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## Autonomous plasmid-like replication of a conjugative transposon

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## Summary

Integrative and conjugative elements (ICEs), a.k.a. conjugative transposons, are mobile genetic elements involved in many biological processes, including pathogenesis, symbiosis, and the spread of antibiotic resistance. Unlike conjugative plasmids that are extra-chromosomal and replicate autonomously, ICEs are integrated in the chromosome and replicate passively during chromosomal replication. It is generally thought that ICEs do not replicate autonomously. We found that when induced, *Bacillus subtilis* ICEBs1 undergoes autonomous plasmid-like replication. Replication was unidirectional, initiated from the ICEBs1 origin of transfer, *oriT*, and required the ICEBs1-encoded relaxase NicK. Replication also required several host proteins needed for chromosomal replication, but did not require the replicative helicase DnaC or the helicase loader protein DnaB. Rather, replication of ICEBs1 required the helicase PcrA that is required for rolling circle replication of many plasmids. Transfer of ICEBs1 from the donor required PcrA, but did not require replication, indicating that PcrA, and not DNA replication, facilitates unwinding of ICEBs1 DNA for horizontal transfer. Although not needed for horizontal transfer, replication of ICEBs1 was needed for stability of the element. We propose that autonomous plasmid-like replication is a common property of ICEs and contributes to the stability and maintenance of these mobile genetic elements in bacterial populations.

## Introduction

Horizontal gene transfer helps drive evolution and is important in pathogenesis, symbiosis, and the spread of antibiotic resistance (Keeling & Palmer, 2008, Ochman *et al.*, 2000). In bacteria, horizontal gene transfer is often mediated by mobile genetic elements, including phages, plasmids, and transposons (Frost *et al.*, 2005). These elements are typically comprised of various functional modules, apparently assembled by acquisition from or exchange with other mobile and non-mobile genetic elements (Osborn & Boltner, 2002, Toussaint & Merlin, 2002, Burrus *et al.*, 2002).

Conjugative plasmids and conjugative transposons, a.k.a., integrative and conjugative elements (ICEs), transfer directly from one cell to another by mating. Many elements transfer single stranded DNA into recipient cells (reviewed in Waters & Guiney, 1993, Lanka & Wilkins, 1995, Llosa *et al.*, 2002). The transferred strand is unwound from a double-stranded circular element starting at a nick in the origin of transfer (*oriT*). A DNA relaxase (nickase) makes the single strand cut and is covalently attached to the 5' end of the strand to be transferred. For plasmids, the transferred strand is replaced by replication in the donor. Although replacement strand synthesis has been proposed as one mechanism to promote plasmid unwinding and single-strand transfer, it has been difficult to address the role of replication in plasmid conjugation (Meyer, 2009), since replication is needed for plasmid maintenance.

Many ICE's and conjugative plasmids appear to use similar machinery and mechanisms to transfer DNA (Toussaint & Merlin, 2002). Both plasmids and ICEs mate as extra-chromosomal elements. However, whereas conjugative plasmids start out as extra-chromosomal elements, ICEs are typically found integrated in the host genome and excise to form a circular extra-chromosomal element prior to mating.

In contrast to plasmids that replicate autonomously to ensure their inheritance during cell growth and cell division, ICEs are generally thought to be incapable of autonomous replication, instead relying on their integration in the host genome and replication and segregation along with the host chromosome for genetic stability (Burrus & Waldor, 2004). However, some reports indicate that certain ICE and ICE-like elements are capable of autonomous replication (Ramsay *et al.*, 2006, Wang *et al.*, 2001, te Poele *et al.*, 2008). We investigated whether *ICEBsI* (Fig. 1A), an ~21 kb conjugative transposon in *Bacillus subtilis* (Auchtung *et al.*, 2005, Burrus *et al.*, 2002), is capable of autonomous replication.

Under normal growth conditions, *ICEBsI* is found stably integrated into a tRNA gene in *B. subtilis* (Fig. 1A). However, *ICEBsI* is activated and can excise from the genome when cells experience DNA damage and activate the *recA*-dependent SOS response, or when the signaling protein RapI is activated in response to a high density of cells lacking *ICEBsI* (Auchtung *et al.*, 2005). Excision of *ICEBsI* leads to the formation of a circular product and if suitable recipients are present, *ICEBsI* can transfer from donor to recipient (Auchtung *et al.*, 2005). Previous observations indicated that *ICEBsI* might be capable of autonomous replication after excision. Under certain conditions, a single donor appeared to transfer *ICEBsI* to more than one recipient (Auchtung *et al.*, 2005). There was a small increase in *ICEBsI* gene copy number after induction of the SOS response (J.D. Wang, ADG, unpublished results), and excision of *ICEBsI* alters the subcellular location of the replication machinery (Berkmen *et al.*, 2009).

Taking advantage of the ability to readily induce excision of *ICEBsI* in the majority of cells in a population (Auchtung *et al.*, 2005, Lee *et al.*, 2007), we found that *ICEBsI* undergoes plasmid-like autonomous replication after induction. *ICEBsI* replication initiated at *oriT* and required the *ICEBsI*-encoded relaxase Nick. *ICEBsI* replication also required the

chromosomally-encoded helicase PcrA that is used by several rolling circle replicating (RCR) plasmids and did not require the helicase DnaC that is needed for chromosomal replication. By controlling excision and replication of *ICEBs1*, we were able to address the role of replication in *ICEBs1* conjugation and stability. We found that replication in the donor is required for genetic stability of *ICEBs1*.

## RESULTS

### Autonomous replication of *ICEBs1* following induction

The cell-cell signaling regulator RapI induces expression of *ICEBs1* genes required for *ICEBs1* excision and conjugation (Auchtung et al., 2005, Lee et al., 2007, Lee & Grossman, 2007, Berkmen et al., 2009, Bose\* et al., 2008). Overproduction of RapI causes *ICEBs1* to excise and form a circle, typically in >95% of cells (Auchtung et al., 2005, Lee et al., 2007). We found that expression of RapI caused *ICEBs1* to replicate. We used DNA microarrays to measure the relative copy number of chromosomal and *ICEBs1* genes with and without induction of *ICEBs1*. In cells carrying an excision-competent *ICEBs1*, the copy number of *ICEBs1* genes increased ~2-5-fold one hour after induction of *rapI* (Fig. 1B, triangles). The *ICEBs1* gene *nicK* had the highest copy number (4.6) and genes located rightward from *nicK* had progressively lower copy numbers (*ydcS-yddD*, copy numbers 4 to 3; *yddF-yddM*, copy numbers <3). Our results indicate that induction of *ICEBs1* leads to its autonomous replication. Replication likely starts near *nicK* and proceeds rightward from *nicK* (Fig. 1A) around the *ICEBs1* circle, sometimes terminating before completing a round of replication to the left of *nicK*.

The copy number of chromosomal genes adjacent to *ICEBs1* also increased after induction of *ICEBs1* (Fig. 1B). The increase was relatively modest (<1.5-fold) and extended far into the

chromosomal region, especially on the right side. Replication of this region required induction with RapI and the presence of an adjacent replication-competent *ICEBsI* (see below). We suspect that replication initiates in *ICEBsI* in some cells before excision, and continues into the flanking chromosomal region.

