = AB77; ICEBs1 Δxis = SAM249; ICEBs1
Δxis + xis (i.e., complementation of Δxis) = SAM388; ICEBs1 Δxis ΔhelP (unable to undergo autonomous rolling circle
replication) = SAM393. Results are the means from at least 3 biological replicates each with two technical replicates for
CFU counts. Error bars indicate standard error of the mean.

https://doi.org/10.1371/journal.pgen.1010524.g003
Activation of ICEBs1 Δxis caused cell death, apparently similar to that observed in ICEBs1 Δxis transconjugants. ICEBs1-containing cells were grown and treated as above and then placed cells on an agarose pad to track the fate of individual cells by time-lapse microscopy (S2 and S3 Movies). We found that during a three hour period following one hour of growth in ICE-inducing conditions, cell lysis occurred in ~70% of cells containing ICEBs1 Δxis compared to ~5% of those containing ICEBs1 xis+.

We found that the loss of viability of cells with ICEBs1 Δxis was due to loss of xis and not some unexpected effect on downstream genes or a cis-acting element in ICEBs1. We provided xis in trans from an ectopic locus (SAM388), thereby restoring excision. Viability was also restored to levels seen for cells with ICEBs1 xis+ (Fig 3), analogous to the restoration of the timing of integration observed when ICEBs1 Δxis transfers into recipients expressing xis (Table 1 and Fig 2A). Together, these results indicate that ICEBs1 Δxis is a suitable proxy for premature integration of ICEBs1 Δxis in transconjugants, that loss of cell viability is due to the inability of the element to excise from the chromosome, and that the terminal phenotype is cell lysis.

Cell death precedes lysis

Although cell lysis was the terminal phenotype, we found that cell death preceded lysis. We used staining with propidium iodide (PI) as an indicator of cell death (Methods; [24]) and tracked the ability of cells to take up PI and cell lysis using microscopy. We found that at the time ICEBs1 Δxis cells (SAM427) became PI-positive there was an appreciable decrease (~25%, n = 20) in cell surface area (S3A and S3B Fig), consistent with cell membrane permeability and loss of the proton motive force [25]. Cell lysis occurred on average within 12 minutes after staining with PI and the decrease in surface area. Together these results indicate that cell death caused by activation of ICEBs1 Δxis occurred before cell lysis. We suspect that loss of the proton motive force likely activates B. subtilis autolysins which then cause cell lysis [26,27].

The inability to excise does not immediately explain the loss of cell viability in ICEBs1 Δxis and indicates that there is another function of ICEBs1 biology that is lethal when the element is activated and unable to excise and when an active element is integrated prematurely in transconjugants.

Activation of ICEBs1 Δxis is lethal due to rolling circle replication of the element in the host chromosome

When activated, ICEBs1 undergoes unidirectional rolling circle replication that initiates from its origin of transfer (oriT) [8,28]. When the element is unable to excise due to deletion of the attachment site at the right end (ΔattR), this replication initiates in the integrated ICE and extends into the chromosome [8]. Replication of ICEBs1 requires three element-encoded functions: the relaxase NicK [15], the origin of transfer (oriT) that is nicked by the relaxase [15] and also functions as an origin of replication [8,28], and the helicase processivity factor HelP [28]. In an ICEBs1 ΔhelP mutant, nicking occurs at oriT [15], but the ICE DNA is not unwound and cannot undergo autonomous rolling circle replication [28].

We found that the lethality caused by activation of ICEBs1 Δxis was likely due to replication of the integrated element. There was no cell death in the ICEBs1 Δxis ΔhelP mutant (SAM393) (Fig 3), indicating that replication, or at least DNA unwinding, was required for cell death following activation of the excision-defective element. These results demonstrate that the loss of viability and subsequent lysis (above) caused by an activated ICEBs1 Δxis was most likely due to rolling circle replication of an element that is unable to excise. Further, cell lysis was the terminal phenotype, consistent with results above for early integration of ICEBs1 Δxis in transconjugants.
Activation of ICEBs1 Δxis induces the SOS response
Rolling circle replication of integrated ICEBs1 originates from oriT, extends into the chromosome, and generates ssDNA [15,28]. Iterative replication, that is multiple initiation events at the newly synthesized oriT, could also lead to a fragile region of the chromosome and potential arrest of chromosome replication forks that had initiated at the chromosomal origin, oriC. Both the increase in ssDNA and arrest or collapse of chromosomal replication forks could trigger the RecA-dependent SOS response.

We monitored induction of the SOS response in single cells using a transcriptional fusion of the reporter gene encoding the fluorescent protein mNeongreen to the promoter for yneA (yneA-mNeongreen), a gene that is repressed by LexA and activated during the SOS response [Methods and [29] and references therein]. We found that the SOS response was activated two hours after induction of ICEBs1 Δxis. The median fluorescence intensity of the population increased ~20-fold compared to the uninduced ICEBs1 Δxis, with ~83% of the population responsible for fluorescence (Fig 4A and 4B). In contrast, we found that two hours after activation of the excision competent ICEBs1 (xis+), the median fluorescence intensity of the population increased ~2-fold compared to the uninduced ICEBs1 xis+, with ~10% of the population responsible for fluorescence (Fig 4A and 4B). These results indicate that activation of ICEBs1 Δxis causes activation of the SOS response in the majority of cells in the population and are consistent with previous findings that ICEBs1 that is unable to excise from the chromosome causes induction of the SOS response [23].

Activation of ICEBs1 Δxis causes chromosome abnormalities
We found that activation of ICEBs1 Δxis caused chromosome abnormalities, analogous to those caused by premature integration of ICEBs1 Δxis in transconjugants. We grew cells containing ICEBs1 xis+ (SAM426) or ICEBs1 Δxis (SAM427) in defined minimal medium, activated ICEBs1 by overexpression of rapI (from PxyI-rapI) for one hour, then removed the activator and placed cells on an agarose pad that supported rapid growth (doubling time of ~20 minutes). To observe the nucleoid and formation of septa, agarose pads contained DAPI (DNA staining) and FM4-64 (membrane staining) and cells were visualized every three minutes for three hours (Methods).

We found that the majority (~92%) of cells with an activated ICEBs1 Δxis had aberrant nucleoids and-or defects in chromosome segregation (Table 2 and Figs 4C and S4A–S4H and S4 Movie), including approximately 30% of cells with elongated nucleoids (S4D–S4F Fig). We defined aberrant chromosome segregation as nucleoids that had an asymmetric distribution between daughter cells. Daughter cells were defined as cells that existed for at least one frame after division and prior to lysis.

In addition to abnormal chromosome segregation and elongated nucleoids, many cells had defects in cell division, including mislocalized septa (septum formation not at mid-cell), guillotined chromosomes (S4D–S4F Fig), and anucleate cells (S4G and S4H Fig). We found that 29% of cells with an activated ICEBs1 Δxis had mislocalized septa, 6% had guillotined chromosomes, and 8% were anucleate. Additionally, approximately 65% of the population lysed during the course of the experiment (Table 2).

Many more cells were affected by activation of ICEBs1 Δxis compared to activation of ICEBs1 that was able to excise (ICEBs1 xis+). Of the cells in which ICEBs1 (xis+) was activated and able to excise (SAM426), only ~5% lysed, <15% had aberrant-looking chromosomes, and <10% had any apparent defect in cell division, compared to ~65% lysed, ~92% with chromosome abnormalities, and ~43% with cell division defects in cells with ICEBs1 Δxis (Table 2).
Together, these results indicate that activation of ICEBs1 Δxis causes severe perturbations in chromosome organization and segregation, cell division, and ultimately causes cell death.

**Chromosome abnormalities and cell lysis caused by activation of ICEBs1 Δxis were not due to activation of phage**

We found that the chromosome abnormalities and cell lysis caused by activation of ICEBs1 Δxis occurred in the absence of the functional phage SPβ and the defective phage PBSX that are present in the *B. subtilis* chromosome. Both SPβ and PBSX are activated during the SOS response [e.g., [29]] and cause cell lysis [30–33]. In a strain devoid of these two elements...
B1Δxis still caused chromosome abnormalities and cell lysis (Table 2 and S5 Movie). Based on these results, we conclude that activation of ICE B1Δxis causes chromosome abnormalities and cell lysis independently of these phage or phage-like elements.

There was always a fraction of cells in our experiments that had no detectable chromosome abnormalities, appeared to grow normally, and in contrast to most other cells in the population, did not lyse. This apparent normalcy could be because these cells were resistant to the effects of ICE B1Δxis, ICE B1Δxis was not activated, or the element had been lost. We used a fluorescent reporter fused to the major ICE B1 promoter (Pxis-mApple; at an ectopic location in the chromosome; i.e., not in ICE B1) that allowed us to distinguish between these possibilities. There is little to no fluorescence from Pxis-mApple in cells with ICE B1 that is integrated in the chromosome and fully repressed (i.e., not activated) due to repression by the element-encoded repressor ImmR. Expression from Pxis is high in cells without ICE B1 and intermediate in cells with ICE B1 that had been activated (de-repressed) (S5 Fig).

We found that virtually all cells that had normal-looking chromosomes and that did not lyse had little or no detectable expression of Pxis-mApple (S6 Movie and S6 Fig), indicating that ImmR was present and fully active. Based on these results, we conclude that these cells still contained ICE B1Δxis, but that the element had not been activated. There were some cells that had activated ICE B1 and had chromosome abnormalities but did not lyse (S6 Fig). It is possible that these cells were destined to lyse, but not during the three hours of our observations.

