Selective Pressures to Maintain Attachment Site Specificity of Integrative and Conjugative Elements

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Abstract

Integrative and conjugative elements (ICEs) are widespread mobile genetic elements that are usually found integrated in bacterial chromosomes. They are important agents of evolution and contribute to the acquisition of new traits, including antibiotic resistances. ICEs can excise from the chromosome and transfer to recipients by conjugation. Many ICEs are site-specific in that they integrate preferentially into a primary attachment site in the bacterial genome. Site-specific ICEs can also integrate into secondary locations, particularly if the primary site is absent. However, little is known about the consequences of integration of ICEs into alternative attachment sites or what drives the apparent maintenance and prevalence of the many ICEs that use a single attachment site. Using ICEBs1, a site-specific ICE from Bacillus subtilis that integrates into a tRNA gene, we found that integration into secondary sites was detrimental to both ICEBs1 and the host cell. Excision of ICEBs1 from secondary sites was impaired either partially or completely, limiting the spread of ICEBs1. Furthermore, induction of ICEBs1 gene expression caused a substantial drop in proliferation and cell viability within three hours. This drop was dependent on rolling circle replication of ICEBs1 that was unable to excise from the chromosome. Together, these detrimental effects provide selective pressure against the survival and dissemination of ICEs that have integrated into alternative sites and may explain the maintenance of site-specific integration for many ICEs.

Introduction

Integrative and conjugative elements (ICEs, also known as conjugative transposons) are mobile genetic elements that encode conjugation machinery that mediates their transfer from cell to cell. Most characterized ICEs were identified because they carry additional genes that confer phenotypes to the host cell. These can be genes involved in pathogenesis, symbiosis, and antibiotic resistances, among others (reviewed in [1]). ICEs are typically found integrated in the host bacterial chromosome and can excise to form a circular product that is the substrate for conjugation. Their ability to spread to other organisms through conjugation makes ICEs important agents of horizontal gene transfer in bacteria, and they appear to be more prevalent than plasmids [2]. ICEs can also facilitate transfer (mobilization) of other genetic elements [1,3,4].

Some ICEs have a specific integration (attachment or insertion) site in the host genome whereas others are more promiscuous and can integrate into many locations. For example, SXT, an ICE in Vibrio cholerae has one primary site of integration in the 5' end of pyC [5]. In contrast, Tn916 has a preference for AT-rich DNA in many different hosts and integrates into many different chromosomal sites [6,7]. Each strategy for integration has its benefits. The more promiscuous elements can acquire a wider range of genes adjacent to the integration sites, and their spread is not limited to organisms with a specific attachment site. On the other hand, site-specific elements are much less likely to disrupt important genes. The attachment site for these elements is typically in a conserved gene, often a tRNA gene [8,9]. If sequences at the end of the integrating element are identical with the 3' end of the gene (which is often the case), then gene function is not disrupted. Integration into conserved genes makes it likely that many organisms will have a safe place for these elements to integrate. We wished to learn more about the ability of site-specific ICEs to integrate into secondary integration (or attachment) sites, particularly if the primary site is not present in a genome. We wondered if an ICE could function normally in a secondary site and if there was any effect on the host.

We used ICEBs1 of Bacillus subtilis to analyze effects of integration into secondary attachment sites. ICEBs1 is a site-specific conjugative transposon that is normally found integrated into a tRNA gene (trnS-leu2) [10,11]. ICEBs1 is approximately 20 kb (Figure 1), and many of its genes are similar to genes in other ICEs, including those in Tn916 [11,12], the first conjugative transposon identified [13,14]. It is not known what properties or advantages ICEBs1 confers on host cells, and naturally occurring ICEBs1 is not known to carry genes involved in antibiotic resistances, virulence, or metabolism. However, because of the conservation of many of its functions, the ease of manipulating B. subtilis, and the high efficiency of experimental induction of gene expression, ICEBs1 is extremely useful for studying basic and conserved properties of ICEs.

Induction of ICEBs1 gene expression leads to excision from the chromosome in >90% of the cells, autonomous rolling-circle
Author Summary

Integrative and conjugal elements (ICEs) are mobile genetic elements that transfer DNA between bacteria, driving bacterial evolution and the acquisition of new traits, including antibiotic resistances. ICEs normally reside integrated in a host genome, but can excise and transfer to recipient cells. Many ICEs are site-specific, predominantly integrating into a single “attachment” site. Others are more promiscuous, capable of integrating into many different sites. Little is known about the consequences of a site-specific ICE integrating into an alternative attachment site, or the selective pressures that maintain the specificity of integration for ICEs with a single attachment site. We found that integration of ICEBs1, a site-specific ICE in Bacillus subtilis, into alternative attachment sites was detrimental to both ICEBs1 and the host cell. Excision of ICEBs1 from alternative attachment sites was reduced or eliminated, thereby limiting mobility of the element. In addition, when ICEBs1 gene expression was activated, cell proliferation and viability dropped if ICEBs1 was in an alternative attachment site. This drop was due to autonomous replication of ICEBs1 that was stuck in the host chromosome. These detrimental effects likely provide strong selective pressure to maintain attachment site specificity of ICEBs1 and likely many other site-specific ICEs.

Results

Identification of secondary sites of integration of ICEBs1

We identified 27 independent insertions of ICEBs1 into secondary integration sites in the B. subtilis chromosome. Briefly, these insertions were identified by: 1) mating ICEBs1 into a recipient strain deleted for the primary attachment site attB (located in the tRNA gene trnS-leu2), 2) isolating independent transconjugants, and 3) determining the site of insertion in each of 27 independent isolates. The frequency of stable acquisition of ICEBs1 by strains missing attB was reduced to ~0.5–5% of that of strains containing attB [Materials and Methods, and [Materials and Methods, and 10]].

There were 15 different secondary integration sites for ICEBs1 among the 27 independent transconjugants (Figure 2). Seven of the 15 sites were described previously [10], and eight additional sites are reported here. There appears to be no absolute bias for replication of ICEBs1, and mating in the presence of appropriate recipients [10,11,15]. After excision from the chromosome, autonomous replication of ICEBs1 is needed for its stability during cell growth and division [15]. In addition, excision is not needed for replication; ICEBs1 that is unable to excise from the chromosome undergoes autonomous unidirectional replication following induction of ICEBs1 gene expression [15]. At least some other ICEs appear to undergo autonomous replication [1,16–18]. In addition, the genes in ICEBs1 that are required for autonomous replication are conserved [19]. Based on these observations and the properties of ICEBs1, we suspect that many ICEs undergo rolling circle replication and use the origin of transfer as an origin of replication and the cognate conjugal relaxase as a replicative relaxase [3,19].

Our aim was to examine the physiological consequences of integration of ICEBs1 into secondary attachment sites. Previous work showed that in the absence of its primary attachment site (attB in the gene for tRNA-leu2), ICEBs1 integrates into secondary attachment sites [10]. Seven different sites were identified and characterized previously, providing insight into the chromosomal sequences needed for integration [10]. Work presented here extends these findings by identifying additional secondary sites, evaluating the ability of ICEBs1 to excise from these sites, and determining the effects of integration at these sites on host cells. Our results indicate that integration of ICEBs1 in secondary integration sites is deleterious to ICEBs1 and to the host cell. Excision and spread of ICEBs1 from the secondary sites was reduced or eliminated and there was a drop in cell viability due to autonomous replication of ICEBs1 that was defective in excision. These effects likely provide strong selective pressure for insertions into sites from which ICEBs1 can excise and against the propagation of insertions in secondary sites.

