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The *Bacillus subtilis* conjugative transposon ICEBs1 mobilizes plasmids lacking dedicated mobilization functions

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running title: Mobilization of plasmids lacking Mob-oriT functions

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Abstract

Integrative and conjugative elements (ICEs, a.k.a., conjugative transposons) are mobile elements that are found integrated in a host genome and can excise and transfer to recipient cells via conjugation. ICEs and conjugative plasmids are found in many bacteria and are important agents of horizontal gene transfer and microbial evolution. Conjugative elements are capable of self-transfer, and also capable of mobilizing other DNA elements that are not able to self-transfer. Plasmids that can be mobilized by conjugative elements are generally thought to contain an origin of transfer (oriT), from which mobilization initiates, and to encode a mobilization protein (Mob, a relaxase) that nicks a site in oriT and covalently attaches to the DNA to be transferred. Plasmids that do not have both an oriT and a cognate mob are thought to be non-mobilizable. We found that Bacillus subtilis carrying the integrative and conjugative element ICEBs1 can transfer three different plasmids to recipient bacteria at high frequencies. Strikingly, none of the plasmids contain dedicated mobilization-oriT functions. Plasmid mobilization required conjugation proteins of ICEBs1, including the putative coupling protein. In contrast, plasmid mobilization did not require the ICEBs1 conjugative relaxase or co-transfer of ICEBs1, indicating that the putative coupling protein likely interacts with the plasmid replicative relaxase and directly targets the plasmid DNA to the ICEBs1 conjugation apparatus. These results blur the current categorization of mobilizable versus non-mobilizable plasmids and indicate that conjugative elements play an even more significant role in horizontal gene transfer than previously recognized.
**Introduction**

Integrative and conjugative elements (ICEs, also known as conjugative transposons) are mobile elements found integrated in a host genome. Under certain conditions, ICEs can excise, circularize, and transfer to recipient cells via conjugation. ICEs and conjugative plasmids are found in many bacterial species and contribute to the acquisition of new traits, including antibiotic resistance.

Conjugative elements encode components of a transmembrane conjugation (mating) apparatus (often called the mating pore formation or Mpf complex) used to translocate DNA from donor to recipient. They also encode a relaxase protein that processes the element's DNA by nicking and covalently attaching to the element's origin of transfer (oriT) creating a relaxosome complex. An element-encoded coupling protein interacts with the relaxosome complex and the mating machinery to recruit (or couple) the substrate DNA to the mating apparatus and to facilitate transfer of the relaxase and a single strand of DNA to a recipient (1, 18, 42, 46, 49).

ICEBs1 (Fig. 1) is a conjugative transposon found integrated at the gene for tRNA-Leu2 (trnS-leu2) in *Bacillus subtilis* (5, 10). ICEBs1 is particularly useful for the study of conjugation. Several of the ICEBs1 genes are similar to genes in other ICEs, including Tn916 (8, 10, 41), the first conjugative transposon identified (17). ICEBs1 gene expression is induced during the RecA-mediated SOS response or when the cell sensory protein RapI is expressed and active (5). Analyses of ICEBs1 functions are facilitated by the ability to induce ICEBs1 gene expression simply by overproduction of RapI from an exogenous promoter, leading to excision of ICEBs1 from the chromosome of >90% of the cells in a population (5, 32). Induction of gene expression also leads to nicking of ICEBs1 by its relaxase (encoded by nickK) at a site in oriT (34). Nicking and subsequent unwinding by a host-encoded helicase (PcrA) are required for conjugative transfer and
for autonomous plasmid-like replication of ICEBs1 (33). Several ICEBs1 genes encode proteins required for transfer from donor to recipient, and high mating efficiencies of 1-10% transconjugants per donor (7, 32, 34) are obtained with B. subtilis recipients that do not contain ICEBs1 (4). ICEBs1 can also transfer to other Gram-positive bacterial species (5).

Many transposons and plasmids are not capable of self-transfer to recipient cells, but can be mobilized by the conjugation machinery of conjugative elements (19, 46). These mobilizable transposons (a.k.a., integrative mobilizable elements) and plasmids typically contain a dedicated oriT and encode a cognate relaxase protein (Mob) for mobilization. Mobilizable transposons excise from the genome prior to transfer, whereas the Mob/oriT-containing plasmids are autonomous genetic elements with their own replication functions (Rep and ori) that are separate from the transfer functions (Mob and oriT). Similar to the relaxase and oriT of conjugative elements, Mob and oriT of mobilizable elements are needed to create a relaxosome that then interacts with a coupling protein that will function to transfer the mobilizable DNA. Coupling proteins are typically encoded by the conjugative element, but can also be encoded by the mobilizable element (11, 46).

We found that B. subtilis ICEBs1 donors are capable of efficiently mobilizing three different plasmids, pC194, pBS42, and pHPl3 (Fig. 2) that replicate by rolling circle replication (RCR) and are typically described as non-mobilizable (30, 43, 44, 46), although very low efficiencies of mobilization of pC194 and a relative of pBS42 by the conjugative transposon Tn916 have been reported (38, 45). The three plasmids used here contain an origin of replication, but are not known to contain an origin of transfer or mobilization functions. For pBS42, we found that the plasmid replicative relaxase was required for plasmid mobilization. Our results indicate that, similar to the ICEBs1
relaxase (33), the plasmid replicative relaxases may function in both replication and DNA transfer. The plasmid relaxase may facilitate DNA transfer by interacting with the conjugation machinery of ICEBs1. These findings indicate that many more plasmids than previously thought might be readily mobilized and disseminated by conjugative elements.

Materials and Methods

Strains and alleles. B. subtilis strains were either cured of ICEBs1 (ICEBs1^0) (5) or carried one of several derivatives of ICEBs1 (Fig. 1B-E). All B. subtilis strains are listed in Table 1 and are derived from the lab strain JH642 (trp phe; not shown in genotypes in Table 1). Chromosomal alleles and plasmid DNA were introduced into B. subtilis by natural transformation (24). Important alleles and plasmids are described below.