**Discussion**

We used a combination of population and single-cell analyses to study integration of ICE B1 in transconjugants. We found that integration occurs several generations after initial transfer, early integration is lethal to most cells, and lethality is likely due to rolling circle replication from ICE B1 that had integrated into the chromosome. Furthermore, based on our results, we conclude that premature integration of ICE B1 in transconjugants causes phenotypes similar to those caused by activating an element that is unable to excise from the chromosome. In both cases, rolling circle replication from ICE B1 in the chromosome causes a host SOS response, chromosome abnormalities, and cell death.

Multiple lines of evidence led to the conclusion that integration of ICE B1 that is undergoing autonomous rolling circle replication causes host cell death. Most dramatically, activation of ICE B1 that is unable to excise from the host genome caused cell death. Cell death was dependent on the element-encoded helicase processivity factor (HelP) that is needed for rolling circle replication (and DNA unwinding) of the element. In contrast, activation of ICE B1 that is capable of excision (and autonomous rolling circle replication) did not cause cell death.

### Table 2. DNA morphology and cell division defects following expression of ICE B1.

| Strain2 | Lysis | Elongated | Asymmetric | Guillotined | Anucleate | Mislocalized | N  |
|---------|-------|-----------|------------|-------------|-----------|--------------|----|
| WT      | 5%    | 6%        | 7%         | 1%          | 2%        | 4%           | 246|
| Δxis    | 65%   | 30%       | 62%        | 6%          | 8%        | 29%          | 191|
| Δxis, Δø | 68%  | 18%       | 66%        | 7%          | 6%        | 21%          | 175|

1 Cultures were grown to OD600 of 0.2 in minimal defined medium with L-arabinose as a carbon source. Expression of rapI was induced by adding medium with 1% D-xylose for 1 hr before visualization by fluorescence microscopy (Methods).

2 Strains included: WT, ICE B1 xis+ (SAM426); Δxis, ICE B1 Δxis (SAM427); and Δxis Δø (SAM520) which is missing the phage SPβ and the defective phage PBSX.

https://doi.org/10.1371/journal.pgen.1010524.t002

(SAM520), activation of ICEB1 Δxis still caused chromosome abnormalities and cell lysis (Table 2 and S5 Movie). Based on these results, we conclude that activation of ICEB1 Δxis causes chromosome abnormalities and cell lysis independently of these phage or phage-like elements.

We used a combination of population and single-cell analyses to study integration of ICEB1 in transconjugants. We found that integration occurs several generations after initial transfer, early integration is lethal to most cells, and lethality is likely due to rolling circle replication from ICEB1 that had integrated into the chromosome. Furthermore, based on our results, we conclude that premature integration of ICEB1 in transconjugants causes phenotypes similar to those caused by activating an element that is unable to excise from the chromosome. In both cases, rolling circle replication from ICEB1 in the chromosome causes a host SOS response, chromosome abnormalities, and cell death.

Multiple lines of evidence led to the conclusion that integration of ICEB1 that is undergoing autonomous rolling circle replication causes host cell death. Most dramatically, activation of ICEB1 that is unable to excise from the host genome caused cell death. Cell death was dependent on the element-encoded helicase processivity factor (HelP) that is needed for rolling circle replication (and DNA unwinding) of the element. In contrast, activation of ICEB1 that is capable of excision (and autonomous rolling circle replication) did not cause cell death.
Further, premature integration of ICEBs1 in a transconjugant caused death of the transconjugant as determined by a drop in the number of viable transconjugants and as visualized by microscopy of single cells. Furthermore, there were chromosome abnormalities in cells in which ICEBs1 was integrated and undergoing autonomous rolling circle replication, but few chromosome abnormalities in cells in which ICEBs1 was integrated and not undergoing autonomous replicating, or in cells in which ICEBs1 was extrachromosomal and undergoing autonomous rolling circle replication.

Together, our results demonstrate that robust viability and fitness of transconjugants depends on repression of replication before or concomitant with integration. Integration and ongoing rolling circle replication causes chromosome abnormalities and death of transconjugants. We conclude that ICEBs1 normally links integration, which is required for stable acquisition and maintenance of an element in a population [19], to the cessation of autonomous ICEBs1 replication. The ability of ICEBs1 to remain extrachromosomal and replicate autonomously is beneficial for the establishment and spread of the element, and for the hosts if the presence of the element confers a fitness advantage. Replication of ICEBs1 upon transfer to a new host allows for the element to segregate during growth and division of the transconjugants and for the transconjugants to become donors as soon as the ICE genes needed for conjugation are expressed. This is especially critical for transfer of the element in chains of cells and cells growing in communities [20,34–36]. Below, we discuss the linkage between integration and cessation of autonomous replication and postulate that it is a conserved regulatory feature of integrative mobile genetic elements, including ICEs and temperate phages that integrate into the host genome.

Excisionases, required in donors, are also important in transconjugants

There is a large body of work demonstrating the importance of excisionases (recombination directionality factors) for the excision of ICEs and prophages from the chromosome of host cells [37]. However, it is largely assumed that excisionases are not important for establishment of an element after it enters a naïve host. We found that in addition to its well-known and expected role in excision from the chromosome [19], the ICEBs1 excisionase is critical following conjugative transfer of the element to a new host (transconjugant) and that it acts to affect the timing of stable integration.

Upon entry into a new host, most ICEBs1 genes are expressed as there is no repressor (ImmR) present and the major promoter Pxis is on (Fig 1C). This prevents integration and enables new transconjugants to serve as donors while the element is still extrachromosomal and expressing genes needed for autonomous rolling circle replication and conjugation, thereby increasing transfer of the element in a population [20,34–36]. This de-repressed gene expression upon transfer to a naïve host is analogous to zygotic induction of phage lambda following its transfer to a new, non-lysogenic host via conjugation [38].

After transfer to a new host, ICEBs1 genes encoding the repressor (immR), anti-repressor (immA), and integrase (int) are also expressed (Fig 1C). Once enough ImmR accumulates, it activates its own promoter and represses transcription from Pxis [18,19]. This regulatory circuit also stimulates transcription of int. For ICEBs1, as with analogous systems, the presence of the excisionase directs the activity of the integrase so that excision is favored. Thus, integration of ICEBs1 would occur following the accumulation of the integrase and depletion of the excisionase. Depletion of the previously made excisionase occurs by dilution during cell growth and degradation if the protein is unstable. Although we have not yet tested it directly, we suspect that Xis is degraded by host proteases, thereby enabling integration shortly after repression of Pxis by ImmR, analogous to degradation of λ Xis [39].
Once repression of Pxis occurs, the ICEBs1 genes required for rolling circle replication are no longer expressed and autonomous replication will cease. In this way, integration of ICEBs1 into the chromosome of its new host is enabled, either concomitant with or shortly after the cessation of replication. This coordinated timing affects the fitness of ICEBs1 and host cells in several ways: 1) It allows replication and maintenance of ICEBs1 in the growing transconjugants prior to integration; 2) It allows early transconjugants to become donors before integration; and 3) It ensures that when the element does integrate into the genome, most of the integrants will be stable and viable with no autonomous replication of the integrated element.

Regulation and timing of integration of other ICEs

It is not known if premature integration of other ICEs causes phenotypes similar to those described here. However, there are several ICEs that are known to undergo autonomous replication, including Tn916 (Enterococcus faecalis) [9], ICEclc (Pseudomonas knackmussii B13) [7,10], ICEHin1056 (Haemophilus influenzae) [40,41], ICEEMISymR7 (Mesorhizobium lotiR7A) [16], SXT/R391 (Vibrio cholerae/Providencia rettgeri) [13], TnGBS1/TnGBS2 (Streptococcus agalactiae) [14], and ICESt1/ICESt3 (Streptococcus thermophilus) [42]. If autonomous replication of any of these ICEs occurs in transconjugants, then it seems likely that replication ceases prior to or concomitant with integration. We suspect that this is also true in the initial host if the element were to excise and then reintegrate. Additionally, some of these, including ICEHin1056 and TnGBS1/TnGBS2, are known to exist transiently as replicating elements in transconjugants, at least for a few generations prior to integration. These properties indicate that integration likely occurs after replication is halted.

ICEHin1056.

Although it was not known to be an ICE at the time, prior work characterizing what is now known as ICEHin1056 revealed that donors, without detectable free plasmid during growth, were able to transfer antibiotic resistance to recipients. The resulting transconjugants transiently contained plasmid DNA and were capable of efficient transfer [40,41]. These initial studies indicated that at least some ICEs likely exist as extrachromosomal plasmids for several generations before integrating into the nascent host chromosome and that while plasmids, they were capable of efficient transfer to other cells.

TnGBS1/TnGBS2.

ICEs in the TnGBS family differ from most characterized ICEs in that they use a DDE transposase rather than a phage-like integrase. The limited host range of TnGBS ICEs was found to be due to the inability of the element to replicate in species other than S. agalactiae. Further, qPCR analysis of the ratio of integrated ICE to circular ICE revealed that TnGBS ICEs remained in the circular form in transconjugants for some time before integration into the chromosome, and that the timing of integration varied between elements [14].

Other ICEs.