Figure 1. Map of ICEBs1 and its derivatives. A. The linear genetic map of ICEBs1 integrated in the chromosome. Open arrows indicate open reading frames and the direction of transcription. Gene names are indicated above or below the arrows. The origin of transfer (oriT) is indicated by a thick black line overlapping the 3’ end of conQ and the 5’ end of nick. oriT functions as both the ICEBs1 origin of transfer and origin of replication [15,23]. The thin black arrow indicates the direction of ICEBs1 rolling-circle replication. The small rectangles at the ends of ICEBs1 represent the 60 bp direct repeats that contain the site-specific recombination sites in the left and right attachment sites, attL and attR, that are required for excision of the element from the chromosome. B–F. Various deletions of ICEBs1 were used in this study. Thin horizontal lines represent regions of ICEBs1 that are present and gaps represent regions that are deleted. Antibiotic resistance cassettes that are inserted are not shown for simplicity. B. rapI and phrI are deleted and a kanamycin resistance cassette inserted. C. The right attachment site (attR) is deleted and a tetracycline resistance cassette inserted. D. The genes from the 5’ end of nick and into yddM are deleted and a chloramphenicol resistance cassette inserted. E. The genes from the 5’ end of ydcS and into yddM are deleted and a chloramphenicol resistance cassette inserted. F. The entire coding sequence of helP (previously known as ydcP) and 35 bp in the helP-ydcQ intergenic region is removed. There is no antibiotic resistance cassette in this construct.

doi:10.1371/journal.pgen.1003623.g001
The slash marks represent the approximate location of the ICE\textsubscript{Bs1} insertion site. The name of the gene near which (gyxA) or into which (all other locations) ICE\textsubscript{Bs1} inserted is indicated on the outside of the circle. The arrows on the inside of the circle indicate the direction of ICE\textsubscript{Bs1} replication for each insertion. *\textit{trans-leu}2 (in bold) contains the primary ICE\textsubscript{Bs1} integration site att\textit{B}. B. DNA sequence of the primary and 15 secondary integration sites. The gene name is indicated on the left, followed by the DNA sequence (chromosomal target). The primary attachment site (att\textit{B}) is a 17 bp sequence with 5 bp inverted repeats (underlined) separated by a 7 bp spacer. Mismatches from att\textit{B} are indicated in bold, capital letters. “mm” indicates the number of mismatches from the primary 17 bp att\textit{B}. “occurrences” indicates the number of independent times an insertion in each site was identified. Percentages of the total (27) are indicated in parenthesis. The * next to yqh\textit{G} indicates that two different ICE\textsubscript{Bs1} insertions were isolated in this gene, once in each orientation. C. Sequence logo of the ICE\textsubscript{Bs1} secondary attachment sites. Using Weblogo 3.3 [21], we generated a consensus motif of the 26 bases surrounding the insertion site of the 15 secondary insertion sites for ICE\textsubscript{Bs1}. For comparison, the primary attachment site for ICE\textsubscript{Bs1} is a 17 bp region with 5 bp inverted repeats and a 7 bp spacer region in the middle [10]. The size of each nucleotide corresponds to the frequency with which that nucleotide was observed in that position in the secondary attachment sites.

doi:10.1371/journal.pgen.1003623.g002

Figure 2. Map and DNA sequence of the primary and 15 secondary integration sites for ICE\textsubscript{Bs1}. A. Approximate position of the primary and 15 secondary ICE\textsubscript{Bs1} integration sites on the \textit{B. subtilis} chromosome. The circle represents the \textit{B. subtilis} chromosome with the origin of replication (ori\textit{C}) indicated by the black rectangle at the top.


doi:10.1371/journal.pgen.1003623.g002
mismatches (out of 17 bp) from the sequence of attB (Figure 2B). These results indicate that in the absence of the primary integration site in trnS-leu2, ICEBs1 can integrate into many different sites throughout the genome, albeit at a lower efficiency [10]. Based on the diversity of the observed secondary attachment sites and the number of sites identified only once, it is clear that we have not identified all of the possible secondary integration sites for ICEBs1.

Integration into the secondary site in ykrM in the presence of a functional attB

We wondered if ICEBs1 could insert into a secondary site in cells in which the primary site, attB, is intact. To test this, ICEBs1 (from donor strain KM250) was transferred by conjugation to an ICEBs1-cured recipient that contained attB (strain KM254). Transconjugants were selected on solid medium and \( \sim 10^8 \) independent transconjugants were pooled. DNA from the pooled transconjugants was then used as a template for quantitative real-time PCR (qPCR) with primers that detected ICEBs1 integrated into ykrM (the most frequently used secondary site). We found that the frequency of integration into ykrM was \( \sim 10^{-3} \) to \( \sim 10^{-2} \) of that into attB. As a control, we performed reconstruction experiments. Known amounts of DNA from two strains, one containing an insertion in ykrM (strain KM72), and the other containing an insertion in attB (strain AG174) were mixed and used as a template in qPCR, analogous to the experiment with DNA from the pooled transconjugants. These reconstruction experiments validated the results determined for the frequency of insertion into ykrM.

Excision of ICEBs1 from secondary integration sites is reduced

We wished to determine if there were any deleterious consequences of integration of ICEBs1 into secondary attachment sites. We found that although ICEBs1 integrated into the secondary integration sites, excision from all of the secondary sites we analyzed was reduced or eliminated. We monitored excision from seven of the secondary sites by overexpressing the activator of ICEBs1 gene expression, RapI, from a regulated promoter (PxyI-rapI) integrated in single copy in the chromosome at the nonessential gene amyE (Materials and Methods). Overproduction of RapI induces ICEBs1 gene expression [11,22] and typically results in excision of ICEBs1 from attB in \( \sim 90\% \) of cells within 1–2 hrs [10,15]. Following a similar protocol as described for monitoring excision from attB [11,15,22], we performed qPCR using genomic DNA as template and primers designed to detect the empty primary attachment site on the circular ICEBs1. The relative amounts of the circular form to the empty attachment site was about the same for insertions in ykrM, mmsA, yycJ, and spoVD, and ykrP, which are predicted to form a heteroduplex and two 5 bp inverted repeats. Integrase-mediated site-specific recombination occurs in the 7 bp spacer (the crossover region) [10] (Figure 3C). If the 7 bp region in a chromosomal attachment site is different from that in ICEBs1, as is the case for mmsA and yycJ, then integration and host replication will create left (attL) and right (attR) ends that have different crossover regions (Figure 3D). Upon excision, these elements are predicted to contain a heteroduplex in the attachment site on the excised circular ICEBs1. Of the four insertions that have readily detectable excision frequencies, two (mmsA and yycJ) are predicted to form a heteroduplex and two (ykrM and sfA4) are not. In the case of mmsA:ICEBs1, the left and right ends are known to have different sequences [10].

Together, our results indicate that excision of ICEBs1 from secondary sites from which a heteroduplex is formed leads to lower excision frequencies. The excision frequencies of ICEBs1 integrated in ykrM and sfA4, the mating efficiencies were approximately what was expected from the reduced excision frequencies.

As expected, the ratio of the amounts of the circular form to the empty attachment site was about the same for insertions in ykrM and sfA4 as for an insertion in attB (Figure 3B). In contrast, the ratio of the circle to the empty attachment site for mmsA and yycJ was significantly less than that for wild type (Figure 3B). Comparing the total amount of the ICEBs1 circle from mmsA and yycJ to that from attB indicated that there was approximately 0.3% as much circle from each site as from attB. This decrease in the amount of ICEBs1 circle is consistent with and likely the cause of the drop in mating efficiency to approximately 0.2% of that of ICEBs1 from attB.

Decreased conjugation of ICEBs1 from secondary sites that generate a heteroduplex

We measured the mating efficiencies of ICEBs1 following excision from the four secondary attachment sites from which excision was reduced but detectable. Excision of ICEBs1 is required for transfer of the element to recipient cells. Thus, if the ICEBs1 circle is stable, then the mating efficiencies should be proportional to the excision frequency. The mating efficiencies of ICEBs1 from ykrM and sfA4 were \( \sim 2\% \) of that of ICEBs1 from attB. Likewise, the excision frequencies of ICEBs1 integrated in ykrM and sfA4 were \( \sim 5\% \) of those of ICEBs1 in attB. These results indicate that for ICEBs1 integrated in ykrM and sfA4, the mating efficiencies were approximately what was expected from the reduced excision frequencies.