Virtually nothing is known about the mechanisms and proteins involved in autonomous replication of ICEs, or the function of replication in their conjugation and stability. It has been generally assumed that ICEs do not replicate autonomously (e.g., Burrus & Waldor, 2004), although a couple of reports indicate this might not be the case for an ICE and ICE-like element that use a single-strand transfer mechanism (Ramsay et al., 2006, Wang et al., 2001). Therefore, we determined the mechanism of replication of *ICEBsI* and the role of replication in its function.

#### ***ICEBsI* replication proceeds unidirectionally from a site in or near *nicK***

Analysis of an excision-defective *ICEBsI* mutant confirmed that replication initiates in or near *nicK* and proceeds rightward. Excision of *ICEBsI* requires site-specific recombination between *attL* and *attR* (Fig. 1A), and deletion of *attR* prevents excision (Lee et al., 2007). The copy number of *nicK* and genes to its right increased following induction of the excision-defective *ICEBsI*  $\Delta attR$  mutant (Fig. 1C, filled triangles). In contrast, there was little or no increase in the copy number of genes to the left of *nicK* in the  $\Delta attR$  mutant (Fig. 1C, open triangles). The copy number of *nicK* and genes to its right was lower in the  $\Delta attR$  mutant (3.3 to 2) than in the WT (4.6 to 3). For elements replicating unidirectionally, a complete round of replication is required to generate two intact origin regions, each capable of reinitiation. *ICEBsI* cannot complete a round of replication without excision and the  $\Delta attR$  mutant has only one replication-competent origin, likely explaining why the copy number of *ICEBsI* genes is lower in the  $\Delta attR$  strain than in the WT. We conclude that replication of genes to the left of *nicK* (*int-*

*ydcQ*) requires excision and circularization of *ICEBsI*, replication is unidirectional, initiates in or near *nicK* and proceeds rightward, and excision is not needed for initiation of *ICEBsI* replication. In addition, autonomous replication of *ICEBsI* was much less processive than normal chromosomal replication, perhaps indicating differences in the replication proteins used (see below).

### **Replication of *ICEBsI* initiates within *oriT* and requires *nicK***

The *ICEBsI* conjugative relaxase encoded by *nicK* was required for *ICEBsI* replication. *NicK* is a homolog of Rep DNA relaxases required for replication of RCR plasmids. *ICEBsI* *NicK* makes a ssDNA break in a GC-rich inverted repeat in *oriT*, in the 5' end of *nicK*, and is required for conjugative transfer of *ICEBsI* from donor to recipient (Lee & Grossman, 2007). There was no increase in *ICEBsI* gene copy number following induction of a  $\Delta nicK$  mutant, nor was there any increase in copy number of the flanking chromosomal genes (Fig. 1D,  $\Delta nicK$ ). These results indicate that *nicK* is required for replication of *ICEBsI* and the flanking chromosomal genes.

*ICEBsI* replication initiated at or near *nic*, the site of nicking in *oriT*. Using quantitative real-time PCR (qRT-PCR), we measured the copy number on each side of *nic* 2 hours after induction of *ICEBsI* (Fig. 2A) in both wild type and excision-defective *ICEBsI*. In the excision-defective ( $\Delta attR$ ) strain, the copy number of the region ~170 bp to the right of *nic* was 3.6 and the copy number ~30 bp to the left of *nic* was <1 (Fig. 2B). These results indicate that, in the  $\Delta attR$  strain, the region to the left of *nic* did not replicate while that to the right of *nic* did. The qRT-PCR assays also showed that there is more replication of the region to the right of *nic* in the WT (copy number 9.3) than in the  $\Delta attR$  mutant (copy number 3.6), confirming that replication of excised circular *ICEBsI* is more productive than that of integrated *ICEBsI*. Based on these results, we



conclude that the unidirectional replication of *ICEBsI* requires NicK and initiates at *nic* in *oriT*. We suspect that *oriT* and NicK recruit replication proteins to *ICEBsI* in a way analogous to that of a dsDNA origin and its cognate Rep protein of RCR plasmids.

### **The catalytic subunit of DNA polymerase (PolC) and single strand DNA binding protein (Ssb) associate with replicating *ICEBsI* in vivo**

We monitored association of various proteins with *ICEBsI* using crosslinking and immunoprecipitation followed by hybridization to DNA microarrays (ChIP-chip). Since *ICEBsI* seems to replicate by a rolling circle mechanism, we tested for association of single strand DNA binding protein (Ssb) and the catalytic subunit of DNA polymerase (PolC) with replicating *ICEBsI* (Fig. 3A, B). We used Ssb-GFP and PolC-GFP fusions and immunoprecipitated with anti-GFP antibodies. After induction, *ICEBsI* DNA was significantly enriched in the immunoprecipitates, indicating that both Ssb-GFP (Fig. 3A) and PolC-GFP (Fig. 3B) were associated with replicating *ICEBsI*. For both Ssb-GFP and PolC-GFP, enrichment of *ICEBsI* DNA was dependent on induction of *ICEBsI*, required addition of specific antibodies, and did not occur in cells without the GFP-tag (data not shown). These results indicate that Ssb and PolC are recruited to *ICEBsI* after activation and associate with *ICEBsI* DNA during replication. Chromosomal genes to the right of *ICEBsI* were modestly enriched in the ChIP samples, indicating that, Ssb and PolC are involved in replication of both *ICEBsI* and the flanking chromosomal genes.

### **PolC and the $\beta$ -clamp (DnaN) of the *B. subtilis* replicative DNA polymerase are required for *ICEBsI* replication**

We found that the catalytic subunit PolC and the  $\beta$ -clamp DnaN (a.k.a. the processivity clamp) of the *B. subtilis* replicative polymerase were required for autonomous replication of

*ICEBsI*. The replication inhibitor hydroxyphenylazo-uracil (HPUra) binds PolC that is associated with DNA and arrests replication (Brown, 1970). There was little or no increase in the copy number of *nicK* when *ICEBsI* was induced shortly after treatment of cells with HPUra (Table 1, line 1). In contrast, similar induction of *ICEBsI* without treatment with HPUra resulted in a copy number of ~6 for *nicK* (Table 1, line 2). In addition, in a *dnaNts* mutant, there was little or no increase in the copy number of *nicK* after induction of *ICEBsI* at non-permissive temperature (47°C) (Table 1, line 3). Similar treatment of wild type (*dnaN<sup>+</sup>*) cells resulted in an increase in the copy number of *nicK* of ~9-fold (Table 1, line 4).

The defects in replication were not a secondary effect of a possible defect in nicking in *oriT*. Primer extension assays showed that the *ICEBsI oriT* was nicked in the *dnaNts* mutant and in the presence of HPUra (data not shown). Defects in excision can cause a reduction in replication, as discussed above, and *ICEBsI* excision frequencies were reduced by HPUra treatment and by the *dnaNts* mutation (24% and 47% excision, respectively). However, these reductions in excision could not account for the blocks in replication, as excision is not needed for replication of the genes to the right of *oriT*, including *nicK* (Fig. 1C). We conclude that both PolC and the  $\beta$ -clamp of the chromosomal replicative DNA polymerase are required for replication of *ICEBsI*.