For some ICEs, for example SXT/R391, ICEEMISymR7A, and ICEBs1, autonomous replication is critical for the maintenance of the element in donors following activation and excision [8,13,16]. In addition, for ICEBs1 and ICEEMISymR7A, replication is important in the generation of stable transconjugants [16,28]. Autonomous replication of ICEclc in donors increases the conjugation efficiency [7,10], and the element remains extrachromosomal in transconjugants for up to 10 hours after transfer [7]. For several ICEs, it has been shown that dysregulation between replication and integration results in instability of the element [8,9,13,16,19]. Together, the knowledge that various ICEs replicate autonomously indicates that proper regulation of integration is likely important for the stability of the element and survival of host cells.
Mechanisms for coupling integration with cessation of autonomous replication

There are at least three strategies used by ICEs to couple integration with cessation of autonomous replication: 1) Coordinated repression of excision and replication functions; 2) Excisionase-mediated activation of genes needed for replication; and 3) Physical separation of replication genes from their promoter upon integration.

1) Coordinated repression of excision and replication functions. ICEBs1 regulatory hierarchy involves the co-transcription of genes needed for excision, replication, and conjugation \[8,17–19,28\]. The gene for the excisionase is upstream of those for replication and conjugation. As of yet, there are no published data on possible differential regulation of expression of excision and replication genes for ICEBs1. However, we note that a review of a transcriptomics study of \(B.\ subtilis\) \[43\] indicates an additional promoter downstream of the excisionase, driving expression of genes needed for replication and conjugation. The presence of this promoter could provide additional regulatory inputs that might affect the timing of integration and cessation of replication. Additionally, we suspect that the ICEBs1 excisionase (and probably others) is unstable, providing tighter temporal linkage between the repression of \(xis\) expression, disappearance of the protein, and the ability of the element to stably integrate.

2) Excisionase-mediated activation of genes needed for replication. ICEMLSym\(^{R7A}\) appears to use a similar regulatory hierarchy as ICEBs1. Variants of ICEMLSym\(^{R7A}\) are found in other \(Mesorhizobium\) species and have a similar regulatory hierarchy \[44,45\]. Additionally, for these ICEs the excisionase has been found to act as a transcription factor, activating the expression of downstream genes required for the transfer of the elements. This indicates that ICEs of the \(Mesorhizobium\) spp., may use a similar method of regulation observed for CTnDOT of \(Bacteroides thetaiotamicron\), in which the excisionases moonlight as transcription factors, activating the expression of genes needed for conjugative replication, thereby directly linking Xis to the regulation of replication \[45,46\].

3) Physical separation of replication genes from their promoter upon integration. Tn916 of \(Enterococcus faecalis\) employs a regulatory hierarchy that involves physical separation of promoters from genes needed for replication and conjugation \[47\]. Excision of the element orients the promoter such that transfer and replication genes are transcribed. Conversely, integration separates the genes needed for replication and conjugation from the promoter(s) that drive their expression, thereby preventing further autonomous replication of the element.

Contrast between ICEs and conjugative plasmids

In contrast to ICEs, conjugative plasmids (reviewed in \[4\]) are typically maintained as extrachromosomal elements. However, conjugative plasmids can integrate into the host chromosome, typically by RecA-mediated homologous recombination between insertion sequences on the plasmid and the chromosome. For example, integration of the \(E.\ coli\) F plasmid into the chromosome generates an Hfr (high frequency of recombination) strain that can transfer chromosomal DNA to recipient cells \[48\]. Unlike ICEs, integration and excision of conjugative plasmids into the chromosome is not required for plasmid stability and is not viewed as a normal part of the plasmid lifecycle. In contrast to the largely uncontrolled replication from \(oriT\) of an integrated ICEBs1 that causes cell death, replication from \(oriT\) in an Hfr causes little or no cell death, but does cause a transient arrest of cell division \[49\]. This difference is likely due to limited nicking of and replication from \(oriT\) in the Hfr.
Conclusions

This linkage between integration and the absence of autonomous replication likely pertains to ICEs that undergo autonomous rolling circle replication and many temperate phages. Temperate phages that infect naïve hosts and integrate into the genome to form lysogens typically do so prior to the start of autonomous replication. This is typically achieved by a hierarchy of phage genes expressed at different times. Phage genes required for replication are typically expressed after the early genes and only if the phage enters the lytic cycle [50–53].

In summary, our work provides a new perspective on ICE regulation as it pertains to transconjugants. We uncovered a critical role for the excisionase in transconjugants and revealed the importance of regulatory mechanisms that delay integration until the cessation of replication. Because of the deleterious effects of integration before the cessation of replication, it is likely that most, and perhaps all integrative elements that undergo autonomous replication have a mechanism that enables integration only after replication is no longer possible. Because of the prevalence of ICEs, their powerful ability to transfer genes, and their recent uses in genetic analyses and engineering [54,55], it is important to understand ICE regulatory mechanisms and events in new hosts. This understanding could inform strategies to reduce the spread of undesirable elements (e.g., encoding clinically relevant antibiotic resistances) and facilitate the use of ICEs for engineering a wide range of microbes.

Methods and materials

Media and bacterial growth

Cells were grown in LB medium or defined S750 minimal medium with 0.1% glutamate [56] and a carbon source, typically 1% L-arabinose, as indicated. Serial dilutions and resuspensions for plating viable CFUs were done in 1x Spizizen salts [57]. For experiments, indicated strains were colony purified from frozen (-80°C) glycerol stocks on LB plates with the appropriate antibiotics. A single colony was picked and used to start a culture which was grown to mid-exponential phase. An aliquot was then diluted into fresh medium and allowed to grow to an appropriate culture density in mid-exponential phase for each experiment. Cells were typically grown in flasks at 37°C with shaking (250 RPM).

Where indicated, ICEBs1 was induced by the addition of D-xylose (1%) to cause overexpression of rapI from a xylose-inducible promoter in strains containing either amyE::(Pxyr-rapI) spc [58] or lacA::(Pxyr-rapI), tet [8]. Where indicated, cells were also grown with L-arabinose to induce expression of the xylose transporter. Expression from the LacI-repressible-IPTG-inducible promoter Pspank was induced with 2 μM IPTG.

Antibiotics used included: kanamycin (5 μg/ml); chloramphenicol (5 μg/ml); tetracycline (12.5 μg/ml); spectinomycin (100 μg/ml); streptomycin (100 μg/ml); and 0.5 μg/ml erythromycin plus 12.5 μg/ml lincomycin to select for macrolide-lincosamide-streptogramin B (MLS) resistance conferred by mls (ermB).

Bacterial strains and alleles

All B. subtilis strains (Table 3) are derivatives of JH642 (AG174; [59,60] and contain trpC and pheA mutations (not indicated in the table). Most strains were constructed by natural transformation to introduce appropriate alleles. In some cases, as indicated, ICEBs1 was transferred into strains by conjugation. ICEBs1 in most strains contained a gene conferring resistance to an antibiotic, often kan (kanamycin resistance), inserted in the rapI-phrI locus [e.g., Δ(rapI-phrI)342:kan] [17]. Strains cured of ICEBs1 [17] are indicated as ICEBs0. Almost all alleles were first constructed in so-called ‘clean’ strains that were typically wild-type B. subtilis with
Table 3. *B. subtilis* strains used.