We measured the relative amounts of circular ICEBs1 after excision from ykrM, sfA4, mmsA, and yycJ, the four insertions with reduced but detectable excision, using qPCR primers designed to detect only the circular form of ICEBs1. The relative amounts of each circle were compared to the relative amount of the empty attachment site from which ICEBs1 excised. Measurements were made two hours after induction of ICEBs1 gene expression (overproduction of RapI).

As expected, the ratio of the amounts of the circular form to the empty attachment site was the same for insertions in ykrM and sfA4 as for an insertion in attB (Figure 3B). In contrast, the ratio of the circle to the empty attachment site for mmsA and yycJ was significantly less than that for wild type (Figure 3B). Comparing the total amount of the ICEBs1 circle from mmsA and yycJ to that from attB indicated that there was approximately 0.3% as much circle from each site as from attB. This decrease in the amount of ICEBs1 circle is consistent with and likely the cause of the drop in mating efficiency to approximately 0.2% of that of ICEBs1 from attB.

The decrease in the amount of circular ICEBs1 from mmsA and yycJ is likely due to the formation of a heteroduplex in the attachment site on the circular ICEBs1. The ICEBs1 attachment site contains a 17 bp sequence with a 7 bp spacer region between 5 bp inverted repeats. Integrase-mediated site-specific recombination occurs in the 7 bp spacer (the crossover region) [10] (Figure 3C). If the 7 bp region in a chromosomal attachment site is different from that in ICEBs1, as is the case for mmsA and yycJ, then integration and host replication will create left (attL) and right (attR) ends that have different crossover regions (Figure 3D). Upon excision, these elements are predicted to contain a heteroduplex in the attachment site on the excised circular ICEBs1. Of the four insertions that have readily detectable excision frequencies, two (mmsA and yycJ) are predicted to form a heteroduplex and two (ykrM and sfA4) are not. In the case of mmsA:ICEBs1, the left and right ends are known to have different sequences [10].

Together, our results indicate that excision of ICEBs1 from secondary sites from which a heteroduplex is formed leads to lower levels of the circular ICEBs1 heteroduplex and a reduction in the ability of ICEBs1 to transfer to other cells. We do not yet know...
Excision of ICEBs1 from secondary attachment sites.

A–B. Excision frequencies and relative amounts of the excision products (circular ICEBs1 and empty chromosomal site) were determined as described in Materials and Methods. Cells were grown in defined minimal medium with arabinose as carbon source. Products from excision were determined two hours after addition of xylose to induce expression of PxyI::rapI to cause induction of ICEBs1 gene expression. Primers for qPCR were unique to each attachment site. Strains used include: wt, that is, ICEBs1 inserted in attB (CAL874); ΔattR, ICEBs1 integrated in attB, but with the right attachment site deleted and ICEBs1 unable to excise (Figure 1) (CAL872); ymmA::ICEBs1 (KM70); ymmB::ICEBs1 (KM72); yrkM::ICEBs1 (KM141); yyrC::ICEBs1 (KM132); yrkP::ICEBs1 (KM77); spoVD::ICEBs1 (KM130); yvbT::ICEBs1 (KM94). Each strain was assayed at least three times (biological replicates) and qPCR was done in triplicate on each sample. Error bars represent standard deviation.

A. Frequency of excision of ICEBs1 from the indicated site of integration. The relative amount of the empty chromosomal attachment site was determined and normalized to the chromosomal gene cotF. Data were also normalized to a strain with no ICEBs1 (JMA222), which represents 100% excision.

B. Relative amount of circular ICEBs1 compared to the amount of empty chromosomal attachment site for the indicated insertions. The relative amount of the ICEBs1 circle, normalized to cotF, was divided by the relative amount of the empty attachment site, also normalized to cotF. These ratios were then normalized to those for wild type.

C. Cartoon of integration of ICEBs1 into its primary bacterial attachment site attB. attB is identical to the attachment site on ICEBs1, attICEBs1. They consist of a 17 bp region with 5 bp inverted repeats (gray boxes) on each side of a 7 bp spacer region (white box). During integration and excision, a recombination event occurs in the 7 bp spacer (crossover) region [38].

D. Cartoon of integration of ICEBs1 into secondary integration sites. A secondary integration site is indicated with a black box. When ICEBs1 integrates into a secondary site, the crossover regions in attICEBs1 and that of the secondary site are not necessarily identical, potentially creating a mismatch. This mismatch, if not repaired, will be resolved by host replication, generating left and right ends with different crossover sequences. Excision would then create a circular ICEBs1 with a heteroduplex in the attachment site on ICEBs1.

Table 1. Summary of properties of several ICEBs1 insertions in secondary attachment sites.

| Insertion site | Excision frequency | Viability | dinC-lacZ |
|----------------|--------------------|-----------|-----------|
| attB           | 1.0                | 1.0       | 1.0       |
| yrkM (2)       | 0.06               | 0.030     | 34        |
| mmsA (3)       | 0.15               | 0.14      | 6.1       |
| srfAA (3)      | 0.04               | 0.10      | 8.7       |
| yycJ (7)       | 0.12               | 0.073     | N.D.      |
| spoVD (8)      | < 10^-4            | 0.010     | N.D.      |
| ykrP (12)      | < 10^-4            | 0.040     | 4.1       |
| yvbT (11)      | < 10^-4            | 0.0038    | 24        |
| ΔattB          | < 10^-4            | 0.092     | 6.7       |

1 site of insertion of ICEBs1; #mm indicates the number of mismatches between the insertion site and attB (illustrated in Figure 2).

2 excision frequency measured as the empty attachment site 2 hrs after induction of ICEBs1 gene expression; normalized to wt; same data as in Figure 3, except that here data is normalized to wt (attB). Excision frequency from attB was 1.

3 cell viability normalized to ICEBs1 at attB; same data as in Figure 4. Viability of ICEBs1 at attB was 0.9 of uninduced.

4 expression of damage inducible gene dinC-lacZ, normalized to that of cells with ICEBs1 in attB; data from Figure 6. ß-galactosidase specific activity of ICEBs1 at attB was 0.3.

doi:10.1371/journal.pgen.1003623.g003
what causes the lower amounts of the ICEBs1 heteroduplex. Loss of the DNA mismatch repair gene mutS did not alter the instability of the ICEBs1 heteroduplex (unpublished results), indicating that mismatch repair is not solely responsible for this effect. Nonetheless, the overall reduction in transfer is due to both decreased excision and further decreased amounts of the excised element. Both of these defects provide barriers to the spread of ICEBs1 from secondary attachment sites.

**ICEBs1 returns to attB following excision and conjugation from secondary sites**

We found that when ICEBs1 excises from a secondary site and transfers to wild type cells via conjugation it tends to integrate in the primary attachment site, attB, and not in a secondary site. Donors with ICEBs1 in ykrM, mms4, yycJ, and sfkA were crossed with a recipient (strain KM110) containing attB (and all known secondary sites). Individual transconjugants from each cross were isolated and tested by PCR for the presence of ICEBs1 in attB. ICEBs1 was present in attB in 9 of 10 transconjugants from ykrM::ICEBs1 donors, 9 of 9 transconjugants from mms4::ICEBs1 donors, 9 of 10 transconjugants from yycJ::ICEBs1 donors, and 10 of 10 transconjugants from sfkA::ICEBs1 donors. In the two cases where ICEBs1 was not in attB, it was not present in the secondary site from which it came. We confirmed, using PCR primers internal to ICEBs1, that ICEBs1 was present in the transconjugants. Thus, we conclude that even if ICEBs1 is able to excise from a secondary attachment site, there is a strong bias in returning to the primary site if that site is present in a transconjugant.