Strains that were used as donors in conjugation experiments contained Pspank(hy)-rapI integrated at amyE, amyE::{(Pspank(hy)-rapI) spc}, for IPTG-inducible overproduction of RapI that causes induction of ICEBs1 gene expression (5). To monitor transfer of ICEBs1, a derivative [ICEBs1 Δ(rapI-phrI)342::kan] (Fig 1B) encoding resistance to kanamycin was typically used, as previously described (5).

ICEBs1 Δ(conG-yddM)319::kan (Fig 1C) is an insertion-deletion that removes several essential conjugation genes and replaces them with the kan cassette. The mutation removes 661 bp from the the 3' end of conG (of the 2,445 bp conG open reading frame), 782 bp from the 5' end of yddM (of the 939 bp yddM open reading frame) and everything between the two genes (34).

ICEBs1 ΔnicK306 (Fig. 1D) is a 519 bp deletion that disrupts the ICEBs1 conjugative relaxase encoded by nicK, but leaves a functional oriT (34).
For this study, a 1,113 bp unmarked, in-frame ICEBs1 deletion ΔconQ848 (Fig. 1E) was constructed that fuses the first two codons to the last 107 codons of conQ (of the 1440 bp conQ open reading frame), using the same method described for construction of Δnick306 (34). The ΔconQ848 allele does not appear to affect the function of oriT, which likely overlaps the 3' end of conQ and the 5' end of nick (34) (Fig. 1A).

Four truncated derivatives of ICEBs1 were inserted at thrC to test for complementation of the ΔconQ848 mutant (Fig. 1F-I). thrC229::[(ICEBs1-303 Δ(conQ-attR)::tet) mls] (Fig. 1I) and thrC229::[(ICEBs1-1637 Δ(conQ-attR)::cat) mls] (Fig. 1H) did not complement the ΔconQ848 deletion as they only contain ICEBs1 genes upstream of conQ, from int to ydcP (Fig. 1H-I). In contrast, thrC229::[(ICEBs1-304 Δ(ydcS-attR)::tet) mls] (Fig. 1F) and thrC229::[(ICEBs1-337 Δ(nick-attR)::cat) mls] (Fig. 1G) both contain wild type conQ and complement the ΔconQ848 mutant. These ICEBs1 derivatives are integrated at thrC and are unable to excise due to loss of attR. Δ(conQ-attR)::tet and Δ(conQ-attR)::cat remove sequences starting with the 109th codon of conQ. Δnick-attR::cat removes sequences starting immediately downstream of the conQ stop codon. Δ(ydcS-attR)::tet removes sequences starting immediately downstream of the nick stop codon. tet was from pDG1513 (22). cat was from pGEM-cat (50). All four alleles at thrC were derived from thrC229::[(ICEBs1 Δ(rapI-phrl)342::kan) mls] (34) and were constructed using the long-flanking homology PCR method (48) or one-step isothermal DNA assembly (20). Introduction of the ΔconQ-attR, Δnick-attR and ΔydcS-attR alleles into thrC229::[(ICEBs1 Δ(rapI-phrl)342::kan) mls] yielded tetracycline-resistant or chloramphenicol-resistant, kanamycin-sensitive transformants due to replacement of the ΔrapI-phrl::kan insertion.

Strain CAL89 is streptomycin-resistant (str-84) and cured of ICEBs1 (ICEBs10) and was used as the recipient in mating experiments. It also contains a conK::spc null
mutation that prevents acquisition of DNA by transformation (natural genetic competence). Results from mobilization experiments with different alleles of ICEBs1 are summarized in Fig. 1.

**Plasmids.** Three different plasmids were used, pC194, pBS42, and pHP13 (Fig. 2). All three plasmids use rolling circle replication and express chloramphenicol-resistance in *B. subtilis*. pC194 is 2.9 kb and from *Staphylococcus aureus* (26) (Fig. 2A). pBS42 (6) (Fig. 2B) and pHP13 (23) (Fig. 2C) are 4.8 kb shuttle vectors designed to replicate in *Escherichia coli* and *B. subtilis*. pBS42 has replicons from pBR322 (*E. coli*) and pUB110 (*S. aureus*/*B. subtilis*). pHP13 has replicons from a pUC plasmid (*E. coli*) and pTA1060 (*B. subtilis*). Although pUB110 from *S. aureus* and pTA1060 from *B. subtilis* are mobilizable plasmids, their Mob/oriT functions are not present on pBS42 and pHP13 (9, 37, 44). The ‘mobU’ sequence in pBS42 (Fig. 2B) is a non-functional portion of the 3’ end of the mobU gene from pUB110.

We constructed two derivatives of pBS42 to test for the requirement of the replicative relaxase in conjugative transfer. In one plasmid, pCAL1738, the plasmid relaxase gene repU is disrupted at the NsiI site (Fig. 2B). To allow for plasmid replication in the absence of functional RepU, the inserted DNA fragment contains the replication origin (oriN) and the cognate replication initiator gene (repN) from plasmid pLS32 of *B. subtilis* subsp. natto (25, 35, 47). oriN-repN support bi-directional theta replication (25, 47). As a control, pCAL1737 contains the intact origin of replication and relaxase gene from pBS42 and the oriN-repN fragment is inserted in the truncated mob (‘mob’) in pBS42 at the NsiI site (Fig. 2B). *B. subtilis* strain CAL1749 contains the control plasmid pCAL1737 and forms smaller colonies and grows 15-20% slower than normal in LB liquid medium supplemented with chloramphenicol. The presence of two active
replicons, oriU-repU and oriN-repN, on pCAL1737 may affect plasmid stability and cell growth.

pCAL1737 and pCAL1738 were constructed using one-step isothermal DNA assembly (20) to piece together three DNA fragments: the 3.13 kb NsiI-NsiI fragment of pBS42; a PCR product with the 1.64 kb NsiI-NsiI fragment of pBS42; and a PCR product with the 1.22 kb oriN-repN region. The assembly reactions were designed to yield plasmids identical to those generated by ligation of the oriN-repN fragment into full length pBS42, linearized at the NsiI restriction site in repU or in ’mob (Fig. 2B). The oriN-repN sequence was obtained from pDL110 (35) and transcription of repN was co-oriented with the disrupted ’mob and repU reading frames. The 1.22 kb insert includes 251 bp upstream and 112 bp downstream of the 861 bp repN open reading frame.