| Strain | Relevant genotype[^1]; note; reference |
|--------|----------------------------------------|
| AG174  | *(H642) trpC2 pheA1*[^59,60]            |
| AB77   | ICEBs11 [ΔrapI-phrI]342:kan amyE::(Pxyl-rapI) cat |
| CAL522 | ICEBs11^ΔattB::cat trnS-leuU522[23]; parent strain for SAM074 |
| ELC69  | ICEBs1Δ amyE::[(lox71/66−P(lox71)]-spc cat]; gift from Emily Bean; reporter for Cre-mediated recombination; sensitive to spectinomycin unless there is Cre-mediated recombination that inverts the promoter fragment causing expression of spc. |
| JMA22  | ICEBs1Δ trpC2 pheA1[^17]               |
| LSF197 | ΔSPB::(lox71-kan-lox66) [61]            |
| LSF198 | ΔPBSX::(lox71-cat-lox66) [61]           |
| SAM005 | ICEBs1 amyE::[(Pxis-gfp) cat]         |
| SAM008 | ICEBs1 lacA::[(Pxyl-rapI) spc]        |
| SAM111 | ICEBs1 amyE::[(Pxis-gfp) cat] lacA::[(Pxyl-rapI) spc] |
| SAM032 | ICEBs1 [ΔrapI-phrI]342:kan amyE::[(Pxis-gfp) cat] lacA::[(Pxyl-rapI) spc] |
| SAM074 | amyE::[attB(1–60) spc] ΔattB::cat ICEBs1[^6]; used as recipient to introduce versions of ICEBs1 into ectopic attachment site (amyE::attB) |
| SAM078 | amyE::ICEBs1 [ΔrapI-phrI]342:kan] spc lacA::[(Pxyl-rapI) tet] ΔattB::cat |
| SAM103 | ICEBs1[^9] yddA::mls (ermB)            |
| SAM137 | ICEBs1 [ΔrapI-phrI]342:kan] yddA::mls |
| SAM168 | ICEBs1 amyE::[(Pxyl-mApple) cat]      |
| SAM198 | ICEBs1 cgeD::[Pspank-xis (lox71-kan-lox66)] |
| SAM207 | amyE::ICEBs1 [Δxis190 ΔrapI-phrI]342:kan] spc ΔattB::cat lacA::[(Pxyl-rapI) tet], cgeD::[Pspank-xis (lox Δkan)] referred to as (Pspank-xis) |
| SAM249 | ICEBs1 [Δxis190 ΔrapI-phrI]342:kan] amyE::[(Pxyl-rapI) cat] |
| SAM268 | amyE::[(Pxyl-rapI) spc] ICEBs1 [ΔrapI-phrI]342::[(Ppen-mNeongreen) kan]] |
| SAM271 | ICEBs1[^9] yddA::mls amyE::[(Ppen-mApple) spc] |
| SAM288 | amyE::ICEBs1 [ΔrapI-phrI]342:kan] ΔattB::cat lacA::[(Pxyl-rapI) tet] |
| SAM301 | ICEBs1 [ΔrapI-phrI]342::[(Pspac-cre) kan] amyE::[(Pxyl-rapI) spc] |
| SAM371 | ICEBs1[^9] yddA::mls cgeD::[Pxis-xis (lox71-kan-lox66)] |
| SAM352 | ICEBs1 [ΔrapI-phrI]342:kan lacA::[(Pxyl-rapI) tet] amyE::[(PyneA-mNeongreen) spc] |
| SAM379 | ICEBs1[^9] yddA::mls cgeD::[Pxis-xis (lox Δkan)] referred to as (Pxis-xis) |
| SAM388 | ICEBs1 [Δxis190 ΔrapI-phrI]342:kan] amyE::[(Pxyl-rapI) cat] cgeD::(Pxis-xis) |
| SAM393 | ICEBs1 [Δxis190 ΔhelP ΔrapI-phrI]342:kan] amyE::[(Pxyl-rapI) spc] |
| SAM426 | ICEBs1 [ΔrapI-phrI]342:kan lacA::[(Pxyl-rapI) tet] amyE::[(PyneA-mNeongreen) spc] |
| SAM427 | ICEBs1 [Δxis190 ΔrapI-phrI]342:kan lacA::[(Pxyl-rapI) tet] amyE::[(PyneA-mNeongreen) spc] |
| SAM456 | ICEBs1 [Δxis190 ΔrapI-phrI]342:kan ΔlacA::[(Pxyl-rapI) tet] amyE::[(Pxyl-rapI) catsp] |
| SAM472 | amyE::ICEBs1 [Δxis190 ΔrapI-phrI]342::[(Ppen-mNeongreen) kan]] ΔattB::cat lacA::[(Pxyl-rapI) tet], cgeD::[(Pspank-xis) |
| SAM520 | ICEBs1 [Δxis190 ΔrapI-phrI]342:kan lacA::[(Pxyl-rapI) tet] ΔPBSX::(lox-cat-lox) ΔSP8::lox; referred to as ΔΔ0 |
| SAM529 | ICEBs1 [Δxis190 ΔrapI-phrI]342:kan lacA::[(Pxyl-rapI) tet] ΔPBSX::(lox71-cat-lox66) ΔSP8::lox Δskin::(lox71-spc-lox66); referred to as ΔΔ0 |
| SAM599 | ICEBs1[^9] yddA::mls amyE::[(lox71-P(off)-lox66] -spc cat]); recipient to monitor Cre-mediated recombination (inversion) between the lax sites. Viable cells with the inversion are resistant to spectinomycin. |
| SAM698 | ICEBs1 [ΔrapI-phrI]342::[(Pspac-cre) kan]], ΔyddA::mls amyE::[(lox-P(on)-lox]-spc cat]; used as PCR control for 100% inversion between the lax sites. |
| SAM828 | ICEBs1 [ΔrapI-phrI]342::[(Pxis-cre) kan]] |
| SAM830 | amyE::ICEBs1 [ΔrapI-phrI]342::[(Pxis-cre) kan]] spc ΔattB::cat lacA::[(Pxyl-rapI) tetR] |

(Continued)
few if any other alleles. These were verified by phenotype, diagnostic PCR, and sometimes sequencing. Alleles were then transferred to other strains to build strains with complex genotypes.

**amyE::attB.** SAM074 contains the chromosomal attachment site for ICEBsI (attB) cloned into the nonessential gene amyE (amyE::attB). It is cured of ICEBsI, and contains a deletion of attB from its normal location (**ΔattB::cat**) that was described previously [23]. amyE::(attB spc) was made by cloning **attB** into the amyE integration vector pDG364 [62]. Oligonucleotides that contained the 60-bp **attB** were annealed and 25-bp of random sequence on the flanking ends were added and cloned into pDG364. The cat gene in pDG364 was then replaced with spc (spectinomycin resistance), that had been amplified from pMMB856 [58]. This plasmid (pSAM071) was then used to introduce **attB** into the *B. subtilis* chromosome at amyE by transformation into strain CAL522 (**ΔattB::cat**), selecting for resistance to spectinomycin.

Various versions of ICEBsI were introduced into SAM074 (**amyE::attB, ΔattB::cat**) by conjugation with the relevant donor and SAM074 as recipient and selecting for transconjugants. Strains were verified by PCR and sequencing of relevant alleles. Strains constructed in this way include: SAM078; SAM207; SAM288; SAM472; SAM830; and SAM892.

**yddS::mls.** yddS is ~5 kb from **attB** and yddS::mls was used to distinguish recipients and transconjugants from donors, with mls serving as a counter-selective marker (MLS resistance) to prevent growth of donors, and a sequence for PCR that was present in only recipients and transconjugants. The allele contains an insertion of mls at base pair 22 of the 1,311 bp yddS open reading frame and was made by PCR amplifying ~2 kb of DNA from upstream and downstream of the insertion site and assembling these (Gibson isothermal assembly [63]) such that they flanked a DNA fragment containing mls that had been amplified from pCAL215 [18]. This fragment was then cloned between the EcoR1 and KpnI sites in pUC19, generating pSAM095, which was then used to transform *B. subtilis* selecting for MLS resistance.

**Ectopic expression of xis.** Constructs for ectopic expression of ICEBsI xis included cgeD::(Pspank-xis) and cgeD::(Pxis-xis). Both were made with the plasmid pSAM178, a vector for integrating cloned DNA at cgeD. pSAM178 contains the gatB/yerO terminator adjacent to a multi-cloning site, kan (flanked by lox71 and lox66 sites for removal of kan by Cre-mediated recombination [64,65] as indicated, between sequences from cgeD in the pMMB124 [18] backbone. Plasmid pSAM189 contains Pspank-xis [19] in the pSAM178 backbone. pSAM276 contains Pxis-xis amplified from its endogenous location from 158 bp upstream of the xis open reading frame to 33 bp into ydzL, the gene downstream from xis, in the pSAM178 backbone. Both constructs were introduced into *B. subtilis* by natural transformation and selection for kanamycin resistance. Strains were confirmed by diagnostic PCR and checking for chloramphenicol sensitivity (loss of the plasmid backbone). kan was then deleted from these strains by introducing cre on a temperature sensitive plasmid that confers MLS resistance and expresses the Cre recombinase (pSAM097). Strains were then cured of pSAM097 by switching to growth
at a non-permissive temperature without selection. Strains with ectopic expression of xis include: SAM198, SAM207, SAM371, SAM379, SAM388, and SAM472.

**Fusions to mNeongreen, gfp, and mApple.** *amyE::PyneA-mNeongreen* (in strains SAM426, SAM427) was used to monitor SOS induction. The fusion contains sequences extending from the first nucleotide to 151 bp upstream of the yneA open reading frame, including the promoter region [66,67]. This was fused to *mNeongreen* and flanked by sequences for recombination into *amyE*, including *spc*, by linear Gibson isothermal assembly [63]. The resulting construct was then transformed into *B. subtilis* selecting for spectinomycin resistance to generate strain SAM352. Genomic DNA from SAM352 was used to move *amyE::[(PyneA-mNeongreen) spc]* into various strains by transformation selecting for spectinomycin resistance.

*Ppen-mNeongreen* in ICEBs1 (*ICEBs1 Δ(rap1-phr1)342::(Ppen-mNeongreen) kan*) in SAM268; SAM288; SAM472 was used to visualize ICEBs1 in single cells. The constitutive promoter *Ppen* was amplified from pMMB1010 [20] (with a *spoVG* ribosome binding site in one of the PCR primers downstream of Ppen for efficient translation of *mNeongreen*) and fused to *mNeongreen*. This fusion was flanked by sequences upstream and downstream from the endpoints of Δ(rap1-phr1)342 and included kan [17] using Gibson isothermal assembly. This was then transformed into *B. subtilis* (ICEBs1+), selecting for kanamycin resistance and verifying that the reporter was in ICEBs1.

*amyE::Pxis-gfp* (in SAM005, SAM011, and SAM032) and *amyE::Pxis-mApple* (in SAM168, SAM456) were used to report on expression levels of Pxis, as a reflection of the amount of active ImmR (the ICEBs1 repressor) in the cells. Pxis was amplified from its endogenous location from 215 bp to 4 bp upstream of the xis open reading frame, fused to gfp or mApple, and cloned into the *amyE* integration vector pDR160 [21], replacing the xylose-inducible cassette, followed by integration of *Ppen-mApple* at *amyE* by double-crossover homologous recombination (between the *amyE* fragments in pDR160) selecting for spectinomycin resistance.