We also found that if attB is not present in recipients during conjugation, then ICEBs1 integrates into a secondary attachment site, but with no apparent bias for the site from which it originated. We crossed donors with ICEBs1 in ykrM, mms4, and sfkA with a recipient missing attB (strain KM111), and tested individual transconjugants for integration into the cognate site from which ICEBs1 excised in the donor. With the ykrM::ICEBs1 donor, 1 of 6 transconjugants had ICEBs1 in ykrM. With the mms4::ICEBs1 donor, none of the 10 transconjugants tested had ICEBs1 integrated in mms4. With the sfkA::ICEBs1 donor, none of the four transconjugants tested had ICEBs1 in sfkA. Together, these results indicate that ICEBs1 has a strong preference to integrate into attB, even when it starts from a secondary site, and that if attB is not available, ICEBs1 tends to go to a secondary site, with no apparent preference for the original location.

**Decreased proliferation and viability of strains in which ICEBs1 has decreased excision**

We found that strains with ICEBs1 in secondary integration sites had a decreased ability to form colonies when ICEBs1 gene expression was induced. We measured colony forming units (CFUs) of several strains with excision-defective (meaning reduced or no detectable excision) ICEBs1 insertions, including ICEBs1 in secondary sites and ICEBs1 ΔattR (in attB), both with and without induction of ICEBs1 gene expression. We also measured CFUs of wild type ICEBs1 (with normal excision frequencies) integrated at attB under similar conditions (Figure 4A). In the absence of RapI expression, when most ICEBs1 genes are repressed, growth and viability of excision-defective strains were indistinguishable from that of excision-competent strains. In contrast, by three hours after induction of ICEBs1 gene expression in excision-defective ICEBs1 strains (ΔattR with ICEBs1 in attB, or insertions in mms4, ykrM, sfkA, yycJ, spoVD, yebT, and ykrP), the number of CFUs was reduced compared to that of the excision-competent ICEBs1 (in attB) (Figure 4A). These results are consistent with previous observations that excision-defective int and xis null mutants have a viability defect when RapI is overproduced [10].

Induction of ICEBs1 in several of the secondary integration sites (insertions in mms4, sfkA, yycJ and ICEBs1 ΔattR in attB) caused a drop in CFU/ml to ~10% of that of strains without ICEBs1 induction or the strain with wild type ICEBs1 at attB (Figure 4A). Induction of ICEBs1 in other insertion sites (yebT, ykrM, spoVD, yebT) caused a more severe drop in viability. The differences in CFU/ml between induced and uninduced cells (three hours after induction) appeared to be the combined effects of both a defect in proliferation (cell division) and cell death (viability). At times ≥3 hrs after induction of ICEBs1 gene expression, the number of CFU/ml dropped to below that before induction of gene expression, indicating that preexisting cells lost viability. For simplicity, we use “viability” to refer to both cell death and the decreased proliferation.

The drop in viability after induction of ICEBs1 in the various insertions did not correlate with dissimilarity of the attachment sites to attB or to the amount of residual excision in the excision-defective strains. For example, the ICEBs1 ΔattR mutant is completely unable to excise, and viability is ~10% three hours after induction of ICEBs1 gene expression. In contrast, ICEBs1 inserted into ykrM has about 5% excision after induction of ICEBs1 gene expression and viability is ~3% (Table 1). Together, these results indicate that something about the specific locations of the insertions is likely causing the more extreme viability defect observed in some of the excision-defective ICEBs1 strains.

One of the most extreme effects on viability after induction of ICEBs1 gene expression is from the insertion in yebT. Within three hours after induction of ICEBs1 gene expression in the yebT::ICEBs1 strain, viability was ~0.3% of that of strains without ICEBs1 induction or of the strain with excision-competent ICEBs1 (Figure 4A). yebT gene product is predicted to be similar to alkana monoxygenases (luciferases). Insertion of ICEBs1 in yebT likely knocks out yebT function, so it seemed possible that the loss of yebT combined with induction of ICEBs1 gene expression was causing the severe drop in viability. To test this hypothesis, we deleted yebT in cells containing ICEBs1 inserted in ykrM and tested for viability after induction of ICEBs1 gene expression. There was no additional drop in viability of the mms4::ICEBs1 yebT null mutant compared to the mms4::ICEBs1 secondary site alone (wild type yebT), either with or without induction of ICEBs1 gene expression. Based on these results, we conclude that the severe defect in viability of the yebT::ICEBs1 secondary site mutant was not due to the loss of yebT function combined with induction of ICEBs1 gene expression. It is also possible the severe drop in viability was due to production of a fragment of the yebT gene product. This possibility seems highly unlikely because the putative fragment alone does not cause a phenotype, rather the drop in viability requires both induction and replication (see below) of ICEBs1. In addition, other insertions also caused a severe drop in viability and it is highly unlikely that each one of these is producing a toxic protein fragment.

We do not know what causes the more severe drop in viability in some insertions. However, the decrease in cell proliferation and viability caused by expression of ICEBs1 in secondary attachment sites should provide selective pressure against the long term survival of these strains. The more severe the loss in viability, the stronger the selective pressure against long term survival of strains with insertions in these sites. Suppressor mutations that alleviate the drop in viability are readily obtained (KLM, C. Lee, ADG, data not shown), although most of these mutations have not been characterized.
ICExs1 replication functions are required for the drop in viability of excision-defective insertions

Because the drop in proliferation and viability in the first few hours after induction of ICExs1 gene expression occurs in ICExs1 excision-defective and not in excision-competent strains, the decreased viability is likely due to a cis-acting property of ICExs1 and not a diffusible ICExs1 product. One of the more dramatic changes following induction of ICExs1 gene expression is induction of multiple rounds of unidirectional rolling circle replication [15]. This replication initiates from the ICExs1 origin of transfer oriT, requires the ICExs1 relaxase encoded by nicK and the helicase processivity factor encoded by helP (previously ydcP) [19]. Rolling circle replication of ICExs1 occurs even when ICExs1 is unable to excise from the chromosome as observed previously for a mutant unable to excise [15]. Therefore, we expected that induction of ICExs1 gene expression in the secondary site insertions would lead to unidirectional rolling circle replication from oriT in the host chromosome (Figure 5). It seemed

two secondary integration sites (ykrP and yvbT) and the excision defective ICExs1 ΔattR. The helP allele is a non-polar deletion [19]. Strains used include: attB::ICExs1 ΔattR::tet (CAL872); attB::ICExs1 ΔhelP ΔattR::tet (KM347); ykrP::ICExs1 (KM77); ykrP::ICExs1 ΔhelP (KM429); yvbT::ICExs1 (KM94); yvbT::ICExs1 ΔhelP (KM459). Data for KM94, KM77, and CAL872 are the same as those shown above in panel A and are shown here for comparison.

doi:10.1371/journal.pgen.1003623.g004
likely that this replication could cause damage to the chromosome and lead to the decrease in cell viability. We tested nicK and the genes downstream for effects on cell viability following induction of ICEBs1 gene expression in the excision-defective insertions. Preliminary experiments indicated that loss of nicK restored viability after induction of ICEBs1 gene expression. However, this effect could have been due to polarity on downstream genes. Unfortunately, nicK null mutants are difficult to fully complement [23], perhaps because NicK might act preferentially in cis. In addition, complementation of other supposedly “non-polar” mutations in ICEBs1 are not complemented fully [19,24]. Therefore, to test if loss of nicK was responsible for the suppression of lethality, or if the suppression was due to loss of expression of a downstream gene, we compared two different deletions in ICEBs1. One deletion removed nicK and most of the downstream genes $\{\Delta(nicK-yddM)\}$ (Figure 1D). In the second deletion, nicK was intact but most of the genes downstream from oriT and nicK were removed $\{\Delta(ydcS-yddM)\}$ (Figure 1E).

We found that deletion of nicK alleviated the growth defect of excision-defective secondary insertions, including mmsA::ICEBs1 and yvrT::ICEBs1 that caused the most severe drop in viability (Figure 4B). Deletion of the genes downstream from nicK did not alleviate the drop in viability (Figure 4B), indicating that expression of these genes (many encoding conjugation functions) was not the cause of the decreased cell viability. In addition, in preliminary experiments, we found that several suppressor mutations that restore viability to an excision-defective ICEBs1 (in this case, at attB) were null mutations in nicK (C. Lee, ADG, unpublished results). Together, these results indicate that a NicK-dependent process is causing the drop in viability of the excision-defective ICEBs1.