Conjugation and mobilization assays. Cells were grown at 37°C in LB medium, supplemented with chloramphenicol when necessary to select for maintenance of the plasmids. Donor cells were induced for ICEBs1 gene expression and conjugation by addition of IPTG for 1 hour. Mixtures of donor and recipient cells were filtered onto nitrocellulose membranes and incubated on agar containing minimal salts as described (32). Cells recovered from the filters after mating were plated onto solid media to select for transconjugants.

Transconjugants containing ICEBs1 (kan) were resistant to kanamycin (from ICEBs1) and streptomycin (from the recipient). Transconjugants containing a plasmid were resistant to chloramphenicol (from a plasmid) and streptomycin (from recipient). Mating efficiencies were calculated as the percent of transconjugant colony forming units (CFU) recovered per donor CFU present in the original mating mixture plus or minus the standard deviation.
Results

In the course of defining the ICEBs1 oriT (34), we found that plasmids previously described as non-mobilizable appeared to be mobilized by ICEBs1. Based on these preliminary findings, we characterized the mobilization of three plasmids, pC194, pBS42, and pHIP13, by ICEBs1. All three plasmids (Fig. 2) use rolling circle replication and express chloramphenicol-resistance in B. subtilis. These plasmids do not have a known oriT and none contain an intact mob gene (21, 30, 44, 46). Thus, pC194, pBS42 and pHIP13 are typically described as non-mobilizable.

Mobilization of plasmids by ICEBs1

We found that all three plasmids were mobilized by ICEBs1 (Table 2, line 1; Fig. 1B). In these experiments, donor strains containing ICEBs1 marked with a gene encoding resistance to kanamycin (kan), with or without the indicated plasmid (all encoding chloramphenicol resistance), were grown in rich medium (LB) and ICEBs1 gene expression was induced by ectopic expression of rapI from a fusion to a LacI-repressible-IPTG-inducible promoter (Pspank(hy)-rapI) for one hour. Production of active RapI induces ICEBs1 gene expression, excision, and conjugation ability (5). The recipients did not contain ICEBs1 (ICEBs1°) and were defective in the development of genetic competence (comK::spc) and hence non-transformable. Activated donors were mixed with recipient cells at a ratio of ~1:1 and mating efficiencies were determined (Materials and Methods).

pBS42 and pC194 were transferred with frequencies of ~3% plasmid-containing transconjugants per donor and pHIP13 was transferred with a frequency of ~0.07% plasmid-containing transconjugants per donor. Plasmid transfer was dependent on the presence of ICEBs1 in the donor as there was no detectable acquisition of chloramphenicol-resistance from cells that did not contain ICEBs1 (Table 2, line 2).
Plasmid transfer required components of the ICEBs1 mating machinery. An ICEBs1 mutant that is missing genes from conG to yddM \( \Delta \text{(conG-yddM)319::kan} \) (Fig. 1C) is defective in ICEBs1 conjugation (34). This mutant was incapable of mobilizing all three plasmids tested (Table 2, line 3; Fig. 1C). Together, these results indicate that ICEBs1 can mobilize the three plasmids pH13, pBS42, and pC194, and that mobilization requires at least some of the ICEBs1 mating components.

Transfer of ICEBs1 itself was not affected by the presence of any of the three plasmids tested. The mating efficiency of ICEBs1 from plasmid-free donors was approximately 6% (Table 3, line 1), similar to that reported previously (32). The mating efficiencies of ICEBs1 from plasmid-containing donors (Table 3, lines 2-4) were indistinguishable from that from the plasmid-free strain.

**Acquisition of both ICEBs1 and a plasmid by a single recipient**

We analyzed transconjugants that acquired a plasmid to determine the frequency that they also acquired ICEBs1. In experiments analogous to those described above (Table 2), single colonies of plasmid-containing transconjugants (chloramphenicol-resistant) were picked and tested for resistance to kanamycin, indicative of acquisition of ICEBs1. Of the transconjugants acquiring pBS42, pC194, or pH13, 19%, 45%, and 35%, respectively (of ≥200 transconjugants tested for each plasmid), also acquired ICEBs1. If transconjugants that acquired both the plasmid and ICEBs1 received the elements from a single donor, then these relatively high frequencies of co-transfer indicate that once a mating pair is formed, it is likely that both elements will be transferred.

In these mating experiments, the ratio of donor to recipient was approximately 1:1, and it seemed possible a single transconjugant could have acquired ICEBs1 from one donor and a plasmid from another. If so, then the frequency of co-transfer should drop
if the ratio of donor to recipient is reduced. We repeated the mating experiments described above using a donor to recipient ratio of 1:100, rather than 1:1. Of the transconjugants that acquired pBS42, pC194, or pHP13, 20-60% (of 100 transconjugants tested for each plasmid) also acquired ICEB_{s1}. Furthermore, of the transconjugants that acquired ICEB_{s1}, between 2-20% also acquired pBS42, pC194, or pHP13 (of ≥100 transconjugants tested for each plasmid). Together, these results indicate that a single donor is capable of transferring both ICEB_{s1} and a plasmid and that the relatively high frequency of co-transfer indicates that once a mating pair is formed, it is likely that both elements will be transferred.