### **The *B. subtilis* replicative helicase, DnaC, is not required for *ICEBsI* replication and does not associate with replicating *ICEBsI* in vivo**

We found that the replicative helicase in *B. subtilis*, DnaC, is not required for autonomous replication of *ICEBsI* and is not associated with replicating *ICEBsI* DNA. We tested for effects of DnaC helicase activity on *ICEBsI* replication using two temperature-sensitive mutations, one in *dnaC* and one in *dnaB*. *dnaB* encodes a component of the helicase loader and is required for DnaC to assemble at *oriC* and at stalled replication forks (Bruand *et al.*, 2001, Velten *et al.*,

2003). *dnaCts* mutations cause a rapid and almost immediate block in replication elongation after shift to non-permissive temperature (Karamata & Gross, 1970). In contrast, at non-permissive temperature, *dnaBts* mutations cause a block in replication initiation and replication does not stop until ongoing rounds have finished (Karamata & Gross, 1970).

To test for a role of the replicative helicase in replication of *ICEBsI*, we shifted *dnaCts* and *dnaBts* mutants to the non-permissive temperature for 5 or 60 minutes, respectively. Maintaining the cells at the non-permissive temperature, we then induced *ICEBsI* by overexpression of RapI. The *dnaCts* mutation did not prevent *ICEBsI* replication (Table 1, line 5). If anything, *ICEBsI* replicated more efficiently in this mutant than in the wild type under similar conditions (Table 1, line 4). We do not know what causes the more efficient replication in the *dnaCts* mutant, but it might be due to SOS induction in the mutant at the non-permissive temperature or perhaps indirect effects on machinery involved in *ICEBsI* replication. *ICEBsI* also replicated in the *dnaBts* mutant. The copy number of *nicK* was ~4 (Table 1, line 6), and was less than that in the comparably treated wild type strain in which the copy number of *nicK* was ~9 (Table 1, line 7). The reduced replication of *ICEBsI* in the *dnaBts* mutant is likely due to a decreased excision frequency (12.5%). We were also unable to detect association of DnaC with replicating *ICEBsI* DNA using ChIP-chip with antibodies to DnaC (Fig. 3C). The same antibodies are able to detect helicase at *oriC* in ChIP experiments (W.K. Smits, A. I. Goranov, ADG, unpublished results). Based on these results, we conclude that neither the replicative helicase DnaC, nor the helicase loader protein DnaB, are required for autonomous replication of *ICEBsI*, and that DnaC is not detectably associated with autonomously replicating *ICEBsI*.

### **The helicase PcrA associates with replicating ICEBsI**

We found that the chromosomally-encoded helicase PcrA associates with replicating ICEBsI DNA. PcrA is required for replication of RCR plasmids in *B. subtilis* and other Gram-positive bacteria (Khan, 2005). Since ICEBsI replication has features similar to that of RCR plasmids and DnaC is not required and none of the ICEBsI genes are predicted to encode a helicase, we decided to test PcrA. We made a *myc-PCR*A fusion and used anti-Myc monoclonal antibodies to immunoprecipitate Myc-PcrA. We found that ICEBsI DNA was highly enriched in the anti-Myc-PcrA immunoprecipitates (Fig. 3D). Enrichment of ICEBsI DNA was dependent on RapI-overexpression and did not occur if cells did not contain the Myc-tagged PcrA (data not shown). These results indicate that, like PolC and Ssb, PcrA is recruited to ICEBsI after induction and associates with ICEBsI DNA during replication. Chromosomal genes to the right of ICEBsI were modestly enriched in the Myc-PcrA immunoprecipitates, indicating that, in addition to mediating the replication of ICEBsI, PcrA may also mediate the replication of flanking chromosomal genes.

### **PcrA is required for ICEBsI replication and mating**

*PCR*A was required for autonomous replication of ICEBsI. *PCR*A is essential, even though it is not required for replication of the *B. subtilis* chromosome (Petit *et al.*, 1998). PcrA appears to be needed to disassemble chromosomal recombination complexes that can block replication (Petit & Ehrlich, 2002, Anand *et al.*, 2007), and the lethality of *PCR*A null mutants is suppressed by null mutations in genes of the *recFOR* recombination pathway. We constructed a *PCR*A null mutation in a *recF* null mutant. After induction, there was no detectable replication of ICEBsI in the *PCR*A *recF* double mutant (Fig. 1E). ICEBsI did replicate after similar induction in wild type (Fig. 1F) and a *recF* single mutant (Fig. 1G). Ectopic expression of *PCR*A (and the upstream

uncharacterized gene *pcrB*) from its native promoter, at a heterologous chromosomal location, restored ICEBs1 replication in the *pcrA recF* double mutant (Fig. 1H), indicating that the defect in ICEBs1 replication in the double mutant was due to loss of *pcrA* and not a polar effect on the downstream genes *ligA* (DNA ligase) and *yerH* (unknown). The defect in replication in the *pcrA recF* double mutant was not due to a defect in nicking of ICEBs1 in *oriT* since primer extension assays showed that ICEBs1 was nicked at the same frequency in the double mutant as in the *recF* single mutant (data not shown). There was a modest reduction of ICEBs1 excision in the *pcrA recF* double mutant (54% excision), but this cannot account for the block in replication. Based on these results, we conclude that the helicase PcrA is required for autonomous replication of ICEBs1. PcrA function in ICEBs1 replication is most likely analogous to its function in plasmid replication.

*pcrA* was required for ICEBs1 conjugation. We mixed different ICEBs1 donor cells with potential recipients and measured mating efficiencies (Fig. 4A). In the conditions used, wild type and *recF* mutant donors had approximately 3% and 8% mating efficiencies (transconjugants per donor), respectively (Fig. 4A). In contrast, the mating efficiency of the *pcrA recF* mutant donor was <0.0001% (Fig. 4A). This mating defect was due to loss of *pcrA* and not a polar effect on the downstream genes as it was fully complemented by ectopic expression of *pcrA* (Fig. 4A). Based on these results, we conclude that the helicase PcrA is required in donor cells for conjugative transfer of ICEBs1. This requirement could reflect a requirement for replication of ICEBs1 in mating, or a requirement for the activity of PcrA independently of ICEBs1 replication.

### **Replication is not required for ICEBs1 mating**

DNA replication of a donor element might play a direct role in conjugative DNA transfer if leading-strand DNA synthesis (dependent on a helicase) from the nicked *oriT* is required to

unwind the DNA template and generate a single DNA strand for transfer (Lanka & Wilkins, 1995, Llosa et al., 2002). Alternatively, a helicase could promote DNA unwinding and conjugation independently of replication (Lanka & Wilkins, 1995). Since replication is required for plasmid maintenance, determining whether or not replication of a plasmid is required for conjugative transfer is complicated (e.g., Meyer, 2009). However, since *ICEBsI* is maintained as an integrated element, and its excision and autonomous replication are not obligatory, we could test whether or not autonomous replication is required to produce a single strand template for transfer.