NicK creates a nick at a specific site in ICEBs1 oriT [23], and nicking is required for ICEBs1 replication (and conjugation) [15]. To determine if the drop in cell viability was due to nicking per se, or to replication, we used a recently defined ICEBs1 gene, helP, which encodes a helicase processivity factor that is needed for ICEBs1 replication but not for nicking [19,23]. Deletion of helP (Figure 1F) is not polar on nicK and does not affect nicking at oriT [19]. Deletion of helP completely alleviated the growth defect associated with induction of ICEBs1 (Figure 4C).

Based on these results, we conclude that unidirectional rolling circle replication from oriT in the chromosome most likely caused the drop in viability of the excision-defective ICEBs1. The decrease in viability could be due to breaks and degradation of chromosomal DNA around the site of insertion and/or disruptions in host chromosomal replication caused by the multiple rounds of rolling circle replication from oriT (Figure 5).

Induction of the SOS response in strains in which ICEBs1 is defective in excision

We found that induction of ICEBs1 gene expression in the excision-defective insertions caused induction of the host SOS response. Like that in other organisms, the SOS response in B. subtilis results in increased expression of a large set of genes in response to DNA damage or replication stress [25]. We used a lacZ fusion to a damage-inducible gene, dinC-lacZ [26,27], to monitor the SOS response in cells following induction of ICEBs1. Without induction of ICEBs1 gene expression, there was no detectable β-galactosidase activity above background levels, indicating that none of the insertions alone caused elevated SOS gene expression. In all of the excision-defective ICEBs1 strains analyzed (ICEBs1 ΔattR in attB, and insertions in mmsA, yvrT, ykrP, sfrA1, and yrkM), there was a ≥3.5-fold increase in β-galactosidase levels from the dinC-lacZ fusion 3 hrs after induction of ICEBs1 gene expression (Figure 6). In contrast, there was no detectable increase in β-galactosidase activity three hrs after induction of ICEBs1 gene expression in the excision-compotent insertion in attB (Figure 6).

There was no apparent correlation between the amount of SOS induction and the severity of the viability defect. For example, one of the strains with the most severe viability defect (ICEBs1 in ykrP) had a relatively low amount of expression of dinC-lacZ (Figure 6). However, the amount of SOS induction could be an underestimate since many cells in the population lose viability.

Induction of dinC-lacZ in the strains with ICEBs1 in secondary attachment sites was consistent with prior preliminary experiments using DNA microarrays that indicated induction of the SOS response in ICEBs1 int and vs mutants that are incapable of excision (N. Kavanaugh, C. Lee, ADG, unpublished results). Based on these results, we conclude that induction of ICEBs1 gene expression in cells in which ICEBs1 is stuck in the chromosome causes DNA damage that induces the host SOS response. However, the SOS response per se is not what caused cell death.

Discussion

We isolated and characterized insertions of the integrative and conjugative element ICEBs1 of B. subtilis into secondary integration (attachment or insertion) sites. Secondary integration sites appear to be used naturally, even in the presence of the primary site, at a frequency of $\sim 10^{-4}$ to $10^{-3}$ of that of the primary site, indicating that approximately 100–1,000 cells in a population of $\sim 10^6$ transconjugants will have ICEBs1 at a secondary site. We found that insertions in secondary sites are detrimental for the propagation of ICEBs1 and detrimental to the survival of the host cells. These detrimental effects likely provide selective pressure to maintain the already established site-specificity. Below we discuss target site selection among ICEs, aspects of ICEBs1 biology that make insertions into secondary sites detrimental, and the more general implications for the evolution of ICEs.
Target site selection and maintenance of tRNA genes as integration sites

We have identified 15 different secondary insertion sites for ICEBs1. Some of these sites are similar to the primary attachment site, but some are quite different. Based on the diversity of sites, and the isolation of only a single insertion in many of them, it is likely that we are nowhere near saturation for identifying all possible sites in non-essential regions. Given that there is some sequence conservation among the secondary sites, DNA sequence is clearly important in the potential function as an integration site. However, we suspect that other factors also contribute. These factors could include possible roles for nucleoid binding proteins, other DNA binding proteins, transcription, and local supercoiling.

Many site-specific ICEs have preferred integration sites in tRNA genes. This preference is thought to occur, at least in part, because tRNA genes are highly conserved and contain inverted repeats that are typically used as integration targets for site-specific recombinases [9]. We postulate that the selective pressure to maintain site-specific integration in a tRNA gene comes from a combination of factors, including: the conservation of tRNAs, the ability of an ICE to efficiently excise from the primary attachment site, and the decreased cell viability and decreased ability of an ICE to spread when excision is reduced due to integration into a secondary site.

Selective pressures against ICEs in secondary attachment sites

Our results indicate that there are at least two main types of selective pressures against propagation of ICEBs1 that has inserted into a secondary integration site. First, there is strong pressure against the spread of that particular element due to the large defect in its ability to excise and the instability of circular ICEBs1 when it forms a heteroduplex. The excised circular form of an ICE is necessary for its complete transfer to a recipient cell. At least one other ICE has a reduced excision frequency from a secondary integration site. Excision of SXT from a secondary attachment site in Vibrio cholerae was reduced 3–4-fold relative to its ability to excise from the primary attachment site [28]. In addition, lysogenic phages can also have reduced excision efficiencies from secondary attachment sites [29]. Insertion of any type of mobile genetic element into a location from which it has trouble getting out will be deleterious to the further horizontal propagation of that element. Based on our results, this is particularly true for ICEBs1.

In addition to the defect in ICEBs1 excision and transfer from secondary integration sites, there is a decrease in cell viability following induction of ICEBs1 gene expression. ICEBs1 gene expression is normally induced under conditions of starvation or cell crowding when the activator RapI is expressed and active, or when the RecA-dependent SOS response is induced [22]. Induction of ICEBs1 gene expression causes rolling circle replication from the ICEBs1 origin of transfer oriT [15,19]. Our results indicate that rolling circle replication from an element that is unable to efficiently excise from the chromosome causes a drop in cell viability. This drop is likely due to chromosomal damage and stalling of the chromosomal replication forks when they reach the complex structure formed by repeated initiation of rolling circle replication from oriT in the chromosome (Figure 5).

We suspect that autonomous replication is a common property of many ICEs but has not been generally observed because of the low frequency of induction and excision of most of these elements. There are indications that some other ICEs undergo autonomous replication [1,16–18]. If autonomous replication of ICEs is widespread, as we postulate [15,19], then there should be selective pressure against viability of cells in which an ICE is induced, replicates, and is unable to excise.

There were at least two different effects caused by replication of excision-defective elements. Replication from ICEBs1 in the chromosome caused a drop in cell viability of at least 10-fold, but sometimes caused a severe drop, 100–1000-fold in about 3 hrs. We do not know what causes this severe drop in viability, but it requires active replication of the ICEBs1 that is unable to excise from a specific chromosomal location. This severe drop in viability could be due to increased dosage of nearby genes or perhaps differential fragility of these chromosomal regions. In any case, the severe drop in viability provides even stronger selective pressure against propagation of the strains with insertions of ICEBs1 in these locations.

The growth defect associated with the secondary insertions is most obvious when ICEBs1 gene expression is induced. Cells with ICEBs1 insertions in secondary attachment sites might be purged from the population under natural conditions of induction, providing selective pressure against maintenance of integrants in secondary sites and favoring a site-specific strategy of integration and excision.