*amyE::Pxis-gfp* (in SAM005, SAM011, and SAM032) and *amyE::Pxis-mApple* (in SAM168, SAM456) were used to report on expression levels of Pxis, as a reflection of the amount of active ImmR (the ICEBs1 repressor) in the cells. Pxis was amplified from its endogenous location from 215 bp to 4 bp upstream of the xis open reading frame, fused to gfp or mApple, and cloned into the *amyE* integration vector pDR160 [21], replacing the xylose-inducible cassette, followed by integration of *Ppen-mApple* at *amyE* by double-crossover homologous recombination (between the *amyE* fragments in pDR160) selecting for spectinomycin resistance.

**ΔSPI::(lox-kan-lox), ΔPBSX::(lox-cat-lox), Δskin::(lox-spc-lox)** in strains SAM520 and SAM529. Deletion-insertion mutations of the prophage SPß and the defective phages PBSX were described [61]. These essentially delete the indicated phage and insert an antibiotic resistance gene flanked by *lox* sites. The Δskin::(lox-spc-lox) mutation was constructed similarly, by PCR amplifying genomic DNA from upstream and downstream of skin and then using Gibson assembly to place these PCR products fragments on either side of *lox-spc-lox*. This was then transformed into *B. subtilis*, selecting for spectinomycin resistance, resulting in deletion of skin and insertion of *lox-spc-lox*. Genomic DNA was used to move alleles by transformation to build strains SAM520 and SAM529. Where indicated, the antibiotic resistance gene was deleted by Cre-mediated recombination as above.

**ICEBs1 Δ(rap1-phr1)342::Pxis-cre kan** in strains SAM828, SAM830, and SAM892. cre, encoding the Cre recombinase, was cloned into ICEBs1 and used to monitor transfer of the element to cells that might otherwise not remain viable. Transconjugants could be detected with a reporter that would recombine in the presence of Cre. To construct Pxis-cre in ICEBs1,
the promoter *Pxis* was amplified from its endogenous location from 158 bp upstream of and to 4 bp into the *xis* open reading frame. *cre* was amplified from pDR244 [68] and the ribosome binding site from *spoVG* [69] was contained on one of the primers and placed upstream of *cre* to increase translation efficiency. Two DNA fragments, one upstream and one downstream of the endpoints of Δ(*rapI-phrI*)342, were amplified, including *kan*, and the four fragments were assembled using linear Gibson isothermal assembly. This was then introduced into ICEBs1 by transformation selecting for kanamycin resistance.

The reporter in recipient cells (SAM599) that was used to indicate acquisition of *cre* and subsequent Cre recombinase activity (S1 Fig) was constructed by Emily Bean (original strain ELC69). It consists of a promoter flanked by *lox* sites [64] such that recombination would result in a stable inversion [65]. The *lox71*-promoter-*lox66* fragment was upstream from *spc*. The initial orientation is such that the promoter is pointed away from *spc* (*P(off)*). Cre-mediated recombination would invert the promoter-containing fragment such that the promoter would be driving expression of *spc* (*P(on)*). Viable transformants that underwent Cre-mediated recombination would be spectinomycin resistant. Recombinants are also easily detectable by PCR with primers diagnostic of the inversion (S1 Fig). This assay does not require cell viability. Strain SAM698 contains the inversion (*P(on)*) in all cells and was used as a standard for qPCR.

Cre-mediated inversion appeared to underestimate the conjugation efficiency by approximately three-fold. In a direct comparison, the acquisition efficiency of ICEBs1 was ~20% transconjugants per recipient after two hours of mating, as measured by CFUs (S2 Fig) compared to ~6% as measured by Cre-mediated recombination (Fig 2C). This discrepancy decreased at later times and after three hours of mating, ~82% of the transconjugants (Fig 2D) had a detectable inversion based on the Cre reporter. Virtually every viable transconjugant (>99.8%) recovered as a colony was resistant to spectinomycin, indicating that Cre catalyzed an inversion in virtually all transconjugants, but that there was a delay between the initial acquisition of ICEBs1 (as measured by CFUs) and the Cre-catalyzed recombination (inversion) event.

**Mating assays**

Cells were grown in 25 ml flasks in LB medium in a water bath at 37°C and 250 RPM shaking until they reached an OD<sub>600</sub> of ~0.12. Arabinose and xylose (1% each) were added to donor strains to induce expression of *Pxyl-rapI*, thereby causing cleavage of the ICEBs1 repressor ImmR [18,22] and de-repression of ICEBs1 gene expression. Where appropriate IPTG (2 μM) was added to induce expression of genes needed for complementation. Cultures were grown for 40 minutes after de-repression of *Pxyl-rapI* to an OD<sub>600</sub> of ~0.3–0.45.

Donors and recipients (in mid-exponential growth) were mixed at ratio of 2:1 (resulting in the equivalent of 3 OD600 units), vacuum-filtered, and filters were transferred to Spizizen minimal salts plates as described previously [17]. We found that these conditions resulted in high conjugation efficiencies that enabled detection of ICEBs1 integration by qPCR. Mating reactions were allowed to go for various amounts of time, as indicated, and were halted by placing filters in 3 ml of Spizizen minimal salts and vigorously vortexing. An aliquot of this mating mix was then used for serial dilutions and plating for colony forming units with appropriate antibiotics for selection of donors, recipients, and transconjugants. The bulk of the mating mix was harvested for extraction of genomic DNA.

**Quantitative PCR assays to measure integration of ICEBs1 and Cre-mediated recombination**

Quantitative PCR was used to measure integration of ICEBs1 in transconjugants. Cells were lysed with 40 mg/ml of lysozyme and genomic DNA was isolated using Qiagen DNeasy kit.
qPCR was done using SSOAdvanced SYBR master mix and CFX96 Touch Real-Time PCR detection system (Bio-Rad).

The relative number of recipients (including transconjugants) in the mating mix was determined using primers to *mls* (*ermB*) that is only present in the recipient population (Δ*yddS: mls*). Primers to *mls* were oSAM403 (5’- GAAGGATTCT ACAAGCGTAC C-3’) and oSAM404 (5’- CTGGAACACTG TGTTGTATGG -3’).

Integration in transconjugants was detected by the presence of the right junction (attR) at the endogenous location of ICE*Bs1* in *tnrS-leu2* (Fig 1B) using primers oSAM390 (5’- GCAAGTCTTCTCCCATAGC-3’) and oSAM399 (5’- GGCTTTTGTA AATAAAGATA TGATTTTACT AGGTTGA-3’). Cre-mediated recombination (S1 Fig) was quantified using primers oSAM776 (5’- CCAGTCACGT TACGTTATTA GTTATAG -3’) and oSAM777 (5’-TACCCGACAG ATGCCTAG TAATAATT -3’). Standard curves for attR and mls were generated using uninduced genomic DNA from SAM137 (mating experiments) or SAM698 (Cre experiments), ICE*Bs1+* strains with copies of the relevant markers.

Serial dilutions were prepared from genomic DNA for each sample. The averages of at least two dilutions with three technical replicates each within the linear range were used to determine relative amount of ICE*Bs1* integration or Cre-mediated recombination by the Δct method. These were normalized to CFUs, as indicated, to determine the fraction of the recipient or transconjugant population with each marker.

**Microscopy and image acquisition**

We used an inverted microscope (Nikon, Ti-E) with a motorized stage placed in a temperature-controlled box (In Vivo Scientific) at 37°C. Images were acquired with a CoolSnap HQ2 camera (Photometrics). Fluorescence was generated using a Nikon Intensilight mercury illuminator through appropriate sets of excitation and emission filters (filter set 49000 for DAPI, 49003 for mNeongreen, and 49008 for mApple; Chroma). Samples were spotted onto agarose pads that were set in a homemade incubation chamber made by stacking two sealable FrameSeal Incubation Chambers (BIO-RAD). Agarose pads contained 1.5% Ultrapure agarose (Thermo Fisher) dissolved in either minimal medium with Spizizen salts or CH medium [57]. Minimal medium was used when assessing fluorescence intensity and CH medium was used for tracking cell viability. For time-lapse experiments, agarose pads were supplemented with the appropriate carbon source at 1% L-arabinose, 1% glucose, and 1% D-xylose w/v. Where applicable, agarose pads were supplemented with 25 ng/ml DAPI, 170 ng/ml FM4-64, and 0.1M propidium iodide. For visualization of cell membranes, cultures were incubated with FM4-64 (170 ng/ml) for 1 hour before imaging.

**Microscopy image analysis**

Images and videos were processed with Fiji (ImageJ) [70]. All images for fluorescent channels were subjected to background subtraction using a rolling ball radius of 10 or 20 pixels (10 pixels was used for images with foci). Cell meshes were acquired using Oufti [71]. Meshes were analyzed in MATLAB using custom scripts.

**Supporting information**

S1 Fig. Conceptual schematic for mobilizable Cre recombinase assay to measure transfer of ICE*Bs1*. Donors containing ICE*Bs1* with *Pxis-cre* were mated with recipients harboring a reporter to indicate Cre-mediated recombination (inversion). Transfer of ICE*Bs1* with *Pxis-cre* and production of Cre results in inversion of the DNA fragment that can be detected by qPCR. The green circle in the cartoon at the bottom left represents ICE*Bs1* that expresses cre.
The blue triangle in recipients and transconjugants indicates the reporter for and result of Cre-mediated inversion. Blue and yellow triangles indicate lox sites as labeled. The region between the blue and yellow triangles contains a promoter, either driving transcription of spc (With Cre; bottom right) or in the opposite orientation (No Cre; top). Blue block arrow represents spc open reading frame. Inversion in the presence of Cre produces lox71/lox66 and loxP sites which are incapable of subsequent recombination [64]. The horizontal black arrows with letters A, B, and C represent primers for PCR to detect the reporter and Cre-mediated inversion. PCR with primers B and C detect the product of Cre-mediated recombination. PCR with primers B and A detect the starting reporter with the promoter in the ‘off’ orientation.