Horizontal transfer of *ICEBsI* appeared to be independent of autonomous replication in the donor. We blocked replication of *ICEBsI* (and the chromosome) in a *dnaNts* mutant. As a control, we compared the mating efficiency to that of a *dnaCts* mutant in which chromosomal, but not *ICEBsI*, replication was blocked. We grew the mutants and wild type strains at permissive temperature (30°C), shifted a portion of each culture to non-permissive temperature (47°C) to inactivate the temperature-sensitive replication components, then induced *ICEBsI* by over-production of RapI. The wild type donor had mating efficiencies of 0.4% and 1% at 30°C and 47°C, respectively (Fig. 4B). The *dnaCts* donor had mating efficiencies of 0.4% both at 30°C and 47°C (Fig. 4B), indicating that replication of the donor chromosome is not required for *ICEBsI* mating. The *dnaNts* donor had mating efficiencies of 0.3% and 1% at 30°C and 47°C, respectively (Fig. 4B). These efficiencies were indistinguishable from those of the wild type, indicating that replication of *ICEBsI* in the donor was not required for mating. To verify that *ICEBsI* did not replicate in the *dnaNts* cells under the conditions used for mating, we measured *ICEBsI* replication using genomic microarrays in donor cells subjected to a mock mating procedure. We found that *ICEBsI* replicated in mock treated WT donor cells incubated at 47°C

(*nicK* copy number 6.7), whereas *ICEBsI* did not replicate in identically treated *dnaNts* donor cells (*nicK* copy number 0.84). The simplest interpretation of these results is that replication of *ICEBsI* is not required for *ICEBsI* mating. We conclude that the loss of *ICEBsI* mating in the *pcrA* null mutant is most likely due to a requirement for the unwinding activity of the PcrA helicase and not due to the defect in *ICEBsI* replication.

### **Replication is required for stability of *ICEBsI* after induction**

We determined the effects of replication of *ICEBsI* on its stability. If *ICEBsI* re-integrates shortly after excision, then autonomous replication of *ICEBsI* should have a relatively small effect on its stability as *ICEBsI* would resume being replicated with the chromosome. However, if re-integration is delayed, occurring several generations after excision, then replication might be important for the maintenance of *ICEBsI*. We found that after induction, autonomous replication of extrachromosomal *ICEBsI* allowed for its stable maintenance and re-integration in a population of growing and dividing cells.

Before induction, all of the replication-competent (*ICEBsI nicK*<sup>+</sup>) and replication-defective (*ICEBsI ΔnicK*) cells tested (>150 for each strain) were resistant to kanamycin, indicating the presence of *ICEBsI*. In addition, PCR analysis indicated that >99% of cells from each strain contained *attL*, the junction between the left end of integrated *ICEBsI* and the chromosome (Fig. 5A), and <0.1% contained *attB*, the empty chromosomal attachment site, indicating that <1 cell in 10<sup>3</sup> had lost *ICEBsI* before induction (Fig. 5B, C).

Two hours after induction (addition of xylose to induce expression of *Pxyl-rapI*), *ICEBsI* had excised from >90% of the cells of both wild type and the *nicK* mutant, as >90% of cells contained *attB* and <10% contained *attL* (Fig. 5BC). At this point, we repressed expression of *Pxyl-rapI* by removal of xylose and addition of glucose.

Several hours after repression by glucose, we observed that the WT replication-competent *ICEBsI* began to re-integrate into *attB*, as the percentage of cells that had *attL* increased while those with *attB* decreased (Fig. 5B). By 6-9 hours after repression of *Pxyl-rapI*, the percentage of cells with the integrated WT *ICEBsI* (*attL*) leveled off at ~95%, and only ~5% still had *attB* (Fig. 5B). In stark contrast, >98% of cells with the replication-defective *ICEBsI* continued to have an empty *attB* long after repression of *Pxyl-rapI* (Fig. 5C). The copy number of the replication-defective *ICEBsI* circular form had decreased in these cells to <0.002 copies/cell, as determined by quantitation of *attICEBsI* (described in Auchtung et al., 2005), while that of the replication-competent *ICEBsI* was ~0.4 copies/cell. Consistent with the PCR results, 7-8 hours after repression, the replication-competent *ICEBsI* was present in >95% of cells as judged by resistance to kanamycin, while the non-replicating *ICEBsI* was present in <0.1% of cells. We also used a *pcrA recF* double mutant to block replication of *ICEBsI*. As with the replication-defective *nicK* mutant, *ICEBsI* (*nicK*<sup>+</sup>) was unstable after excision in the *pcrA recF* double mutant, but stable in the *recF* single mutant (data not shown). Together, our results indicate that autonomous replication is critical for maintenance of *ICEBsI* in a population of cells, and that re-integration, at least under the conditions tested, begins several generations after removal of the inducing signal.

## DISCUSSION

We found that *ICEBsI* replicates autonomously by a plasmid-like rolling circle mechanism. Replication initiates at the *ICEBsI* origin of transfer and requires the element-encoded DNA relaxase *NicK*. Replication also requires the catalytic subunit of the host DNA polymerase *PolC*, the host processivity clamp *DnaN*, and the host-encoded helicase *PcrA*. *ICEBsI* replication does



not require the host replicative helicase DnaC. Autonomous replication of *ICEBsI* is important for its stable maintenance and re-integration in a population of growing cells, but does not appear to be required for mating.

### **Rolling circle replication and conjugation**

Rolling circle replication initiates when a DNA relaxase nicks the double strand origin of replication and covalently attaches to the 5'-end of the nicked DNA (Khan, 2005). It has been proposed that conjugation evolved from adaptation of a protein secretion system to transport an RCR DNA relaxase and the attached DNA strand (Llosa et al., 2002). Consistent with this idea, *oriT* and *NicK* are required for both conjugative transfer (Lee & Grossman, 2007) and unidirectional replication of *ICEBsI*. Furthermore, *PcrA*, which is required for replication of RCR plasmids in *B. subtilis* and other Gram-positive bacteria (Khan, 2005), is required for replication and conjugation of *ICEBsI*. *PcrA* is recruited to the nicked origin of RCR plasmids by direct interaction with the cognate Rep DNA relaxase and unwinds the DNA template for leading strand synthesis from the nicked 3'-end (reviewed in Khan, 2005). *NicK* is homologous to Rep DNA relaxases and is likely involved in recruiting *PcrA* to the nicked *ICEBsI* template. *PcrA* likely unwinds the double-stranded *ICEBsI* template to generate the single-strand DNA substrate needed for transfer through the *ICEBsI* mating pore.

Early studies of *E. coli* F and other conjugative plasmids demonstrated that there is conjugation-dependent replication in donor cells (Ohki & Tomizawa, 1968, Rupp & Ihler, 1968). Since then, a direct role for DNA replication in conjugation has remained an intriguing possibility, especially for RCR conjugative plasmids (Lanka & Wilkins, 1995, Llosa et al., 2002). Although some experiments indicate that replication is not required for conjugation

(Sarathy & Siddiqi, 1973, Kingsman & Willetts, 1978), others demonstrate the difficulty of totally abolishing replication of conjugative plasmids (Meyer, 2009).

We found that *B. subtilis* DnaN is required for replication of ICEBs1 but is not required for conjugation. Therefore, we propose that DNA replication is not required for ICEBs1 conjugation and that the *B. subtilis* PcrA helicase, not rolling circle replication, mediates unwinding and generation of a single-strand of ICEBs1 DNA for conjugation. It is theoretically possible that ICEBs1 is able to replicate in a subpopulation of the *dnaNts* mutant cells at the non-permissive temperature, that we would not be able to detect this, and that these cells would be the donors for mating. This seems highly unlikely as the mating efficiency of this subpopulation would have to be considerably higher than what we typically observe for WT cells under the conditions tested.