We estimated the effects of insertions in secondary sites in populations without experimentally induced activation of ICEBs1. The “spontaneous” activation and excision frequency of ICEBs1 in a population of cells is estimated to be approximately one cell in $10^{-3}$ to $10^{-1}$ [10,22,30]. Assuming a frequency of activation of ICEBs1 of $\sim 10^{-3}$ per generation, and that all activated cells with ICEBs1 in a secondary site die, we estimate that it would take ~23,000 generations for a population of cells with ICEBs1 in a secondary site to be 0.1 times the size of a population of cells with ICEBs1 in the primary site. The activation frequency increases under several conditions likely to be more relevant than growth in the lab, including: the presence of cells without ICEBs1, entry into stationary phase, and during the SOS response [10,11,22]. For example, if activation of ICEBs1 actually occurs in 0.1% of cells, then it would take ~2,300 generations for the secondary site insertion population to be 0.1 times the population of cells with ICEBs1 in the primary site. These effects are difficult to measure experimentally, but easy to see when ICEBs1 is efficiently induced.

ICEs with single versus multiple integration sites

ICEs of the Tn916/Tn1545 family can integrate into multiple sites in many organisms, yet they are not known to cause a defect in cell growth when gene expression is induced. Tn916 and most family members contain tetM, a gene encoding resistance to tetracycline. Expression of tetM and Tn916 genes is induced in the presence of tetracycline [31]. Tn916 has two helP (helicase processivity) homologues and we predict that it undergoes autonomous rolling circle replication [19]. Despite relatively low excision frequencies, tetracycline-induced Tn916 gene expression is not known to cause a drop in cell viability. Tetracycline induces expression of several Tn916 genes, including those needed for excision. However, the Tn916 relaxase (orf20), the two helP homologues (orf22 and orf23), and the conjugation genes are not expressed until Tn916 excises and circularizes [31]. Based on analogy to ICEBs1, we have postulated that Tn916 is capable of autonomous rolling circle replication [15] and that the relaxase (orf20) and at least one of the helP homologues are likely needed for this replication [19]. The regulation of Tn916 gene expression specifically prevents expression of these putative replication functions until after excision. Consequently, rolling circle replication of Tn916 cannot occur while the element is integrated in the chromosome. We speculate that some of the evolutionary pressures to establish and maintain a high degree of site specificity
is lost when expression of ICE replication functions does not occur until after excision from the host genome.

Materials and Methods

Media and growth conditions

*Bacillus subtilis* was grown at 37°C in LB or defined S79 minimal medium with arabinose (1%) as carbon source. Antibiotics and other chemicals were used at the following concentrations: isopropyl β-D-1-thiogalactopyranoside (IPTG) (1 mM), chloramphenicol (*cat*, 5 μg/ml), kanamycin (*kan*, 5 μg/ml), spectinomycin (*spc*, 100 μg/ml), erythromycin (0.5 μg/ml) and lincomycin (12.5 μg/ml) together, to select for macrolide-lincosamide-streptogramin B resistance (MLS or erm).

*Bacillus subtilis* strains and alleles

*B. subtilis* strains used are listed in Table 2. All except BTS14 are derived from AG174 [JH642] and contain mutations in *tpc* and *pheA* (not shown). Most of the strains were constructed using natural transformation or conjugation, as described above. Many alleles were previously described, *dnClR:Cm or atm7* is an insertion in the damage-inducible gene *dnClC and creates a transcriptional fusion to lacZ* [27]. Most ICEBs1 strains contained a macrolide-resistance cassette {Δ*rapI-phrI342::kan*} [11]. ICEBs1 was induced by overexpression of *rapI* from a xylose-inducible promoter using *amyE::(Pxyl-rapI-lacZ)* [24] or from an IPTG-inducible promoter using *amyE::(Pspank(hy)-rapI-lacZ)*, *spc* [11]. *ΔattR100::tet* deletes 216 bp spanning the junction between the right end of ICEBs1 and the chromosome [10]. Δ*helP155* is an unmarked 413-bp deletion that removes the entire coding sequence and the 35 bp *helP* yedQ intergenic region (Figure 1F) [19].

*ΔattB* mutant with a compensatory mutation in *trnS-leu1*. *ΔattB:*cat is a deletion-insertion that is missing ICEBs1 and removes 185 bp that normally contains the primary chromosomal ICEBs1 attachment site, resulting in the loss of a functional *trnS-leu2* [10]. Although *trnS-leu2* is non-essential [10,32], cells with *ΔattB* do not grow as well as wild type. To improve the growth of *ΔattB:*cat, we used a compensatory mutation in *trnS-leu1* that changes the anti-codon to that normally found in *trnS-leu2* (C. Lee, & ADG), analogous to the *attF1* mutation previously described [32]. The compensatory mutation was constructed by site-directed mutagenesis using the overlap-extension PCR method [33]. Because *trnS-leu1* and *ΔattB:*cat are genetically linked, we selected for chloramphenicol resistant colonies and screened for the single bp mutation in *trnS-leu1* by sequencing. In addition to the mutant *trnS-leu1* allele (*trnS-leu1-522*), the strain had an additional mutation, (5'-CAAAAAACTAA to 5'-CAAAAAAATAG) in the non-coding region between *ΔattB:*cat and *yedN*. Growth of the resulting strain, CAL522, was indistinguishable from that of wild type. This strain stably acquired ICEBs1 in conjugation experiments at a frequency ~0.5% of that of wild type, approximately 10-fold lower than the strain without the compensatory mutation in *trnS-leu1* [10]. We do not understand the cause of this reproducible difference.

Deletion of *nick* and downstream genes. We constructed two large deletion-insertion mutations in ICEBs1, one removing *nick* and all downstream genes, Δ*nick-yddM::cat*, and the other leaving *nick* intact, but removing the downstream genes, Δ*ydhS-ydhM::cat*. Both deletions leave the ends of ICEBs1 intact (Figure 1D, E). Figure 1D, E), have *cat* (chloramphenicol resistance) from pGEMcat [34], and were constructed using long-flanking homology PCR [35]. The Δ*nick-yddM::cat* allele contains the first 127 bp in the 5' end of *nick*. The Δ*ydhS-ydhM::cat* allele contains the first 29 bp in the 5' end of *ydhS*. Both deletions (Figure 1) extend through the first 170 bp in *yddM*. The alleles were first transformed into wild type strain AG174. Chromosomal DNA was then used to transfer the alleles into other strains, including KM70 (*mmxA::ICEBs1*), KM94 (yebT::ICEBs1), KM77 (yebP::ICEBs1), KM141 (sfl4A::ICEBs1), and CAL574 (ICEBs1 at *attB*). In all cases, the incoming deletion associated with *cat* replaced the Δ*rapI-phrI342::kan* allele present in *ICEBs1* in the recipient.

Deletion of *yebT* in *mmxA::ICEBs1*. We constructed a deletion-insertion that removes the 19 bases before *yebT* and the first 808 bp of *yebT*, leaving the last 200 bp intact. The sequence from *yebT* was replaced with *cat*, from pGEMcat [34], using long-flanking homology PCR [35]. The insertion-deletion was verified by PCR and the mutation was introduced into strain KM70 (*mmxA::ICEBs1*) by transformation.

Isolation and identification of secondary ICEBs1 integration sites

Mating ICEBs1 into a *ΔattB* recipient. Mating assays were performed essentially as described [10,11]. Excision of a kanamycin resistant ICEBs1 (ICEBs1 Δ*rapI-phrI342::kan*) was induced in the donor cells by overproduction of RapI from Pspank(hy)-rapI. Donors (resistant to kanamycin and spectinomycin) were mixed with an approximately equal number of recipients (resistant to chloramphenicol) and filtered on sterile cellulose nitrate membrane filters (0.2 μm pore size). Filters were cut into 8 pieces (so that transconjugants were independent isolates), placed on Petri plates containing LB and 1.5% agar, and incubated at 37°C for 3 hours. Cells from each piece of filter were streaked for independent transconjugants by selecting for the antibiotic resistance conferred by the incoming ICEBs1 (kanamycin) and the resistance unique to the recipient (chloramphenicol). The recipient used in this report {Δ*attB::cat trnS-leu1-522*} is different from the recipient {Δ*attB::cat*} used previously [10]. The *trnS-leu1-522* confers normal growth to the *ΔattB* (*ΔattB::att*2) mutant (see above).