(TIF)

S2 Fig. Acquisition efficiency per recipient as measured by CFUs. Acquisition of ICEBs1 by recipients was measured by selective plating and normalized to the total number of recipients. The identity of donor and recipient strain is indicated below the x-axis. Donors: WT (SAM830); Δxis (SAM892). Recipients: WT (SAM599). Circles indicate the results from independent experiments (at least three). Error bars indicate standard error of the mean. Results are from the averages of at least two technical replicates for CFUs.

(TIF)

S3 Fig. Cell death precedes cell lysis. Cells were (ICEBs1 Δxis; SAM427) grown at 37°C in defined minimal medium with L-arabinose. Expression of rapI (Pxyl-rapI) was induced by the addition of D-xylose. After 60 mins, cells were washed and spotted onto agarose pads containing CH medium, DAPI, and propidium iodide. A) Cell 3 mins prior to PI uptake. The white arrows indicate the axis across which cell length was measured and the cell length is indicated under the arrow. DAPI is pseudo-colored blue. Propidium iodide is pseudo-colored red. B) Cell from A, at the time of uptake of propidium iodide and 3 mins prior to lysis.

(TIF)

S4 Fig. Activation of ICEBs1 Δxis causes chromosome abnormalities and defects in cell division. Cells (ICEBs1 Δxis; SAM427) were grown at 37°C in defined minimal medium with L-arabinose. Expression of rapI (Pxyl-rapI) was induced by the addition of D-xylose. After 60 mins, cells were washed and spotted onto agarose pads containing CH medium, DAPI, and FM4-64. A-C) Cell with asymmetric chromosome segregation and mislocalized septum formation. D-F) Cell with an elongated nucleoid and a guillotined chromosome. G-I) Cell with asymmetric chromosome segregation and anucleate division. A, D, and G) Composite image of phase, FM4-64 and DAPI channels. B, E, and H) FM4-64 channel. C, F, and I) DAPI channel.

(TIF)

S5 Fig. Expression from Pxis in cells with and without ICEBs1. Cells with the reporter Pxis-gfp were grown at 37°C in a defined minimal medium with L-arabinose. Expression of rapI (Pxyl-rapI) was induced by the addition of D-xylose. After 2 hrs cells were spotted onto agarose pads and imaged for fluorescence. Each data point represents the fluorescent intensity from a single cell. The longest horizontal line represents the mean and bars above and below represent the standard deviation. No reporter is the parental strain with no genetic markers (AG174). ICEBs1Δ is a strain cured of ICEBs1 (SAM011) and expression from Pxis is in the absence of the repressor ImmR. ICEBs1 OFF is a strain containing ICEBs1 Δ(rap1-phr1)::kan (SAM032) without exogenous activation of the element. These cells produce active ImmR and transcription from Pxis is repressed and there is only basal expression of ICEBs1. ICEBs1 ON is the same strain (SAM032) except that ICE has been activated by virtue of
expression of rapI and subsequent inactivation of the repressor ImmR. Pxis is expressed under these conditions.

(TIF)

S6 Fig. Cells that continue to grow normally following activation of ICEBsI Δxis in the bulk culture still have the element. Cells containing ICEBsI Δxis and the reporter Pxis-mApple (SAM456) were grown at 37°C in a defined minimal medium with L-arabinose. Expression of rapI (Pxyl-rapI) was induced by the addition of D-xylose. After 60 mins cells were washed and spotted onto agarose pads with CH medium and DAPI. The image shown is after 3 hrs on the agarose pad and is a composite of the red fluorescence (Pxis-mApple) and DAPI channels. Red fluorescence is pseudo-colored red and DAPI is pseudo-colored blue. The white arrow points to chain of normally growing cells that have ICEBsI but the element has not been activated. Had they lost ICEBsI, or had ICEBsI been activated, then Pxis-mApple would be expressed.

(TIF)

S1 Movie. The fate of ICEBsI Δxis transconjugants. Donors (ICEBsI Δxis, SAM472) harboring ICEBsI with constitutively expressed mNeongreen were mated with recipients (SAM271) constitutively expressing mApple for 4 h. Following mating, samples were spotted onto nutrient-rich agarose pads containing erythromycin and lincomycin (recipients and transconjugants contain mls and are resistant to these two antibiotics). Donors appear pale yellow. Recipients appear red. Transconjugants are both red and yellow and appear orange or bright yellow.

(AVI)

S2 Movie. Activation of ICEBsI Δxis (unable to excise) causes cell lysis. Cells (SAM427) were grown at 37°C in a defined minimal medium with L-arabinose. Expression of rapI (Pxyl-rapI) was induced by the addition of D-xylose. After 60 mins cells were washed and spotted onto to nutrient-rich agarose pads and imaged every 3 mins for 3 hrs.

(AVI)

S3 Movie. Activation of WT ICEBsI does not cause cell lysis. Cells (SAM426) were grown at 37°C in a defined minimal medium with L-arabinose. Expression of rapI (Pxyl-rapI) was induced by the addition of D-xylose. After 60 mins cells were washed and spotted onto nutrient-rich agarose pads and imaged every 3 mins for 3 hrs.

(AVI)

S4 Movie. Activation of ICEBsI unable to excise causes chromosome abnormalities. Cells (SAM427) were grown at 37°C in a defined minimal medium with L-arabinose. Expression of rapI (Pxyl-rapI) was induced by the addition of D-xylose. After 60 mins, cells were washed and seeded to nutrient-rich agarose pads containing DAPI and imaged every 15 mins for 45 mins and then every 3 mins for 2.25 hrs.

(AVI)

S5 Movie. Chromosome abnormalities caused by activation of ICEBsI that is unable to excise are not due to SPβ, PBSX, or skin. Cells (SAM529) were grown at 37°C in a defined minimal medium with L-arabinose. Expression of rapI (Pxyl-rapI) was induced by the addition of D-xylose. After 60 mins cells were washed and spotted onto nutrient-rich agarose pads containing DAPI and imaged every 15 mins for 45 mins and then every 3 mins for 2.25 hrs.

(AVI)
S6 Movie. Cells containing ICEBs1 Δxis that survive induction are those that have not activated the element. Cells (SAM456) were grown at 37˚C in a defined minimal medium with L-arabinose medium. Expression of rapI (Pxyr-rapI) was induced by the addition of D-xylose. After 60 mins cells were washed and spotted onto nutrient-rich agarose pads containing DAPI and imaged every 15 mins for 45 mins and then every 3 mins for 2.25 hrs.

(MP4)

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

(XLSX)

Acknowledgments
We thank Mary Anderson and Alam Garcia Heredia for comments on the manuscript and Emily L. Bean for the reporter system for detecting Cre-mediated recombination.

Author Contributions

Conceptualization: Saria A. McKeithen-Mead, Alan D. Grossman.

Funding acquisition: Saria A. McKeithen-Mead, Alan D. Grossman.

Investigation: Saria A. McKeithen-Mead.

Methodology: Saria A. McKeithen-Mead.

Project administration: Saria A. McKeithen-Mead, Alan D. Grossman.

Supervision: Alan D. Grossman.

Validation: Saria A. McKeithen-Mead.

Visualization: Saria A. McKeithen-Mead.

Writing – original draft: Saria A. McKeithen-Mead.

Writing – review & editing: Saria A. McKeithen-Mead, Alan D. Grossman.

References

1. Johnson CM, Grossman AD. Integrative and conjugative elements (ICEs): What they do and how they work. Annu Rev Genet. 2015; 49(1):577–601. https://doi.org/10.1146/annurev-genet-112414-055018 PMID: 26473380

2. Goessweiner-Mohr N, Arends K, Keller W, Grohmann E. Conjugative type IV secretion systems in Gram-positive bacteria. Plasmid. 2013 Nov 1; 70(3):289–302. https://doi.org/10.1016/j.plasmid.2013.09.005 PMID: 24129002

3. Grohmann E, Muth G, Espinosa M. Conjugative plasmid transfer in Gram-positive bacteria. Microbiol Mol Biol Rev. 2003 Jun 1; 67(2):277–301. https://doi.org/10.1128/MMBR.67.2.277-301.2003 PMID: 12794193

4. Virolle C, Goldlust K, Djermoun S, Bigot S, Lesterlin C. Plasmid transfer by conjugation in Gram-negative bacteria: from the cellular to the community level. Genes. 2020 Oct 22; 11(11). https://doi.org/10.3390/genes11111238 PMID: 33105635

5. Burrus V, Waldor MK. Genome plasticity and the evolution of microbial genomes: shaping bacterial genomes with integrative and conjugative elements. Res Microbiol. 2004 Jun 1; 155(5):376–86.