#### **Autonomous replication of integrative and conjugative elements**

Similar to ICEBs1, the 501.8 kb ICEMISymR7A element of *Mesorhizobium loti* R7A may have a bi-functional *oriT* and DNA relaxase that allows it to replicate autonomously and be stably maintained in growing cells (Ramsay et al., 2006). Autonomous replication may be a common, if not universal, feature of ICEs that is partly derived from the evolutionary relationship between conjugal relaxases and replication relaxases. It may be difficult to detect replication of other ICEs since many excise at very low frequencies and may only replicate transiently. However, sensitive and quantitative PCR-based methods might be used to detect the unique junctions formed in the chromosome (*attB*) and in the circularized element (*attP*) after excision. An increase in the copy number of *attP* vs *attB* would indicate that the excised ICE replicates more frequently than the chromosome (AB & ADG, unpublished results).

### **A regulatory switch controls the mode of ICEBsI replication**

ICEs appear to be comprised of discrete functional modules (Toussaint & Merlin, 2002, Burrus et al., 2002), and typically excise from the host chromosome to form a circle before conjugative transfer. ICEBsI contains a phage-like module required for integration, excision, and regulation (Auchtung *et al.*, 2007, Lee et al., 2007, Bose\* et al., 2008), a plasmid-like module involved in DNA replication and transfer (Lee & Grossman, 2007, Berkmen et al., 2009), and a cell-cell signaling module (Auchtung et al., 2005). The particular combination of modules present in ICEBsI allows it to switch from an integrated element to a replicating plasmid and back. This conversion is controlled by a phage-like proteolytic regulatory switch. In one state, the repressor ImmR accumulates and prevents expression of excisionase and ICEBsI replication and transfer functions, maintaining ICEBsI in its integrated form. Induction of ICEBsI causes the second state by triggering the degradation of ImmR, thereby de-repressing ICEBsI gene expression (Bose\* et al., 2008), leading to excision and autonomous replication. DNA damage or the presence of a high density of neighboring cells that lack ICEBsI independently activate this regulatory switch (Auchtung et al., 2005, Bose\* et al., 2008). We found that the kinetics of re-integration of ICEBsI after removal of an inducing signal are slow. The delay in re-integration is likely due to the time it takes to deplete the excisionase and re-establish repression. Autonomous replication of ICEBsI contributes to its overall stability within a population. Since many ICEs are related to conjugative plasmids, and excise from the host chromosome prior to conjugative transfer, we suspect that this switch between an integrated passively replicating state and the extra-chromosomal autonomously replicating state is widespread among ICEs.

## EXPERIMENTAL PROCEDURES

### Bacterial strains and growth conditions

Strains were constructed by natural transformation and grown in LB or defined minimal medium (Harwood & Cutting, 1990). All strains (Table 2) contain *pheA1 trpC2*, except for CAL1161, which is *trp*<sup>+</sup> (Berkmen & Grossman, 2007). Most ICEBs1 strains contained a kanamycin-resistance cassette  $\{\Delta(\textit{rapI-phrI})342::\textit{kan}\}$  (Auchtung et al., 2005). ICEBs1 was induced by over-expression of *rapI* from a xylose-inducible promoter using *amyE*:: $\{(P_{\textit{Xyl}}-\textit{rapI}), \textit{spc}\}$  (Berkmen et al., 2009) or *lacA*:: $\{(P_{\textit{Xyl}}-\textit{rapI}), \textit{tet}\}$ , or from an IPTG-inducible promoter using *amyE*:: $\{(P_{\textit{spank(hy)}}-\textit{rapI}), \textit{spc}\}$  (Auchtung et al., 2005). Temperature-sensitive replication mutations included: *dnaB134(ts)* (Mendelson & Gross, 1967), linked to *zhh83*::Tn917(*mls*) (Rokop et al., 2004); *dnaC30(ts)* (Karamata & Gross, 1970) linked to *mls* between *yybS* and *cotF* (Lin & Grossman, 1998); and *dnaN5(ts)* (Karamata & Gross, 1970).

*polC-gfpmut2 (cat)* is a single cross-over insertion of pCAL862 at *polC* and is the only functional copy of *polC* in the cell and is expressed from the endogenous promoter. It is analogous to the *polC-gfpmut2* constructs described previously (Lemon & Grossman, 1998, Berkmen & Grossman, 2006) except for the antibiotic marker.

*ssb-mGFPmut2* is expressed from *lacA*:: $\{(rpsF \textit{ssb-mgfpmut2}) \textit{tet}\}$  in cells that also contain wild type *ssb* (Berkmen & Grossman, 2006).

The alleles *amyE*:: $\{(P_{\textit{spank(hy)}}), \textit{spc}\}$  (empty vector) (Auchtung et al., 2005), *thrC1165*::*cat* and *thrC1167*::*phl* (phleomycin) were used as controls for antibiotic resistance and threonine auxotrophy in mating experiments (Fig. 4). *thrC1165*::*cat* and *thrC1167*::*phl* were constructed using long-flanking homology PCR (Wach, 1996).

We deleted the entire *pcrA* open reading frame, except for the last 44 codons, and inserted a chloramphenicol-inducible *cat* lacking its transcription terminator, using long-flanking homology PCR. We introduced  $\Delta pcrA1021::cat$  into strains carrying  $\Delta recF::spc$ , (Sciocchetti *et al.*, 2001). Growth of the  $\Delta pcrA \Delta recF$  mutant requires chloramphenicol, likely due to chloramphenicol-dependent transcription of *ligA* from the upstream *cat*. *ligA* is downstream from *pcrA* and encodes DNA ligase.

For complementation of  $\Delta pcrA$ , we cloned *pcrA*, the upstream gene *pcrB* and another 341 bp upstream including the endogenous promoter, into a *thrC* vector to generate pCAL1126. This plasmid was used to introduce *thrC::(pcrB pcrA mls)1126* into the chromosome.

Myc-tagged PcrA was expressed from Pspank-*myc-pcrA* (*cat*), a single crossover insertion of pCAL984 into *pcrA* that fuses a 3xMyc epitope tag to the N-terminal end of PcrA. The *myc-pcrA* fusion is expressed from the IPTG-inducible promoter Pspank with an optimized ribosome-binding site and is the only functional copy of *pcrA*.

### **Preparation of DNA for analysis of ICEBs1 and chromosome replication**

Cells growing in minimal medium were mixed with an equal volume of cold (-20°C) methanol, harvested by centrifugation, and cell pellets were frozen at -80°C. Cells from 5 ml of culture (OD<sub>600</sub>~1) were resuspended in 0.3 ml of A buffer (Lin & Grossman, 1998) containing 1 mg/ml lysozyme, lysed by incubation at 37°C for 30 min, followed by room temperature additions of 3 µl 100 mg/ml RNase A for 5 min, 0.3 ml 2 x IP buffer (Lin & Grossman, 1998) for 5 min, then addition of 15 µl 10% SDS and 3 µl Proteinase K (Qiagen, stock solution ≥600 mAU/ml) at 37°C for 30 min. Cell lysates were extracted twice, once with phenol:chloroform:isoamyl alcohol (1:1:48) and once with chloroform alone. Genomic DNA was precipitated from 0.5 ml of extracted lysate by addition of 15 µl of 5M NaCl and 1.25 ml of

cold (-20°C) ethanol and resuspended in 10mM Tris-HCl 0.1mM EDTA pH 8. For microarray analysis, genomic DNA was digested with *HaeIII*, run on MinElute PCR Purification columns, washed twice with PB, once with PE (Qiagen), then eluted with water.