**Plasmid mobilization does not require the ICEB_{s1} relaxase NicK or transfer of ICEB_{s1}**

There are several mechanisms by which plasmids and transposons can be mobilized. Mobilizable elements typically contain an oriT and a gene (mob) that encodes a conjugative relaxase (40, 46). The mob gene product nicks a site in oriT and is required for mobilization. Plasmids (or transposons) lacking mob functions can sometimes be mobilized by cross-recognition of an oriT site on the mobilizable element {e.g., (13)}. Plasmids (or transposons) lacking both mob and oriT functions can sometimes be mobilized when the plasmid integrates into a conjugative element and is transferred in cis as a co-integrate with the conjugative element {e.g., (12)}. In these situations, mutations in the relaxase gene of the conjugative element prevent transfer of the conjugative element and also prevent mobilization of the plasmid (or transposon). Since the plasmids used here do not contain mob and a cognate oriT, we tested whether the ICEB_{s1} relaxase was required for mobilization of these plasmids.

Using an ICEB_{s1} nicK null mutant, we found that plasmid mobilization was independent of the ICEB_{s1} relaxase and of ICEB_{s1} transfer. Although the ICEB_{s1}
relaxase encoded by nicK is essential for ICEBs1 transfer (34), it was not required for plasmid mobilization. There was no detectable decrease in mobilization of pH13, pBS42, and pC194 from ICEBs1 donors lacking nicK (Table 4, lines 1, 2; Fig. 1D). In the same experiment, there was no detectable transfer of ICEBs1 (<0.00002% transconjugants per donor), as previously reported (34). These results indicate that the ICEBs1 relaxase NicK is not needed for plasmid mobilization. Thus, transfer is not occurring by cross-recognition of an oriT on the mobilized plasmids by the ICEBs1 relaxase. Furthermore, since the relaxase mutant is incapable of transferring ICEBs1, these results demonstrate that plasmid mobilization does not require co-transfer with ICEBs1. Instead, plasmid mobilization by ICEBs1 is likely mediated by direct transfer of the plasmid DNA through the ICEBs1 conjugation machinery.

**The plasmid replicative relaxase RepU is required for pBS42 plasmid mobilization**

Since ICEBs1-mediated plasmid mobilization did not require the ICEBs1-conjugative relaxase, it seemed possible that mobilization would require the plasmid replicative relaxase. To test this, we disrupted the relaxase gene (repU) of pBS42 by inserting a DNA fragment into the NsI site in repU (Fig. 2B). Since repU is needed for replication of pBS42, the inserted fragment contained an origin of replication (oriN) and the gene (repN) encoding the cognate replication initiator. oriN-repN support bi-directional theta replication (25, 47). As a control, we also inserted the oriN-repN fragment into the NsiI site in the fragment of mobU that is present on pBS42 (Fig 2B).

We found that the replicative relaxase of pBS42 is needed for ICEBs1-mediated mobilization of pBS42. The plasmid with repU disrupted, pCAL1738 [pBS42 repU::(oriN-repN)] was not detectably mobilized by ICEBs1 (<10^{-5} % plasmid-containing transconjugants per donor). Transfer of ICEBs1 was normal from these donors (~6% ±
2% transconjugants/donor) indicating that the ICEBs1 transfer machinery was functional. In contrast, the control plasmid pCAL1737 \{pBS42 \textit{mob::(oriN-repN)}\} in which \textit{repU} is not disrupted was still mobilized (~0.7% ± 0.1% plasmid-containing transconjugants/donor), indicating that the presence of \textit{oriN-repN} did not prevent mobilization. Based on these results, we conclude that the plasmid replicative relaxase \textit{RepU} is required for pBS42 mobilization and is most likely functioning as both a replicative and a conjugative relaxase.

The putative coupling protein of ICEBs1 is required for conjugation and plasmid mobilization

Transfer of conjugative elements typically requires a coupling protein, an ATPase that interacts with the relaxosome (relaxase attached to DNA) and the conjugation apparatus, coupling the two complexes and enabling transfer of the relaxase and the covalently attached substrate DNA (36). The coupling proteins typically have an FtsK-like motor domain needed for function and are encoded adjacent to or very near the gene encoding the relaxase (28, 39, 46). \textit{conQ} (previously called \textit{ydcQ}) of ICEBs1 (Fig. 1) encodes the putative coupling protein (28).

We found that \textit{conQ} was required for transfer of ICEBs1. We made an in-frame deletion in \textit{conQ} and integrated this into ICEBs1 (Fig. 1E). Following overproduction of RapI and induction of ICEBs1, the \textit{conQ} null mutant was unable to transfer ICEBs1 to recipients (<10\(^{-5}\) % transconjugants per donor, CAL848) (Fig. 1E).

We also found that the \textit{conQ} deletion does not significantly affect \textit{oriT} function nor was it polar on the downstream genes needed for conjugation. The inability of the \textit{conQ} mutant to transfer was largely relieved when \textit{conQ}, along with all the ICEBs1 genes upstream of \textit{conQ} were expressed in trans (Fig. 1G) (mating efficiency of ~1% transconjugants per donor, JT339). The upstream genes were provided in addition to...
conQ because we commonly find that complementation (and presumably protein production) is more efficient when upstream genes are included [e.g., (7)]. The control that provided all the upstream genes, but not a functional conQ, was unable to restore conjugation to the ICEB1 ΔconQ mutant (<10^5 % transconjugants per donor, JT338; Fig. 1H). We conclude that conQ is required for ICEB1 conjugation, that the ΔconQ mutation is not polar on downstream conjugation genes, and that it does not affect oriT function or nicking of oriT by the ICEB1 relaxase.

conQ was also required for mobilization of pHP13, pBS42 and pC194, none of which encode their own dedicated coupling protein. When the conQ null mutant was used as donor, there was no detectable transfer of any of the three plasmids to recipient cells (Table 4, line 3; Fig. 1E). The inability of the conQ mutant to mobilize the plasmids was largely relieved when conQ, along with all the ICEB1 genes upstream of and one gene (nicK) downstream of conQ were expressed from an ectopic locus (Table 4, line 4; Fig. 1F). The control that provided all the upstream genes, but not a functional conQ, was unable to restore mobilization to the conQ mutant (Table 4, line 5; Fig. 1I). Since nicK is not needed for plasmid mobilization (Table 4, line 2), these results indicate that the defect in mobilization was due to loss of conQ and not a polar effect on downstream genes, and that the putative coupling protein of ICEB1 is likely needed to recruit a plasmid-associated relaxasome complex to the ICEB1 mating machinery.