Inverse PCR to identify the site of insertion of independent transconjugants. Identification of integration sites was done essentially as described previously [10]. Briefly, we used inverse PCR to amplify the junction between the chromosome and the right (*yddM*) end of ICEBs1 integrated into various secondary sites. Chromosomal DNA was digested with HindIII and approximately 50 ng was ligated in a 100 μl reaction to favor circularization of DNA fragments. One-fourth of the ligation reaction was used in inverse PCR with either of two primer pairs (CLO17-CLO58 or CLO50-qJMA97) designed to amplify the ICEBs1 and chromosomal sequences flanking *yddM*. PCR products were sequenced with primers CLO17, CLO50, qJMA207, and CLO14 (primers are described in Table 3). Comparison to the *B. subtilis* genome sequence indicated where ICEBs1 had integrated.

Backcross of ICEBs1 insertions. Seven of the 15 different insertions of ICEBs1 in secondary attachment sites were initially chosen for further study. These were first backcrossed into a strain cured of ICEBs1 ([JMA222]. *Psky-rapI (amyE::(Pspank(hy)-rapI-*spc*) was introduced into these strains by transformation and selection for spectinomycin resistance using chromosomal DNA from strain MM8069. We verified that ICEBs1 was still at the original secondary attachment site using PCR with site-specific primers. The final strains from these crosses include: KM70 (*mmxA::ICEBs1*), KM94 (yebT::ICEBs1), KM77 (yebP::ICEBs1), KM130 (sfl4D::ICEBs1), KM141 (sfl4A::ICEBs1), and KM132 (yycJ::ICEBs1).
| Strain | relevant genotype (comment and/or reference) |
|--------|---------------------------------------------|
| AG174  | phe trp [39]                               |
| AG1624 | zby-b2;Tn917 [insertion at 65'] [40]        |
| BTS13  | PY79 (trp+ phe+) ΔmutSL;spc [41]           |
| CALS22 | trnS-leu1-522 ΔattB;cat                     |
| CALS72 | yomR572::ICEB1 Δ(rapl-phen342::kan) ΔattB;cat comK;cat;spc [10] |
| CALS75 | yvh5755::ICEB1 Δ(rapl-phen342::kan) ΔattB;cat comK;cat;spc [10] |
| CALS76 | yvhG576::ICEB1 Δ(rapl-phen342::kan) ΔattB;cat comK;cat;spc [10] |
| CALS77 | yvhJ577::ICEB1 Δ(rapl-phen342::kan) ΔattB;cat comK;cat;spc [10] |
| CALS78 | Intergenic ygaA mmd-155-578::ICEB1 Δ(rapl-phen342::kan) ΔattB;cat comK;cat;spc [10] |
| CAL872 | ΔattR1000: tet Δ(rapl-phen342::kan amyE::(Ppxyl-rapl spc) |
| CAL874 | Δ(rapl-phen342::kan amyE::(Ppxyl-ralp spc) [15] |
| JMA168 | Δ(rapl-phen342::kan amyE::(Ppxyl-ralp spc) [23] |
| J3     | srfAA3::ICEB1 Δ(rapl-phen342::kan) trnS-leu1-522 ΔattB;cat |
| J4     | yycJ4::ICEB1 Δ(rapl-phen342::kan) trnS-leu1-522 ΔattB;cat |
| J9     | ykmM9::ICEB1 Δ(rapl-phen342::kan) trnS-leu1-522 ΔattB;cat |
| J11    | yphG11::ICEB1 Δ(rapl-phen342::kan) trnS-leu1-522 ΔattB;cat |
| J12    | yisQ12::ICEB1 Δ(rapl-phen342::kan) trnS-leu1-522 ΔattB;cat |
| J14    | mmsA14::ICEB1 Δ(rapl-phen342::kan) trnS-leu1-522 ΔattB;cat |
| J16    | ykrP16::ICEB1 Δ(rapl-phen342::kan) trnS-leu1-522 ΔattB;cat |
| JMA222 | ICEB1Δ cured of ICEB1 [11] |
| K1254  | dinC18; Tn917lac; allele originally from YBS018 [27] |
| KM5    | yphL5::ICEB1 Δ(rapl-phen342::kan) trnS-leu1-522 ΔattB;cat |
| KM8    | spoVD8::ICEB1 Δ(rapl-phen342::kan) trnS-leu1-522 ΔattB;cat |
| KM10   | ydbb10::ICEB1 Δ(rapl-phen342::kan) trnS-leu1-522 ΔattB;cat |
| KM70   | mmsA15::ICEB1 Δ(rapl-phen342::kan) amyE::(Ppxyl-ralp spc) |
| KM72   | ykmM9::ICEB1 Δ(rapl-phen342::kan) amyE::(Ppxyl-ralp spc) |
| KM77   | ykrP16::ICEB1 Δ(rapl-phen342::kan) amyE::(Ppxyl-ralp spc) |
| KM94   | yvbT575::ICEB1 Δ(rapl-phen342::kan) amyE::(Ppxyl-ralp spc) |
| KM110  | ICEB1Δ zby-b2; Tn917 (insertion at 65') |
| KM111  | ICEB1Δ zby-b2; Tn917 trnS-leu1-522 ΔattB;cat |
| KM130  | spoVD8::ICEB1 Δ(rapl-phen342::kan) amyE::(Ppxyl-ralp spc) |
| KM132  | yycJ4::ICEB1 Δ(rapl-phen342::kan) amyE::(Ppxyl-ralp spc) |
| KM141  | srfAA3::ICEB1 Δ(rapl-phen342::kan) amyE::(Ppxyl-ralp spc) |
| KM250  | ICEB1Δ Δ(rapl-phen342::kan amyE::(Ppxyl-ralp) cat) |
| KM252  | mmsA15::ICEB1 Δ(rapl-phen342::kan) amyE::(Ppxyl-ralp) cat |
| KM268  | mmsA15::ICEB1 Δ(rapl-phen342::kan) amyE::(Ppxyl-ralp) cat ΔmutSL;spc |
| KM304  | mmsA15::ICEB1 Δ(rapl-phen342::kan) amyE::(Ppxyl-ralp) cat Δyvb5;cat |
| KM358  | mmsA15::ICEB1 Δ(ydcS-yddM336::cat) amyE::(Ppxyl-ralp) spc |
| KM362  | yvbT575::ICEB1 Δ(ydcS-yddM336::cat) amyE::(Ppxyl-ralp) spc |
| KM366  | mmsA15::ICEB1 Δ(nicK-yddM334::cat) amyE::(Ppxyl-ralp) spc |
| KM369  | yvbT575::ICEB1 Δ(nicK-yddM334::cat) amyE::(Ppxyl-ralp) spc |
| KM384  | srfAA3::ICEB1 Δ(ydcS-yddM336::cat) amyE::(Ppxyl-ralp) spc |
| KM386  | srfAA3::ICEB1 Δ(nicK-yddM334::cat) amyE::(Ppxyl-ralp) spc |
| KM388  | ykrP16::ICEB1 Δ(ydcS-yddM336::cat) amyE::(Ppxyl-ralp) spc |
| KM389  | ykrP16::ICEB1 Δ(nicK-yddM334::cat) amyE::(Ppxyl-ralp) spc |
| KM390  | Δ(rapl-phen342::kan amyE::(Ppxyl-ralp) spc) dinC18:: Tn917lacZ mls |
| KM392  | ΔattR1000:: tet Δ(rapl-phen342::kan amyE::(Ppxyl-ralp) spc) dinC18:: Tn917lac |
| KM394  | mmsA15::ICEB1 Δ(rapl-phen342::kan) amyE::(Ppxyl-ralp) spc dinC18:: Tn917lac |
| KM396  | yvbT575::ICEB1 Δ(rapl-phen342::kan) amyE::(Ppxyl-ralp) spc dinC18:: Tn917lac |
Assays for excision and integration of ICEBs1

 Detecting excision from secondary insertions. Excision of ICEBs1 from a chromosomal attachment site creates an extrachromosomal ICEBs1 circle and an “empty” attachment site (also called “repaired chromosomal junction”). Each product was measured using specific primers for quantitative real time PCR (qPCR), using a LightCycler 480 Real-Time PCR system with Syber Green detection reagents (Roche), essentially as described [15]. Cells were grown in defined minimal medium with arabinose as carbon source. Products from excision were determined two hours after addition of xylose to induce expression of Pxyl-rapI to cause induction of ICEBs1 gene expression.