6. Wawrzyniak P, Plucieniczak G, Bartosik D. The different faces of rolling-circle replication and its multifunctional initiator proteins. Front Microbiol. 2017;8. https://doi.org/10.3389/fmicb.2017.02353 PMID: 29259047

7. Delavat F, Moritz R, van der Meer JR. Transient replication in specialized cells favors transfer of an integrative and conjugative element. mBio. 2019 Jun 11; https://doi.org/10.1128/mBio.01133-19 PMID: 31186329
8. Lee CA, Babic A, Grossman AD. Autonomous plasmid-like replication of a conjugal transposon. Mol Microbiol. 2010 Jan 1; 75(2):268–79. https://doi.org/10.1111/j.1365-2958.2009.06985.x PMID: 19943900

9. Wright LD, Grossman AD. Autonomous replication of the conjugal transposon Tn916. J Bacteriol. 2016 Dec 15; 198(24):3355–66. https://doi.org/10.1128/JB.00639-16 PMID: 27698087

10. Delavat F, Mitr S, Pelet S, van der Meer JR. Highly variable individual donor cell fates characterize robust horizontal gene transfer of an integrative and conjugal element. Proc Natl Acad Sci. 2016 Jun 14; 113(24). https://doi.org/10.1073/pnas.1604479113 PMID: 27247406

11. Burrus V. Mechanisms of stabilization of integrative and conjugal elements. Curr Opin Microbiol. 2017 Aug; 38:44–50. https://doi.org/10.1016/j.mib.2017.03.014 PMID: 28482230

12. Delavat F, Miyazaki R, Carraro N, Pradervand N, van der Meer JR. The hidden life of integrative and conjugal elements. FEMS Microbiol Rev. 2017 Jul 1; 41(4):512–37. https://doi.org/10.1093/femsre/fux008 PMID: 28369623

13. Carraro N, Poulin D, Burrus V. Replication and active partition of integrative and conjugal elements (ICEs) of the SXT/R391 family: the line between ICEs and conjugal plasmids is getting thinner. PLOS Genet. 2015 Jun 10; 11(6):e1005298. https://doi.org/10.1371/journal.pgen.1005298 PMID: 26061412

14. Guérillot R, Da Cunha V, Sauvage E, Bouchier C, Glaser P. Modular evolution of Tn916 Bs1 of Bacillus subtilis. Mol Microbiol. 2007 Dec 1; 66(6):1356–69. https://doi.org/10.1111/j.1365-2958.2007.05748.x PMID: 17511812

15. Lee CA, Grossman AD. Identification of the origin of transfer (oriT) and DNA relaxase required for conjugation of the integrative and conjugal element ICEBs1 of Bacillus subtilis. J Bacteriol. 2007 Oct 15; 189(20):7254–61. https://doi.org/10.1128/JB.00932-07 PMID: 17693500

16. Ramsay JP, Sullivan JT, Stuart GS, Ronson CW. Excision and transfer of the Mesorhizobium loti R7A symbiosis island requires an integrase IntS, a novel recombination directionality factor RdS, and a putative relaxase RtxS. Mol Microbiol. 2006; 62(3):723–34. https://doi.org/10.1111/j.1365-2958.2006.05396.x PMID: 17076666

17. Auchtung JM, Lee CA, Monson RE, Lehman AP, Grossman AD. Regulation of a Bacillus subtilis mobile genetic element by intercellular signaling and the global DNA damage response. Proc Natl Acad Sci. 2005 Aug 30; 102(35):12554–9. https://doi.org/10.1073/pnas.0505835102 PMID: 16105942

18. Auchtung JM, Lee CA, Garrison KL, Grossman AD. Identification and characterization of the immunity repressor (ImmR) that controls the mobile genetic element ICEBs1 of Bacillus subtilis. Mol Microbiol. 2007 Jun 1; 64(6):1515–28. https://doi.org/10.1111/j.1365-2958.2007.05748.x PMID: 17511812

19. Lee CA, Auchtung JM, Monson RE, Grossman AD. Identification and characterization of int (integrase), xis (excisionase) and chromosomal attachment sites of the integrative and conjugal element ICEBs1 of Bacillus subtilis. Mol Microbiol. 2007 Dec 1; 66(6):1356–69. https://doi.org/10.1111/j.1365-2958.2007.06005.x PMID: 18005101

20. Babic A, Berkmen MB, Lee CA, Grossman AD. Efficient gene transfer in bacterial cell chains. mBio. 2011 Apr 29; 2(2):e00027-11. https://doi.org/10.1128/mBio.00027-11 PMID: 21406598

21. Bose B, Auchtung JM, Lee CA, Grossman AD. A conserved anti-repressor controls horizontal gene transfer by proteolysis. Mol Microbiol. 2008 Nov 1; 70(3):570–82. https://doi.org/10.1111/j.1365-2958.2008.06414.x PMID: 18761623

22. Bose B, Grossman AD. Regulation of horizontal gene transfer in Bacillus subtilis by activation of a conserved site-specific protease. J Bacteriol. 2011 Jan 1; 193(1):22–9. https://doi.org/10.1128/JB.01143-10 PMID: 21036995

23. Menard KL, Grossman AD. Selective pressures to maintain attachment site specificity of integrative and conjugal elements. PLOS Genet. 2013 Jul 18; 9(7):e1003623. https://doi.org/10.1371/journal.pgen.1003623 PMID: 23874222

24. Boulos L, Prévost M, Barbeau B, Coallier J, Desjardins R. LIVE/DEAD BacLight: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. J Microbiol Methods. 1999 Jul 1; 37(1):77–86. https://doi.org/10.1016/s0167-7012(99)00048-2 PMID: 10395466

25. Barns KJ, Weishaar JC. Real-time attack of LL-37 on single Bacillus subtilis cells. Biochim Biophys Acta. 2013 Jun 1; 1828(6):1511–20. https://doi.org/10.1016/j.bbamem.2013.02.011 PMID: 23454064

26. Lamsa A, Liu WT, Dorrestein PC, Pogliano K. The Bacillus subtilis cannibalism toxin SDP collapses the proton motive force and induces autolysis. Mol Microbiol. 2012; 84(3):486–500. https://doi.org/10.1111/j.1365-2958.2012.08038.x PMID: 22469514
27. Smith TJ, Blackman SA, Foster SJ. Autolysins of *Bacillus subtilis*: multiple enzymes with multiple functions. Microbiology. 2000 Feb 1; 146(2):249–62. https://doi.org/10.1099/00221287-146-2-249 PMID: 10708363

28. Thomas J, Lee CA, Grossman AD. A conserved helicase processivity factor is needed for conjugation and replication of an integrative and conjugative element. PLOS Genet. 2013 Jan 10; 9(1):e1003198. https://doi.org/10.1371/journal.pgen.1003198 PMID: 23326247

29. Goranov AI, Kuester-Schoeck E, Wang JD, Grossman AD. Characterization of the global transcriptional responses to different types of DNA damage and disruption of replication in *Bacillus subtilis*. J Bacteriol. 2006 Aug 1; 188(15):595–605. https://doi.org/10.1128/JB.00342-06 PMID: 16855250

30. Toyofuku M, Cárcamo-Oyarce G, Yamamoto T, Eisenstein F, Hsiao CC, Kurosawa M, et al. Prophage-triggered membrane vesicle formation through peptidoglycan damage in *Bacillus subtilis*. Nat Commun. 2017 Sep 7; 8(1):1–10. https://doi.org/10.1038/s41467-017-00492-w PMID: 28883390

31. Krogh S, Jørgensen ST, Devine KM. Lysis Genes of the *Bacillus subtilis* defective prophage PBSX. J Bacteriol. 1998 Apr; 180(8):2110–7.

32. Kohm K, Hertel R. The life cycle of SPβ and related phages. Arch Virol. 2021 Aug 1; 166(8):2119–30. https://doi.org/10.1007/s00705-021-05116-9 PMID: 34100162

33. Warner FD, Kitos GA, Romano MP, Hemphill HE. Characterization of SPβ: a temperate bacteriophage from *Bacillus subtilis* 168M. Can J Microbiol. 1977 Jan; 23(1):45–51. https://doi.org/10.1139/m77-006

34. Jones JM, Grinberg I, Eldar A, Grossman AD. A mobile genetic element increases bacterial host fitness by manipulating development. eLife. 2021 Mar 3; 10:e65924. https://doi.org/10.7554/eLife.65924 PMID: 33655883

35. Lécuyer F, Bourassa JS, Gélinas M, Charron-Lamoureux V, Burrus V, Beauregard PB. Biofilm formation drives transfer of the conjugative element ICEBs1 in *Bacillus subtilis*. mSphere. 2018 Oct 31; 3(5). https://doi.org/10.1128/mSphere.00473-18 PMID: 30258041

36. Bourassa JS, Jeanotte G, Lebel-Beaucage S, Beauregard PB. Second-generation transfer mediates efficient propagation of ICEBs1 in biofilms. J Bacteriol. 2022 Sep 15; 0(0):e00181–22. https://doi.org/10.1128/JB.00181-22 PMID: 36106856

37. Lewis JA, Hatfull GF. Control of directionality in integrase-mediated recombination: examination of recombination directionality factors (RDFs) including Xis and Cox proteins. Nucleic Acids Res. 2001 Jun 1; 29(11):2205–16. https://doi.org/10.1093/nar/29.11.2205 PMID: 11376138

38. Hoekstra WPM, de Haan PG. Host controlled modification of phage lambda in zygotic induction in *Escherichia coli*. B. Antonie Van Leeuwenhoek. 1963 Dec 1; 29(1):292–6. https://doi.org/10.1007/BF02046071 PMID: 14068461

39. Leffers GG, Gottesman S. Lambda Xis degradation in vivo by Lon and FtsH. J Bacteriol. 1998 Mar 15; 180(6):1573–7.

40. Stuy JH. Plasmid transfer in *Haemophilus influenzae*. J Bacteriol. 1979 Aug; 139(2):520–9. https://doi.org/10.1128/JB.139.2.520-529.1979