Discussion

Experiments described here indicate that ICEB1 of B. subtilis is capable of mobilizing at least three different plasmids, pC194, pBS42, and pHP13. Mobilizable plasmids are thought to require dedicated mobilization functions; a conjugative
relaxase (Mob) and a cognate oriT, that are separate from the replication functions.

None of the plasmids used here have dedicated mobilization functions. Mobilization by ICEBs1 requires the ICEBs1 conjugation machinery and the putative coupling protein, ConQ. In characterized systems, the coupling protein is needed to link the relaxasome complex, which contains a conjugative relaxase attached to the 5′ end of a single strand of DNA, to the conjugation machinery (14, 36, 46). Plasmid mobilization by ICEBs1 did not require the ICEBs1 relaxase encoded by nicK indicating that mobilization was not due to cross-recognition of a cryptic oriT on the plasmids or co-transfer of ICEBs1 and plasmid DNA. Mobilization of pBS42 required the replicative relaxase RepU of pBS42, indicating that this replicative relaxase can also function as a conjugative relaxase. These findings have practical applications for the characterization of ICEBs1 genes and the genetic manipulation of heterologous bacteria. In addition, our findings indicate that there is more potential for horizontal gene transfer of non-conjugative plasmids than previously recognized.

**Practical applications of plasmid mobilization for genetic studies**

Plasmid mobilization can be used to help characterize genes involved in conjugation. For example, some of the genes required for conjugation of ICEBs1 were needed for plasmid mobilization, including at least some of the genes encoding the conjugation machinery and the putative coupling protein. Other functions required for ICEBs1 conjugation, including the relaxase and the ability to excise from the chromosome (data not shown), were not needed for plasmid mobilization. These differences between transfer of ICEBs1 and mobilization of plasmids can be used to help delineate the steps at which different ICEBs1 or host gene products act.

Plasmid mobilization by ICEBs1 could also be a useful and efficient way to introduce DNA to strains that are difficult to transform. ICEBs1 can be transferred to
other organisms (5), and at least some of the plasmids used here are capable of replicating in other organisms. Cloning and genetic manipulations could be done with plasmids in *E. coli* or *B. subtilis* and then the desired plasmids could be mobilized from *B. subtilis* by ICEBs1 into other Gram-positive organisms that are suitable recipients for conjugation. Other conjugative elements have been used for plasmid mobilization (16), but the high efficiencies of mobilization by ICEBs1 and the ability to easily manipulate *B. subtilis* make mobilization by ICEBs1 an attractive system for use with Gram positive bacteria.

**Functional relationship between replicative and conjugative relaxases**

Duplication of pC194, pBS42, and pHPl3 requires a plasmid origin of replication (ori) and a cognate replicative relaxase (Rep) that enables rolling circle replication (29). The first steps in production of a substrate for conjugation and for rolling circle replication are similar. Both require a relaxase (a conjugative or replicative relaxase) that nicks a site in an origin (origin of transfer or origin of replication). The relaxase becomes covalently attached to the cognate origin and serves to mark the site for transfer or replication. Following nicking, a helicase is required for unwinding the double-stranded DNA substrate for either conjugation or replication. Many rolling circle replicating plasmids in Gram positive organisms use the host-encoded helicase PcrA for replication (29). Similarly, ICEBs1 uses PcrA both for replication and for conjugation, although replication is not required for conjugation (33).

The conjugative relaxases are similar to the replicative relaxases, although they are generally thought to belong to different sub-types of the relaxase family (19, 27, 31). However, recent work demonstrated that the ICEBs1 conjugative relaxase NicK also functions as a replicative relaxase using a single origin for both conjugative transfer and replication (33). Results presented here indicate that at least three different replicative
relaxases, from pC194, pBS42 (pUB110), and pH13 (pTA1060), likely also function as conjugative relaxases. This is in contrast to the prevalent view that mobilizable plasmids have separate replication (Rep/ori) and mobilization (Mob/oriT) functions (21, 46).

Previous studies found that certain plasmids from B. thuringiensis or B. subtilis could be mobilized in the absence of mobilization functions (2, 3, 38, 45). For example, the conjugative transposon Tn916 can mobilize pC194 from B. subtilis to B. thuringiensis at a low frequency (38), even though pC194 is still described as not being mobilizable. In addition, mobilization of pUB110 by the conjugative transposon Tn916 from B. subtilis into B. thuringiensis did not require the pUB110 mob gene (45). It was proposed that pUB110 might contain a Tn916-like oriT that could be recognized by the Tn916 conjugative relaxase. Based on results presented here, we think a more likely possibility is that the replicative relaxases from pUB110 and pC194 also function as conjugative relaxases and that plasmid mobilization by Tn916 is likely independent of the Tn916 relaxase.

**Likely mechanism of plasmid mobilization in the absence of dedicated mobilization functions**

Plasmid mobilization mediated by ICEBs1 probably occurs by a mechanism similar to transfer of ICEBs1. We propose that the plasmid replicative relaxasome, consisting of the replicative relaxase attached to plasmid DNA, and perhaps associated with the helicase PcrA, interacts with the putative coupling protein from ICEBs1, ConQ. This interaction might be analogous to the interactions between coupling proteins and the cognate relaxasomes from ICEs and conjugative plasmids (14, 15, 36). The coupling protein ConQ would then recruit the plasmid relaxasome to the ICEBs1 conjugation machinery at the cell membrane. Interactions between the coupling protein and the
helicase PcrA and/or the target DNA could also be involved, either in addition to or instead of interactions with the replicative relaxase. However, the lower efficiency of pH13 mobilization by ICEBs1 as compared to pC194 and pBS42 mobilization argues against mobilization primarily occurring through interactions between the coupling protein and the helicase PcrA. We postulate that the specificity comes from protein-protein interaction between the relaxase and the coupling protein. In this case, the lower efficiency of pH13 mobilization may be due to a lower affinity of the pH13 replicative relaxase for the ICEBs1 coupling protein and for components of the ICEBs1 conjugation machinery