To verify that both ssDNA and dsDNA were recovered, we added purified <sup>32</sup>P-labelled ssDNA or dsDNA fragments to cell suspensions and monitored <sup>32</sup>P levels at each step of purification. There was no difference in the recovery of a 7.3 kb fragment of *ICEBs1* (*ydcS-yddG'*), whether single-stranded or double-stranded.

### **Chromatin immunoprecipitation**

Chromatin immunoprecipitation (ChIP) of DNA bound to epitope-tagged and untagged proteins was done essential as described (Lin & Grossman, 1998, Breier & Grossman, 2007). Tests using unfixed cell lysates were conducted to determine the amount of antibodies needed to immunoprecipitate each epitope-tagged or untagged protein from cell lysates.

### **DNA microarrays for analysis of DNA synthesis and ChIP-chip**

Chromosomal DNA or DNA from ChIP samples was labeled and hybridized to spotted DNA microarrays as described (Breier & Grossman, 2007). Two types of microarrays were used, those prepared from PCR products representing > 99% of the open reading frames in the *B. subtilis* genome and 295 intergenic regions (Breier & Grossman, 2007), and those prepared from a set of synthetic oligonucleotides designed to correspond to all annotated *B. subtilis* ORFs (Auchtung et al., 2005).

*HaeIII* digested genomic DNA samples were labeled with Cy5 and mixed with a reference genomic DNA labeled with Cy3, as described previously (Wang *et al.*, 2007). By comparing the signal from the Cy5 sample to the Cy3 reference from replicate sets of test samples to that of

similarly labeled uninduced IRN342 control samples, we determined the copy number of each gene in the genomic sample relative to the uninduced control.

ChIP DNA samples were labeled with Cy5 and mixed with the corresponding total DNA sample labeled with Cy3. The ratio of Cy5 ChIP to Cy3 total signals indicated the fold enrichment of genes due to immunoprecipitation. The raw Cy5 and Cy3 signals from the genomic microarrays and the ChIP microarrays were normalized as previously described (Breier & Grossman, 2007).

### **Quantitative PCR assays for *ICEBsI* excision and replication**

qRT-PCR was used to measure *ICEBsI* excision and replication from purified DNA samples or from crude cell lysates. In the latter case, cells were lysed by suspension in water containing 0.05 mg/ml of lysozyme, incubation at 37°C for 30 minutes followed by heating at 105°C for 15 minutes, cooling to 4°C for 5 minutes, heating again to 105°C for 3 minutes, then finally cooling again to 4°C. The cell lysates were centrifuged for 10 minutes at room temperature and the cleared supernatant was assayed by qRT-PCR. We used the LightCycler 480 Real-Time PCR system with Syber Green detection reagents (Roche).

*ICEBsI* excision and re-integration were measured using primer pair AB023-ABO24, to quantitate the left-hand integrated *ICEBsI*-chromosomal junction (*attL*), and primer pair CLO261–CLO262, to quantitate the unoccupied attachment site (*attB*) that is formed by excision. Primer pair CLO284-CLO285 was used to quantitate a control chromosomal region outside of *ICEBsI* in *ydbT*, which is located 15 kb to the left of *attL*. Standard curves for *attL* and *ydbT* were generated using genomic DNA from uninduced cells of IRN342 in which *ICEBsI* is integrated in single copy in the chromosome. Standard curves for *attB* and *ydbT* were also

generated using genomic DNA from JMA222, an *ICEBsI*-cured strain that simulates 100% excision.

Replication of *ICEBsI* regions to the left and right of the *nic* site in *oriT* were measured using primer pairs CLO274-CLO275 (left) and CLO280-CLO281 (right). Standard curves for these *ICEBsI* sites and the *ydbT* control site were generated using genomic DNA from uninduced cells of IRN342, described above. Primer sequences are available upon request.

### ***ICEBsI* nicking and mating assays**

Nicking of *ICEBsI* at *oriT* was assayed using purified genomic DNA digested with *HaeIII* (see above) in a primer extension assay with <sup>32</sup>P-labelled primer CLO76, essentially as described (Lee & Grossman, 2007). The frequency of nicking in each sample was calculated by quantitating the amount of primer extension products terminating at the nicked site and at the *HaeIII* restriction site ~50 bp farther upstream in *ydcQ*. Primer extension into *ydcQ* would only occur if an *oriT* template is not nicked by NickK.

*ICEBsI* mating assays were conducted using donor cells activated by expression of *rapI* from an IPTG-inducible promoter Pspank(hy) or from a xylose-inducible promoter P<sub>xyI</sub>, essentially as described (Lee et al., 2007).

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**Table 1. PolC and DnaN are required for replication of ICEBs1.**

line	Strain <sup>a</sup>	Treatment <sup>b</sup>	<i>nicK</i> copy number <sup>c</sup>
1	WT (Pxyl- <i>rapI</i> )	HPUra 5 min; xylose 2 hr	1 ± 0.4
2	WT (Pxyl- <i>rapI</i> )	xylose 2 hr	6 ± 2
3	<i>dnaNts</i> (Pspank(hy)- <i>rapI</i> )	47°C 5 min; IPTG 90 min	0.9 ± 0.1
4	WT (Pspank(hy)- <i>rapI</i> )	47°C 5 min; IPTG 90 min	9 ± 3
5	<i>dnaCts</i> (Pspank(hy)- <i>rapI</i> )	47°C 5 min; IPTG 90 min	27 ± 9
6	<i>dnaBts</i> (Pxyl- <i>rapI</i> )	47°C 1 hr; xylose 2 hr	4 ± 1
7	WT (Pxyl- <i>rapI</i> )	47°C 1 hr; xylose 2 hr	9 ± 2

<sup>a</sup> Strains containing Pxyl-*rapI* were CAL874 (WT, lines 1, 2, 7) and CAL934 (*dnaBts*, line 6). Pspank(hy)-*rapI* strains were JMA168 (WT, line 4), CAL1161 (*dnaNts*, line 3), and CAL1035 (*dnaCts*, line 5).

<sup>b</sup> Prior to the indicated treatment, all cells were grown at 30°C in defined minimal medium with L-arabinose as carbon source. Replication was blocked with HPUra (line 1) or shift of *ts* mutants to non-permissive temperature (lines 3, 5, 6) for the indicated amount of time, and then ICEBs1 was induced by induction of *rapI* with xylose or IPTG, as indicated. Full induction of ICEBs1 with Pxyl-*rapI* takes a bit longer than that with Pspank(hy).

<sup>c</sup> Copy number was calculated relative to uninduced IRN342 (no *rapI*) controls (average of 2) grown at the same temperatures with the addition of xylose (30°C) or IPTG (47°C). Data are the average and standard deviation from two to four independent experiments. Samples from strains with Pxyl-*rapI* were hybridized to DNA microarrays of spotted PCR products. Samples from strains with Pspank(hy)-*rapI* were hybridized to DNA microarrays of spotted oligonucleotides. The difference is simply due to the arrays that were available at the time of the experiments.