 The amount of each empty attachment site was compared to a chromosomal reference gene, cotF, measured with primers CLO257-CLO258. The amount of empty attachment site from each of the secondary sites was normalized to strain JMA222, an ICEBs1-cured strain that simulates 100% excision. Standard curves for qPCR with cotF and the repaired junction for each secondary insertion were generated using genomic DNA from JMA222. Primers (in parentheses) for empty secondary attachment sites are presented in Table 3.

 Detecting integration at yrkM in a pool of transconjugants. ICEBs1 was transferred from donor strain KM250 to recipient KM524 by conjugation, selecting for resistance to kanamycin and MLS antibiotics. Approximately 10^5 transconjugants were collected from four separate conjugation experiments (done on filters placed on agar plates). Cells were washed off of all four filters with a total of 10 ml of minimal salts and aliquots of 0.2 ml were spread on selective plates to give ~2×10^6 transconjugants per plate. After overnight growth, plates (~50) with the transconjugants were flooded with minimal salts, cells were scraped, collected, and transconjugants from all plates were pooled.

 DNA was isolated from the pool of transconjugants and used as a template for qPCR with primers to detect the junction between yrkM and ICEBs1 (primers CLO116 and KM76). Values from this qPCR were compared to qPCR values for a reference gene (cotF). Values were normalized to a strain (KM72) that contains yrkM:ICEBs1 and represents 100% integration at yrkM. Values for yrkM:ICEBs1 in the pool of transconjugants were in the linear range of the qPCR and ≥3-fold above the background signal from the negative control (JMA222, which is cured of ICEBs1). DNA used for standard curves was from strain KM27 yrkM:ICEBs1. The frequency of integration at attB was determined by qPCR with primers CLO273 and CLO264. Values were compared to cotF and normalized to a strain with ICEBs1 at attB (strain AG174 or CAL874). DNA used for standard curves was from AG174 or CAL874. The entire experiment was done twice with similar results.

 Detecting integration at secondary sites after mating from a secondary site. Independent transconjugants, from donors with ICEBs1 at secondary attachment sites, were analyzed for the location of ICEBs1. Sites analyzed and primers used included: yrkM (CLO116-CLO17 or oJMA141-CLO17); mmsA (CLO109-ABO18, mmsA (CLO109-ABO18), mmsA (KM18-KM19), mmsA (KM22-KM25), spoVD (KM20-KM21), spoT (ABO14-ABO15), spoP (KM154-KM16), and attB (CLO261-CLO262).

 The amount of ICEBs1 circle that forms after excision from the chromosome was measured with primers AB019-CLO114. The amount of excised circle was compared to the chromosomal reference cotF (primers CLO257-CLO258), and normalized to the amount of excised circle from attB (strain CAL874). Standard curves for qPCR for cotF and the excised circle were generated using genomic DNA from RapI-induced CAL874. Primer sequences are presented in Table 3.

 Detecting integration at yrkM in a pool of transconjugants. ICEBs1 was transferred from donor strain KM250 to recipient KM524 by conjugation, selecting for resistance to kanamycin and MLS antibiotics. Approximately 10^5 transconjugants were collected from four separate conjugation experiments (done on filters placed on agar plates). Cells were washed off of all four filters with a total of 10 ml of minimal salts and aliquots of 0.2 ml were spread on selective plates to give ~2×10^6 transconjugants per plate. After overnight growth, plates (~50) with the transconjugants were flooded with minimal salts, cells were scraped, collected, and transconjugants from all plates were pooled.

 DNA was isolated from the pool of transconjugants and used as a template for qPCR with primers to detect the junction between yrkM and ICEBs1 (primers CLO116 and KM76). Values from this qPCR were compared to qPCR values for a reference gene (cotF). Values were normalized to a strain (KM72) that contains yrkM:ICEBs1 and represents 100% integration at yrkM. Values for yrkM:ICEBs1 in the pool of transconjugants were in the linear range of the qPCR and ≥3-fold above the background signal from the negative control (JMA222, which is cured of ICEBs1). DNA used for standard curves was from strain KM27 yrkM:ICEBs1. The frequency of integration at attB was determined by qPCR with primers CLO273 and CLO264. Values were compared to cotF and normalized to a strain with ICEBs1 at attB (strain AG174 or CAL874). DNA used for standard curves was from AG174 or CAL874. The entire experiment was done twice with similar results.

 Cell viability assays

 Strains were grown in defined minimal medium with arabinose and expression of Pxyl-rapI was induced with 1% xylose at OD600 of 0.05. The number of colony forming units (CFU) was determined 3 hours after addition of xylose. For each strain, the number of CFU/ml 3 hrs after expression of Pxyl-rapI was compared to the number of CFU/ml without expression of Pxyl-rapI. All experiments were done at least twice.

 β-galactosidase assays

 Cells were grown and treated as described for viability assays. Samples were taken 3 hours after induction of Pxyl-rapI. All experiments were done at least twice, β-galactosidase assays were done essentially as described [36,37]. Specific activity is expressed as the ΔΔA420 per min per ml of culture per OD600 unit×1000.

 Modeling competition between cells with ICEBs1 in the primary attachment site versus cells with ICEBs1 in a secondary attachment site

 We calculated the predicted population size P after G generations for cells in which ICEBs1 is integrated into a
which ICE

D. The estimate of dead cells is based on the fraction of cells in secondary attachment site, with an estimated fraction of dead cells, previously to be between $10^{-2}$ to $10^{-2}$. Population size $P = P_0 e^{G(1-D)G}$, where $P_0$ is the initial population size. The ratio $R$ of the number of cells with ICEBs1 to the number of cells with ICEBs1 in a secondary site is given by $R = P_0 e^{2G}$ (for ICEBs1 in attB and assuming no killing upon induction).
\[ P_{\text{ICD}}^{2}\Delta t \left( 1 - D \right)^{2} \] (for ICEBs-I in a secondary site). This equation reduces to \( R = 1/\left( 1 - D \right)^{2} \). This gives 
\[ G = \log \left( 1/R \right)/\log \left( 1 - D \right) \]
for the number of cells with ICEBs-I in a population to be 10-fold greater than the number of cells with ICEBs-I in a secondary site (\( R = 10 \)) and if the frequency of death if \( 10^{-5} \) for the secondary site insertions, then the number of generations to achieve \( R = 10 \) is:
\[ G = \log(0.1)/\log(0.9999) \] which is \( \approx 23,000 \).

Acknowledgments

We thank J. Wilks for preliminary isolation of some of the insertions in secondary attachment sites, C. Lee for helpful discussions and construction of the compensatory mutation in trnS-Ir1 in the \( \Delta \text{intB} / \Delta \text{trmS-Ir2} \) strain, C. Bonilla and A. Babić for preliminary characterization of some of the secondary-site insertions, N. Kavanaugh for preliminary analysis of mRNA levels in ICEBs-I mutants defective in excision, and A. Columbus and E. Capra for discussions on modeling competition. We thank C. Johnson, C. Lee, J. Thomas, and T. Washington for comments on the manuscript.

Author Contributions

Conceived and designed the experiments: KLM ADG. Performed the experiments: KLM. Analyzed the data: KLM ADG. Contributed reagents/materials/analysis tools: KLM. Wrote the paper: KLM ADG.

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