**Evolutionary implications**

The evolutionary and functional relationship between conjugative and replicative relaxases likely enables direct mobilization of certain rolling circle-replicating plasmids by conjugative elements. Two lines of evidence blur the distinction between conjugative and replicative relaxases. 1) At least three plasmids that are mobilized by the ICEBs1 conjugation machinery encode a single relaxase that may mediate both replication and mobilization. 2) The relaxase from ICEBs1 is clearly bifunctional, serving as a conjugative relaxase and a replicative relaxase for ICEBs1 conjugation and rolling circle replication (33). We suspect that many, and perhaps most, conjugative relaxases can function as replicative relaxases with the cognate oriT functioning as an origin of replication. Similarly, many replicative relaxases may function in conjugation. The key distinguishing feature between conjugative and non-conjugative replicative relaxases might be the ability to interact with a coupling protein, necessary for conjugation but not replication.

The persistence of plasmids in bacterial populations is likely due to benefits they confer on the host cell or to their efficient dissemination to new hosts by horizontal
transfer. Otherwise, the burden placed on the host by the plasmid is thought to result in loss of the plasmid. Based on the lack of a *mob* gene, approximately 60% of 1,730 sequenced plasmids are inferred to be non-mobilizable (46). Because of this inference, it was proposed that persistence of many of these "non-mobilizable" plasmids is due to unknown benefits conferred upon the host (46). The ability of ICEBs1 to mobilize three plasmids lacking dedicated *mob* functions indicates that many "non-mobilizable" plasmids may in fact be mobilizable. This could account for the persistence of so many "non-mobilizable" plasmids, indicating that the impact of conjugation on plasmid mobilization and persistence may be much greater than previously thought.

Acknowledgments

We thank M. Berkmen for helpful discussions and M. Berkmen and K. Menard for comments on the manuscript. This work was supported, in part, by NIH grant GM50895.

References


9. **Bacillus subtilis**


<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype (reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAL13</td>
<td>ICEBs1'' amyE::[(Pspank(hy)-rapl) spc] (34)</td>
</tr>
<tr>
<td>CAL89</td>
<td>ICEBs1'' comK::spc str-84 (5)</td>
</tr>
<tr>
<td>CAL306</td>
<td>ΔnicK306 Δ(rapl-phrI)342::kan amyE::[(Pspank(hy)-rapl) spc] (34)</td>
</tr>
<tr>
<td>CAL421</td>
<td>Δ(conG-yddM)319::kan amyE::[(Pspank(hy)-rapl) spc] (34)</td>
</tr>
<tr>
<td>CAL848</td>
<td>ΔconQ848 Δ(rapl-phrI)342::kan amyE::[(Pspank(hy)-rapl) spc]</td>
</tr>
<tr>
<td>CAL1034</td>
<td>Δ(conG-yddM)319::kan amyE::[(Pspank(hy)-rapl) spc]; pH13 (cat mls)</td>
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<tr>
<td>CAL1392</td>
<td>ICEBs1'' amyE::[(Pspank(hy)-rapl) spc]; pBS42 (cat)</td>
</tr>
<tr>
<td>CAL1393</td>
<td>Δ(rapl-phrI)342::kan amyE::[(Pspank(hy)-rapl) spc]; pBS42 (cat)</td>
</tr>
<tr>
<td>CAL1395</td>
<td>ΔnicK306 Δ(rapl-phrI)342::kan amyE::[(Pspank(hy)-rapl) spc]; pBS42 (cat)</td>
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<td>CAL1396</td>
<td>Δ(conG-yddM)319::kan amyE::[(Pspank(hy)-rapl) spc]; pBS42 (cat)</td>
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<td>CAL1397</td>
<td>ICEBs1'' amyE::[(Pspank(hy)-rapl) spc]; pH13 (cat mls)</td>
</tr>
<tr>
<td>CAL1398</td>
<td>Δ(rapl-phrI)342::kan amyE::[(Pspank(hy)-rapl) spc]; pH13 (cat mls)</td>
</tr>
<tr>
<td>CAL1400</td>
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<td>CAL1532</td>
<td>ICEBs1'' amyE::[(Pspank(hy)-rapl) spc]; pC194 (cat)</td>
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<tr>
<td>CAL1533</td>
<td>Δ(rapl-phrI)342::kan amyE::[(Pspank(hy)-rapl) spc]; pC194 (cat)</td>
</tr>
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<td>CAL1535</td>
<td>ΔnicK306 Δ(rapl-phrI)342::kan amyE::[(Pspank(hy)-rapl) spc]; pC194 (cat)</td>
</tr>
<tr>
<td>CAL1548</td>
<td>Δ(conG-yddM)319::kan amyE::[(Pspank(hy)-rapl) spc]; pC194 (cat)</td>
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<td>CAL1590</td>
<td>ΔconQ848 thrC229::[(ICEBs1-303 ΔconQ-attR::tet) mls] Δ(rapl-phrI)342::kan amyE::[(Pspank(hy)-rapl) spc]</td>
</tr>
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<td>CAL1591</td>
<td>ΔconQ848 Δ(rapl-phrI)342::kan amyE::[(Pspank(hy)-rapl) spc]; pBS42 (cat)</td>
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<td>CAL1592</td>
<td>ΔconQ848 Δ(rapl-phrI)342::kan amyE::[(Pspank(hy)-rapl) spc]; pC194 (cat)</td>
</tr>
<tr>
<td>CAL1593</td>
<td>ΔconQ848 thrC229::[(ICEBs1-304 ΔydcS-attR::tet) mls] Δ(rapl-phrI)342::kan amyE::[(Pspank(hy)-rapl) spc]; pBS42 (cat)</td>
</tr>
<tr>
<td>CAL1594</td>
<td>ΔconQ848 thrC229::[(ICEBs1-304 ΔydcS-attR::tet) mls] Δ(rapl-phrI)342::kan amyE::[(Pspank(hy)-rapl) spc]; pC194 (cat)</td>
</tr>
<tr>
<td>CAL1595</td>
<td>ΔconQ848 thrC229::[(ICEBs1-303 ΔconQ-attR::tet) mls] Δ(rapl-phrI)342::kan amyE::[(Pspank(hy)-rapl) spc]; pBS42 (cat)</td>
</tr>
<tr>
<td>Strain</td>
<td>Description</td>
</tr>
<tr>
<td>------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>CAL1596</td>
<td>ΔconQ848 thrC229::{(ICEBs1-303 ΔconQ-attR::tet) mls} Δ(rapl-phrl)342::kan amyE::{(Pspank(hy)-rapl) spc}; pC194 (cat)</td>
</tr>
<tr>
<td>CAL1597</td>
<td>ΔconQ848 Δ(rapl-phrl)342::kan amyE::{(Pspank(hy)-rapl) spc}; pH13 (cat mls)</td>
</tr>
<tr>
<td>CAL1598</td>
<td>ΔconQ848 thrC229::{ICEBs1-304 ΔydcS-attR::tet) mls} Δ(rapl-phrl)342::kan amyE::{(Pspank(hy)-rapl) spc}; pH13 (cat mls)</td>
</tr>
<tr>
<td>CAL1599</td>
<td>ΔconQ848 thrC229::{ICEBs1-303 ΔconQ-attR::tet) mls Δ(rapl-phrl)342::kan amyE::{(Pspank(hy)-rapl) spc} }; pH13 (cat mls)</td>
</tr>
<tr>
<td>CAL1749</td>
<td>Δ(rapl-phrl)342::kan amyE::{(Pspank(hy)-rapl) spc}; pCAL1737 {pBS42 cat 'mob::(oriN-repN)}</td>
</tr>
<tr>
<td>CAL1751</td>
<td>Δ(rapl-phrl)342::kan amyE::{(Pspank(hy)-rapl) spc}; pCAL1738 {pBS42 cat repU::(oriN-repN)}</td>
</tr>
<tr>
<td>JMA168</td>
<td>Δ(rapl-phrl)342::kan amyE::{(Pspank(hy)-rapl) spc} (5)</td>
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<tr>
<td>JT298</td>
<td>ΔconQ848 thrC229::{(ICEBs1-304 ΔydcS-attR::tet) mls} Δ(rapl-phrl)342::kan amyE::{(Pspank(hy)-rapl) spc}</td>
</tr>
<tr>
<td>JT338</td>
<td>ΔconQ848 thrC229::{ICEBs1-1637 ΔconQ-attR::cat) mls} Δ(rapl-phrl)342::kan amyE::{(Pspank(hy)-rapl) spc}</td>
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<tr>
<td>JT339</td>
<td>ΔconQ848 thrC229::{ICEBs1-337 ΔnicK-attR::cat) mls} Δ(rapl-phrl)342::kan amyE::{(Pspank(hy)-rapl) spc}</td>
</tr>
</tbody>
</table>