**Table 2. *Bacillus subtilis* strains used.**

Strain	Relevant genotype (reference)
CAL108	$\Delta(\text{rapI-phrI})342::kan \Delta\text{attR100}::tet amyE::\{(\text{Pspank}(\text{hy})-\text{rapI}) spc\}$ (Lee et al., 2007)
CAL306	$\Delta(\text{rapI-phrI})342::kan \Delta\text{nicK306} amyE::\{(\text{Pspank}(\text{hy})-\text{rapI}) spc\}$ (Lee & Grossman, 2007)
CAL419	ICEBsI <sup>0</sup> <i>str-84 comK::cat</i> (Auchtung et al., 2005)
CAL635	$\Delta(\text{rapI-phrI})342::kan amyE::\{(\text{Pxyl}-\text{rapI}) spc\} lacA::\{rpsF (ssb-mgfpmut2) tet\}$
CAL874	$\Delta(\text{rapI-phrI})342::kan amyE::\{(\text{Pxyl}-\text{rapI}) spc\}$
CAL879	$\Delta(\text{rapI-phrI})342::kan amyE::\{(\text{Pxyl}-\text{rapI}) spc\} \{(\text{polC}::\text{polC-gfpmut2}) cat\}$
CAL934	$\Delta(\text{rapI-phrI})342::kan amyE::\{(\text{Pxyl}-\text{rapI}) spc\} dnaB134(\text{ts}) zhb83::Tn917(\text{mls})$
CAL992	$\Delta(\text{rapI-phrI})342::kan amyE::\{(\text{Pxyl}-\text{rapI}) spc\} pcrA::\{\text{Pspank}-(\text{myc-}pcrA), cat\}$
CAL1035	$\Delta(\text{rapI-phrI})342::kan amyE::\{(\text{Pspank}(\text{hy})-\text{rapI}) spc\} dnaC30(\text{ts}) \Delta449::mls$
CAL1148	$\Delta(\text{rapI-phrI})342::kan lacA::\{(\text{Pxyl}-\text{rapI}) tet\} \Delta\text{recF}::spc \Delta\text{pcrA}::cat thrC::\{(pcrB-pcrA)1126 mls\}$
CAL1161	$\Delta(\text{rapI-phrI})342::kan amyE::\{(\text{Pspank}(\text{hy})-\text{rapI}) spc\} dnaN5(\text{ts}) (\text{ypjG-hepT})122$
CAL1173	$\Delta(\text{rapI-phrI})342::kan lacA::\{(\text{Pxyl}-\text{rapI}) tet\} amyE::\{(\text{Pspank}(\text{hy})) spc\} thrC::cat$
CAL1174	$\Delta(\text{rapI-phrI})342::kan lacA::\{(\text{Pxyl}-\text{rapI}) tet\} \Delta\text{recF}::spc thrC::cat$
CAL1175	$\Delta(\text{rapI-phrI})342::kan lacA::\{(\text{Pxyl}-\text{rapI}) tet\} \Delta\text{recF}::spc \Delta\text{pcrA1021}::cat thrC::phl$
CAL1215	$\Delta(\text{rapI-phrI})342::kan \Delta\text{nicK306} amyE::\{(\text{Pxyl}-\text{rapI}) spc\}$
IRN342	$\Delta(\text{rapI-phrI})342::kan$ (Auchtung et al., 2005)
JMA168	$\Delta(\text{rapI-phrI})342::kan amyE::\{(\text{Pspank}(\text{hy})-\text{rapI}) spc\}$ (Auchtung et al., 2005)
JMA222	ICEBsI <sup>0</sup> (Auchtung et al., 2005)

## FIGURE LEGENDS

**Figure 1. Unidirectional replication of ICEBsI is activated by RapI and requires *nicK* and *pcrA*.**

**A.** Schematic diagram of ICEBsI integrated in the *B. subtilis* chromosome. Genes (open arrows), the left and right attachment sites (*attL*, *attR*), origin of transfer (*oriT*), the site in *oriT* nicked by NicK (*nic*), and the location and direction of replication initiation (dashed arrow) are indicated.

**B-H.** The effect of RapI-overexpression on gene copy number was determined using genomic microarrays, comparing induced vs uninduced samples, and plotted versus position in the chromosome. Values for ICEBsI genes are represented as large triangles, with open triangles representing genes to the left of *nicK* (*int-ydcQ*) and closed triangles representing *nicK* and genes to its right (*ydcS-yddM*). Values for non-ICEBsI chromosomal genes are shown as gray dots. For simplicity, we plot ICEBsI genes in their integrated chromosomal location even though, once excised, ICEBsI is no longer co-linear with the chromosome. Data for genes from 100 to 1000 kb to the right of the *B. subtilis* *oriC* are shown.

**B-D.** Strains were grown in minimal D-glucose medium at 37°C and expression of RapI (Pspank(hy)-*rapI*) was induced by addition of IPTG for 1 hr. Uninduced *rapI*-null cells (IRN342) were grown under the same conditions and used as controls. Cy-labeled samples were hybridized to DNA microarrays of spotted PCR products. Data are the averages of two induced and uninduced samples. **B.** wild type (JMA168); **C.**  $\Delta attR$  (CAL108); **D.**  $\Delta nicK$  (CAL306).

**E-H.** Strains were grown in minimal L-arabinose medium at 30°C and expression of RapI (Pxyl-*rapI*) was induced by addition of D-xylose for 2 hours. Uninduced *rapI*-null cells (IRN342) were grown under the same conditions. Cy-labeled samples were hybridized to DNA

microarrays of spotted oligonucleotides. Data are from the averages of three induced and two uninduced samples. Replication of *ICEBsI* was lower than observed in previous experiments (Fig. 1, Table 1). Activation of *ICEBsI* by RapI expression from *lacA::{(P<sub>xyl</sub>-rapI) tet}* is less efficient than from the other RapI-expression constructs (B. Bose, ADG unpublished results). **E.**  $\Delta recF \Delta pcrA$  (CAL1175); **F.** wild type (CAL1173); **G.**  $\Delta recF$  (CAL1174); **H.**  $\Delta recF \Delta pcrA + pcrB pcrA$  (CAL1148).

**Figure 2. Unidirectional replication of *ICEBsI* initiates within *oriT*.**

**A.** Schematic diagram of *ICEBsI oriT*. The long horizontal line represents the 0.8 kb *oriT* region. The arrow indicates the site (*nic*) on the top strand of *oriT* that is nicked by NickK. The thick double lines depict the two regions of *oriT* assayed by qRT-PCR. Values above the brackets indicate the distance between the ends of the two regions assayed.

**B.** Copy number of sites flanking *nic*. Strains JMA168 (WT) and CAL108 ( $\Delta attR$ ) were grown in minimal D-glucose medium at 37°C and expression of RapI (P<sub>spank(hy)</sub>-*rapI*) was induced by addition of IPTG for 2 hours. Cell lysates were prepared and assayed by qRT-PCR for the left PCR site, the right PCR site, and a control PCR site outside of *ICEBsI* (*ydbT*). Copy number values were normalized to qRT-PCR assays of an uninduced cell lysate of IRN342. Data are the average and standard deviation from four independent biological replicates.