41. Stuy JH. Chromosomally integrated conjugative plasmids are common in antibiotic-resistant *Haemophilus influenzae*. J Bacteriol. 1980 Jun; 142(3):925–30. https://doi.org/10.1128/jb.142.3.925-930.1980

42. Carraro N, Libante V, Morel C, Decaris B, Charron-Bourgoin F, Leblond P, Guedon G. Differential regulation of two closely related integrative and conjugative elements from *Streptococcus thermophilus*. BMC Microbiol. 2011 Oct 24; 11:238. https://doi.org/10.1186/1471-2180-11-238 PMID: 22024428

43. Poulsen P, Ørskov I. Lambda Xis manages the attachment of phage lambda in zygotic induction in *Escherichia coli*. B. Antonie Van Leeuwenhoek. 1963 Dec 1; 29(1):292–6. https://doi.org/10.1007/BF02046071 PMID: 14068461

44. Leffers GG, Gottesman S. Lambda Xis degradation in vivo by Lon and FtsH. J Bacteriol. 1998 Mar 15; 180(6):1573–7.

45. Stuy JH. Plasmid transfer in *Haemophilus influenzae*. J Bacteriol. 1979 Aug; 139(2):520–9. https://doi.org/10.1128/JB.139.2.520-529.1979

46. Stuy JH. Chromosomally integrated conjugative plasmids are common in antibiotic-resistant *Haemophilus influenzae*. J Bacteriol. 1980 Jun; 142(3):925–30. https://doi.org/10.1128/jb.142.3.925-930.1980

47. Carraro N, Libante V, Morel C, Decaris B, Charron-Bourgoin F, Leblond P, Guedon G. Differential regulation of two closely related integrative and conjugative elements from *Streptococcus thermophilus*. BMC Microbiol. 2011 Oct 24; 11:238. https://doi.org/10.1186/1471-2180-11-238 PMID: 22024428

48. Poulsen P, Ørskov I. Lambda Xis manages the attachment of phage lambda in zygotic induction in *Escherichia coli*. B. Antonie Van Leeuwenhoek. 1963 Dec 1; 29(1):292–6. https://doi.org/10.1007/BF02046071 PMID: 14068461

49. Leffers GG, Gottesman S. Lambda Xis degradation in vivo by Lon and FtsH. J Bacteriol. 1998 Mar 15; 180(6):1573–7.

50. Stuy JH. Plasmid transfer in *Haemophilus influenzae*. J Bacteriol. 1979 Aug; 139(2):520–9. https://doi.org/10.1128/JB.139.2.520-529.1979
48. Low KB. Conjugational methods for mapping with Hfr and F-prime strains. Methods Enzymol. 1991; 204:43–62. https://doi.org/10.1016/0076-6879(91)04005-9 PMID: 1943783

49. Sarathy PV, Siddiqi O. Effect of mating on cell division in Escherichia coli Hfr. Mol Genet Genomics. 1973 Jun 1; 125(2):133–7. https://doi.org/10.1007/BF00268866 PMID: 4590268

50. Clokie MR, Millard AD, Letarov AV, Heaphy S. Phages in nature. Bacteriophage. 2011; 1(1):31–45. https://doi.org/10.4161/bact.1.1.14942 PMID: 21687533

51. Fornelos N, Browninger DF, Pavlin A, Podlesek Z, Hodnik V, Salas M, et al. Lytic gene expression in the temperate bacteriophage GIL01 is activated by a phage-encoded LexA homologue. Nucleic Acids Res. 2018 Oct 12; 46(19):9432–43. https://doi.org/10.1093/nar/gky646 PMID: 30053203

52. Brady A, Felipe-Ruiz A, Gallego del Sol F, Marina A, Quiles-Puchalt N, Penades JR. Molecular basis of lysis–lysogenic decisions in Gram-positive phages. Annu Rev Microbiol. 2021 Oct 8; 75(1):563–81. https://doi.org/10.1146/annurev-micro-033121-020757 PMID: 34343015

53. Casjens SR, Hendrix RW. Bacteriophage lambda: Early pioneer and still relevant. Virology. 2015; 479–480:310–30. https://doi.org/10.1016/j.virol.2015.02.010 PMID: 25742714

54. Bean EL, Herman C, Anderson ME, Grossman AD. Biology and engineering of integrative and conjugative elements: construction and analyses of hybrid ICEs reveal element functions that affect species-specific efficiencies. PLOS Genet. 2022 May 18; 18(5):e1009998. https://doi.org/10.1371/journal.pgen.1009998 PMID: 35584135

55. Brophy JAN, Triassi AJ, Adams BL, Renberg RL, Stratis-Cullum DN, Grossman AD, et al. Engineered integrative and conjugative elements for efficient and inducible DNA transfer to undomesticated bacteria. Nat Microbiol. 2018 Sep; 3(9):1043–53. https://doi.org/10.1038/s41564-018-0216-5 PMID: 30127494

56. Grossman AD, Losick R. Extracellular control of spore formation in Bacillus subtilis. Proc Natl Acad Sci. 1988 Jun 1; 85(12):4369–73. https://doi.org/10.1073/pnas.85.12.4369 PMID: 31327111

57. Harwood CR, Cutting SM. Molecular biological methods for Bacillus. Chichester, New York: Wiley; 1990.

58. Berkmen MB, Lee CA, Loveday EK, Grossman AD. Polar positioning of a conjugation protein from the integrative and conjugative element ICEBs1 of Bacillus subtilis. J Bacteriol. 2010 Jan 1; 192(1):38–45. https://doi.org/10.1128/JB.00860-09 PMID: 19734305

59. Perego M, Spiegelman GB, Hoch JA. Structure of the gene for the transition state regulator, abrB: regulator synthesis is controlled by the spo0A sporulation gene in Bacillus subtilis. Mol Microbiol. 1988 Nov; 2(6):689–99. https://doi.org/10.1111/j.1365-2958.1988.tb0079.x PMID: 3145384

60. Smith JL, Goldberg JM, Grossman AD. Complete genome sequences of Bacillus subtilis subsp. subtilis laboratory strains JH642 (AG174) and AG1839. Genome Announc. 2014 Aug 28; 2(6):689–99. https://doi.org/10.1128/genomeA.00664-10 PMID: 24994804

61. Schons-Fonseca L, Lazova MD, Smith JL, Anderson ME, Grossman AD. Beneficial and detrimental genes in the cellular response to replication arrest. PLOS Genet. 2022 Dec 27; 18(12):e1009564. https://doi.org/10.1371/journal.pgen.1009564 PMID: 36652224

62. Karmazyn-Campelli C, Fluss L, Leighton T, Strager P. The spoIIA27(ts) mutation affects the FtsA protein of Bacillus subtilis. Biochimie. 1992 Aug; 74(7–8):689–94. https://doi.org/10.1016/0300-9084(92)90141-z PMID: 1391048

63. Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA, Smith HO. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods. 2009 May; 6(5):343–5. https://doi.org/10.1038/nmeth.1318 PMID: 19363495

64. Albert H, Dale EC, Lee E, Ow DW. Site-specific integration of DNA into wild-type and mutantlox sites placed in the plant genome. Plant J. 1995; 7(4):649–59. https://doi.org/10.1046/j.1365-313X.1995.7040649.x PMID: 7742860

65. Araki K, Okada Y, Araki M, Yamamura K. Comparative analysis of right element mutantlox sites on recombination efficiency in embryonic stem cells. BMC Biotechnol. 2010 Mar 31; 10(1):29. https://doi.org/10.1186/1472-6879(10)00664-4 PMID: 20356367

66. Kawai Y, Moriya S, Ogasawara N. Identification of a protein, YneA, responsible for cell division suppression during the SOS response in Bacillus subtilis. Mol Microbiol. 2003; 47(4):1113–22. https://doi.org/10.1046/j.1365-2958.2003.03360.x PMID: 12581363

67. Au N, Kuester-Schoeck E, Mandava V, Bothwell LE, Canny SP, Chachu K, et al. Genetic composition of the Bacillus subtilis SOS System. J Bacteriol. 2005 Nov; 187(22):7655–66. https://doi.org/10.1128/JB.187.22.7655-7666.2005

68. Meisner J, Montero Llopis P, Sham LT, Garner E, Bernhardt TG, Rudner DZ. FtsEX is required for Cwo peptidoglycan hydrolase activity during cell wall elongation in Bacillus subtilis. Mol Microbiol. 2013; 89(6):1069–83. https://doi.org/10.1111/mmi.12330 PMID: 23857774
69. Jürgen B, Schweder T, Hecker M. The stability of mRNA from the gsIB gene of Bacillus subtilis is dependent on the presence of a strong ribosome binding site. Mol Gen Genet MGG. 1998 Jun; 258(5):538–45. https://doi.org/10.1007/s004380050765 PMID: 9669336

70. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods. 2012 Jul; 9(7):676–82. https://doi.org/10.1038/nmeth.2019 PMID: 22743772

71. Paintdaki A, Parry B, Campos M, Irnov I, Elf J, Surovtsev I, et al. Oufti: an integrated software package for high-accuracy, high-throughput quantitative microscopy analysis. Mol Microbiol. 2016 Feb 1; 99(4):767–77. https://doi.org/10.1111/mmi.13264 PMID: 26538279