*Strains are derived from laboratory strain JH642 and contain trpC and pheA mutations.*
Table 2. Mobilization of plasmids by ICEBs1.

<table>
<thead>
<tr>
<th>ICEBs1 in donor&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Plasmid mobilization (%)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS42</td>
</tr>
<tr>
<td>1  Δ(rapI-phrI)::kan</td>
<td>2.8 ± 1.6</td>
</tr>
<tr>
<td>2  None</td>
<td>&lt;0.00002</td>
</tr>
<tr>
<td>3  Δ(conG-yddM)::kan</td>
<td>&lt;0.00002</td>
</tr>
</tbody>
</table>

<sup>a</sup> Donor strains contained the indicated plasmid (chloramphenicol resistant) and amyE::[Pspank(hy)-rapI spc] for overproduction of RapI to induce ICEBs1. Donor strains contained PBS42, PC194, and PHP13 and ICEBs1 Δ(rapI-phrI)342::kan (CAL1393, CAL1533, and CAL1398, respectively); no ICEBs1 (CAL1392, CAL1532 and CAL1397, respectively); and ICEBs1 Δ(conG-yddM)::kan (CAL1396, CAL1548 and CAL1034, respectively).

<sup>b</sup> The recipient strain in the mating experiments was CAL89 (ICEBs1<sup>0</sup> comK::spc str). Values for the Δ(rapI-phrI)::kan donors are the means from 9 (PBS42), 4 (PC194), and 5 (PHP13) independent mating assays. Mating assays with donors defective for plasmid mobilization yielded no detectable transconjugants (<0.00002%) in at least two independent experiments. Plasmid mobilization was calculated as percent Cm<sup>R</sup> Str<sup>R</sup> transconjugant CFU per donor CFU in the original mating mixture plus or minus the standard deviation.
Table 3. Transfer of ICEBs1 is not affected by the presence of plasmids.

<table>
<thead>
<tr>
<th>Plasmid (strain)</th>
<th>Percent transfer of ICEBs1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 None (JMA168)</td>
<td>6.3 ± 3.5</td>
</tr>
<tr>
<td>2 pBS42 (CAL1393)</td>
<td>9.9 ± 5.1</td>
</tr>
<tr>
<td>3 pCI94 (CAL1533)</td>
<td>7.0 ± 3.6</td>
</tr>
<tr>
<td>4 pHPI3 (CAL1398)</td>
<td>7.3 ± 3.1</td>
</tr>
</tbody>
</table>

* Donor strains contained the indicated plasmid, ICEBs1 with the Δ(rapI-phrI)342::kan allele and amyE::Pspank(hy)-rapI spc for overproduction of RapI to induce ICEBs1.