**Figure 3. DNA polymerase PolC, single-strand binding protein Ssb and alternative helicase PcrA associate with *ICEBsI* during replication, but the replicative helicase DnaC does not.** The association of various proteins with DNA was assessed by chromatin immunoprecipitation followed by hybridization to DNA microarray (ChIP-chip). Strains were

grown in defined minimal L-arabinose medium at 30°C and expression of RapI (Pxyl-*rapI*) was induced by addition of D-xylose for 2 hours. Cells were fixed with formaldehyde and protein-DNA complexes were immunoprecipitated with antibodies to GFP (A, B) DnaC (C) or c-Myc (D). Cy-labeled samples were hybridized to DNA microarrays of spotted PCR products. Fold-enrichment of DNA by immunoprecipitation is plotted versus position in the chromosome. Data for genes from 100 to 1000 kb to the right of *oriC* are shown. Values for ICEBs1 genes are represented as large closed circles and non-ICEBs1 genes are dark gray dots. Data presented are from one representative experiment. **A.** Ssb-GFP (CAL635); **B.** PolC-GFP (CAL879); **C.** DnaC (CAL635); **D.** Myc-PcrA (CAL992).

**Figure 4. Effects of mutations in *pcrA*, *dnaC*, and *dnaN* on ICEBs1 mating efficiency.**

Efficiencies of transfer of ICEBs1 (kanamycin-resistant) from the indicated donor strains into the recipient CAL419 (streptomycin-resistant) were calculated from the number of kanamycin-resistant, streptomycin-resistant trans-conjugants per initial donor X 100%.

**A.** Wild type (CAL1173), *recF* (CAL1174), *recF pcrA* (CAL1175), and *recF pcrA +pcrA* (CAL1148) donors were grown in minimal L-arabinose medium at 30°C and expression of RapI (Pxyl-*rapI*) was induced by addition of xylose for 2 hours. Mating mixtures were incubated at 37°C for 3 hours. Gray bars show the average and standard deviation from two independent mating assays. The asterisk indicates that the mating efficiency of the *recF pcrA* double mutant was <0.0001%.

**B.** Wild type (JMA168), *dnaCts* (CAL1035), and *dnaNts* (CAL1161) donors were grown in minimal L-arabinose medium at 30°C. A portion was shifted to 47°C for 5 minutes, and then IPTG was added to all donor cultures at 30°C and 47°C for 90 minutes to induce expression of



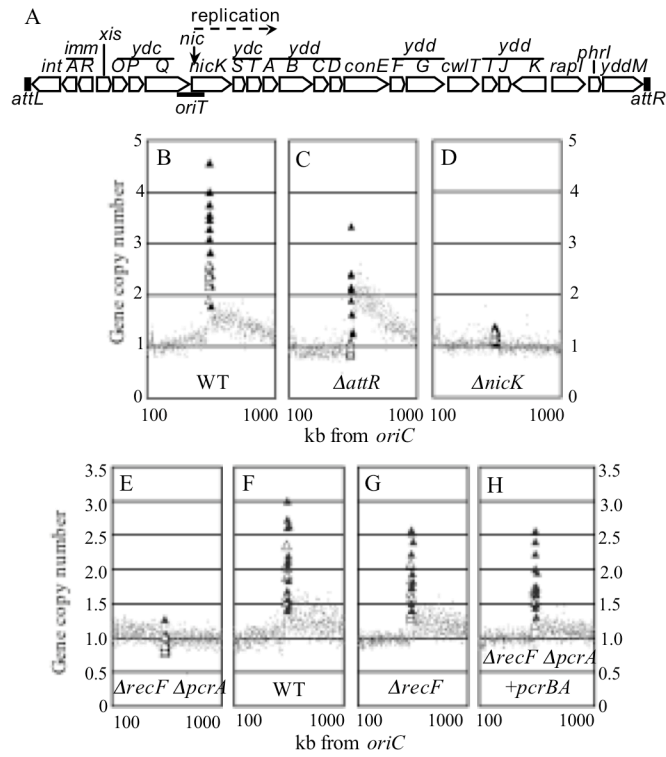
RapI (Pspank(hy)-*rapI*). Mating mixtures were incubated at 30°C or 47°C for 3 hours. Speckled (30°C) and black (47°C) bars show the average and standard deviation from two to four independent mating experiments.

**Figure 5. NickK-dependent replication is required for stability of ICEBsI after excision.**

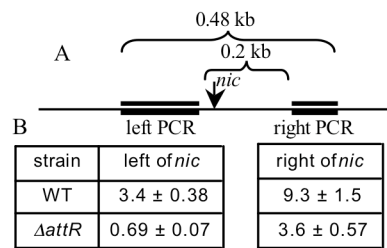
**A.** Schematic for detecting *attL*, the junction between the chromosome and the left end of *ICEBsI*, and *attB*, the empty attachment site in the bacterial chromosome formed after excision of *ICEBsI*. *ICEBsI* is indicated by the gray line in the drawing on the left and is not shown after excision on the right. qRT-PCR using primer pairs **a** and **b** or **a'** and **c** detected *attL* and *attB*, respectively.

**B, C.** Replication-competent wild type, CAL874 (**B**), and replication-defective  $\Delta nicK$ , CAL1215 (**C**), strains were grown in minimal L-arabinose medium at 37°C and expression of *Pxyl-rapI* was induced during exponential growth ( $OD_{600} \sim 0.2$ ) by addition of D-xylose for 2 hours. Expression of *Pxyl-rapI* was then repressed by removal of xylose and resuspension (and dilution) into minimal D-glucose medium at an  $OD_{600}$  of  $\sim 0.025$ . Samples were taken at the indicated times, covering  $\sim 7$  doublings as cells entered stationary phase, and qRT-PCR was used to determine the copy number of *attL* and *attB* relative to that of a nearby gene (*ydbT*) outside of *ICEBsI*. The relative copy number is plotted as a function of time after addition of xylose to induce *ICEBsI*. The arrow at 2 hours indicates the shift to glucose (Glu) to repress expression of *Pxyl-rapI*. Data presented are from one representative experiment.

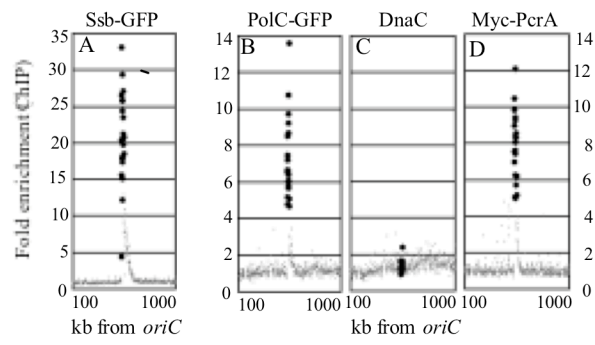
Lee Fig1



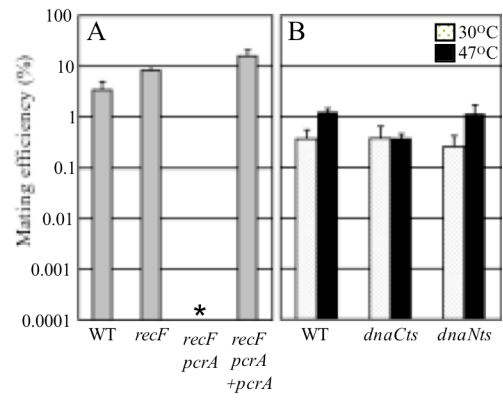
Lee Fig2



Lee Fig3



Lee Fig4



Lee Fig5