* The recipient strain in the mating experiments was CAL89 (ICEBs1<sup>0</sup> comK::spc str). Transconjugants containing ICEBs1 were selected as resistant to kanamycin (from ICEBs1) and streptomycin (from the recipient). Values for percent transfer of ICEBs1 are the means from 5 independent mating assays (9 for pBS42) and are calculated as the percent Kan<sup>R</sup> Str<sup>R</sup> colony-forming units (CFU) per donor CFU in the original mating mixture plus or minus the standard deviation.
Table 4. Plasmid mobilization does not require ICEBs1 nickase NicK, but does require the putative coupling protein ConQ.

<table>
<thead>
<tr>
<th>ICEBs1 in donora</th>
<th>Plasmid mobilization (%)b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pBS42 (n)</td>
</tr>
<tr>
<td>1 Δ(rapI-phrI)::kan</td>
<td>2.8 ± 1.6 (9)</td>
</tr>
<tr>
<td>2 ΔnicK306</td>
<td>3.6 ± 2.0 (4)</td>
</tr>
<tr>
<td>3 ΔconQ848</td>
<td>&lt;0.00005 (4)</td>
</tr>
<tr>
<td>4 ΔconQ848/thrC::conQ+</td>
<td>2.7 ± 1.8 (2)</td>
</tr>
<tr>
<td>5 ΔconQ848/thrC::conQΔ</td>
<td>&lt;0.00005 (2)</td>
</tr>
</tbody>
</table>

a Donor strains contained the indicated plasmid (chloramphenicol-resistant), amyE::(Pspank(hy)-rapI) spc, the ICEBs1 Δ(rapI-phrI)342::kan allele (present in all donors but not indicated in lines 2-5), mutations in nicK or conQ (lines 2-5), and trans-acting ICEBs1 genes at thrC (lines 4 and 5). Donor strains contained pBS42, pC194, and pH13 and: ICEBs1 Δ(rapI-phrI)342::kan (CAL1393, CAL1533, and CAL1398, respectively, as in Table 2); ΔnicK306 Δ(rapI-phrI)342::kan (CAL1395, CAL1535, and CAL1400, respectively); ΔconQ848 Δ(rapI-phrI)342::kan (CAL1591, CAL1592, and CAL1597, respectively); ΔconQ848 Δ(rapI-phrI)342::kan thrC::conQ+ (CAL1593, CAL1594, and CAL1598, respectively); and ΔconQ848 Δ(rapI-phrI)342::kan thrC::conQΔ (CAL1595, CAL1596, and CAL1599, respectively).

Although plasmid mobilization did not require nicK, nicK is required for transfer of ICEBs1 from plasmid-containing donors. There was no transfer of ICEBs1 from plasmid-containing ΔnicK306 donors (data not shown).

The thrC::conQ+ (line 4) used for plasmid mobilization contains the ICEBs1 genes upstream from and including conQ and nicK (Fig. 1). Since nicK is not needed for mobilization, the defect in the ΔconQ848 mutant is due to loss of conQ and not a polar effect on downstream genes. The thrC::conQΔ (thrC::[ICEBs1 ΔconQ-attR::tet]) (line 5) contains all ICEBs1 genes upstream of conQ, but not a functional conQ (Fig. 1). This was used as a control for the complementation (line 4).

b Percent plasmid mobilization was determined as described for Table 2. Values are the mean from ≥2 independent experiments ± the standard deviation. The number of independent experiments for each strain (n) is indicated in parentheses.
Figure 1. Map of ICEBs1 and various mutants.

A. Genetic map of ICEBs1. ICEBs1 is shown in its linear integrated form. Open arrows indicate open reading frames and the direction of transcription. Gene names are indicated above 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 is needed for ICEBs1 transfer (34) and replication (33). 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-E. Schematic diagram of deletion mutations in ICEBs1 in donor strains. Thin horizontal lines represent the regions of ICEBs1 that are present in the donor strains. Gaps in the line represent the regions of ICEBs1 that are deleted. Antibiotic-resistance markers that are inserted in some of the alleles are not shown. Δ(rapIphrI) (B, D, E) (5) and Δ(conG-yddM) (C) (34) are deletion-insertion mutations with a kanamycin-resistance gene inserted (not shown). ΔnicK (E) and ΔconQ (F) are unmarked in-frame deletion mutations that do not disrupt the expression of downstream genes and do not disrupt oriT.

F-I. Schematic diagram of truncated ICEBs1 derivatives integrated at thrC. These constructs cannot excise due to deletion of attR and were used to provide ICEBs1 genes in trans for complementation tests of the ICEBs1 ΔconQ mutant. Thin horizontal lines represent the regions of ICEBs1 that are present at thrC.

B-I. The ability of plasmids to be mobilized or of ICEBs1 to be transferred from various donor strains are summarized as + or -. nd indicates not determined.
Figure 2. Plasmid maps.

Schematic diagrams of plasmids pC194 (A), pBS42 (B), and pH13 (C), all mobilized by ICEBs1. The approximate size of each plasmid is indicated under the plasmid name. Circles represent each plasmid and are not shown to scale. Thin black arrows and gene names within each circle represent functional genes or, in the case of ‘mobU (B), a non-functional 3’ fragment of mobU (missing the 5’end). Antibiotic resistance genes include cat (chloramphenicol resistance) and mls (macrolide, lincosamide, and streptogramin resistance). Small open arrows on each circle represent the origin of replication, which contains the site for nicking by the cognate replicative relaxase (encoded by rep, repU, and rep60). The sequences flanking the nic sites in each plasmid ori are highly conserved. The orientation of the open arrows indicates the direction of leading strand synthesis for rolling circle replication. pBS42 (B) and pH13 (C) are composites of segments from several plasmids (6, 23). The junctions and sources of the different segments are indicated by the intersecting lines and plasmid names on the perimeter of each circle. For pBS42 (B) the location of two NsiI restriction sites is indicated with a line and an "N". The three plasmids have a 0.98 kb sequence in common that contains cat. Other DNA sequences common to all three plasmids are ≤12 bp.
Figure 1
Figure 2