The *Bacillus subtilis* Conjugative Transposon ICEBs1 Mobilizes Plasmids Lacking Dedicated Mobilization Functions

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Integrative and conjugative elements (ICEs, also known as 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 nonmobilizable. We found that *Bacillus subtilis* carrying the integrative and conjugative element ICEBs1 can transfer three different plasmids to recipient bacteria at high frequencies. Strikingly, these plasmids do not have 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 cotransfer 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 and nonmobilizable plasmids and indicate that conjugative elements play a role in horizontal gene transfer even more significant than previously recognized.

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 (see 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 *nick*) 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 recip-

ient, and high mating efficiencies of 1 to 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 (also known as 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 pHP13 (see Fig. 2), that replicate by rolling circle replication (RCR) and are typically described as nonmobilizable (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

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FIG 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 to 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(*rapI-phrI*) (B, D, and E) (5) and Δ(*conG-yddM*) (C) (34) are deletion-insertion mutations with a kanamycin resistance gene inserted (not shown). Δ*nicK* (D) and Δ*conQ* (E) are unmarked in-frame deletion mutations that do not disrupt the expression of downstream genes and do not disrupt *oriT*. (F to 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 Δ*conQ* mutant. Thin horizontal lines represent the regions of ICEBs1 that are present at *thrC*. (B to I) The abilities or inabilities of plasmids to be mobilized or of ICEBs1 to be transferred from various donor strains are summarized as + or −, respectively. nd, not determined.

of replication but are not known to contain an origin of transfer or have 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 either were cured of ICEBs1 (ICEBs1⁰) (5) or carried one of several derivatives of ICEBs1 (Fig. 1B to E). All *B. subtilis* strains are listed in Table 1 and were 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 (isopropyl-β-D-thiogalactopyranoside)-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) conferring resistance to kanamycin was typically used, as previously described (5).

ICEBs1 Δ(*conG-yddM*)319::kan (Fig. 1C) has an insertion-deletion that removes several essential conjugation genes and replaces them with the *kan* cassette. The mutation removes 661 bp from 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 Δ*nicK*306 (Fig. 1D) has a 519-bp deletion that disrupts the ICEBs1 conjugative relaxase encoded by *nicK* but leaves a functional *oriT* (34).

For this study, we made a 1,113-bp unmarked, in-frame ICEBs1 Δ*conQ*848 deletion (Fig. 1E); in this mutant, the first two codons were fused to the last 107 codons of *conQ* (of the 1,440-bp *conQ* open reading frame), using the same method described for construction of the

Δ*nicK*306 mutant (34). The Δ*conQ*848 allele does not appear to affect the function of *oriT*, which likely overlaps the 3' end of *conQ* and the 5' end of *nicK* (Fig. 1A) (34).

Four truncated derivatives of ICEBs1 were inserted at *thrC* to test for complementation of the Δ*conQ*848 mutant (Fig. 1F to I). *thrC*229::[(ICEBs1-303 Δ(*conQ-attR*)::tet) *mls*] (Fig. 1I) and *thrC*229::[(ICEBs1-1637 Δ(*conQ-attR*)::cat) *mls*] (Fig. 1H) did not complement the Δ*conQ*848 deletion, as they contain only ICEBs1 genes upstream of *conQ*, from *int* to *ydcP*. In contrast, *thrC*229::[(ICEBs1-304 Δ(*ydcS-attR*)::tet) *mls*] (Fig. 1F) and *thrC*229::[(ICEBs1-337 Δ(*nicK-attR*)::cat) *mls*] (Fig. 1G) both contain wild-type *conQ* and complement the Δ*conQ*848 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*. Δ*nicK-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 *thrC*229::[(ICEBs1 Δ(*rapI-phrI*)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 Δ*conQ-attR*, Δ*nicK-attR*, and Δ*ydcS-attR* mutations into *thrC*229::[(ICEBs1 Δ(*rapI-phrI*)342::kan) *mls*] yielded tetracycline-resistant or chloramphenicol-resistant, kanamycin-sensitive transformants due to replacement of the Δ*rapI-phrI*::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 *comK*::*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, pC194, pBS42, and pHP13, were used (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* (Fig. 2A) (26). pBS42 (Fig. 2B) (6) and pHP13 (Fig. 2C) (23) 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

TABLE 1 *B. subtilis* strains^a

Strain	Relevant genotype (reference)
CAL13	ICEBs1 ⁰ amyE::[(Pspank(hy)-rapI) spc] (34)
CAL89	ICEBs1 ⁰ comK::spc str-84 (5)
CAL306	ΔnicK306 Δ(rapI-phrI)342::kan amyE::[(Pspank(hy)-rapI) spc] (34)
CAL421	Δ(conG-yddM)319::kan amyE::[(Pspank(hy)-rapI) spc] (34)
CAL848	ΔconQ848 Δ(rapI-phrI)342::kan amyE::[(Pspank(hy)-rapI) spc]
CAL1034	Δ(conG-yddM)319::kan amyE::[(Pspank(hy)-rapI) spc]; pHP13 (cat mls)
CAL1392	ICEBs1 ⁰ amyE::[(Pspank(hy)-rapI) spc]; pBS42 (cat)
CAL1393	Δ(rapI-phrI)342::kan amyE::[(Pspank(hy)-rapI) spc]; pBS42 (cat)
CAL1395	ΔnicK306 Δ(rapI-phrI)342::kan amyE::[(Pspank(hy)-rapI) spc]; pBS42 (cat)
CAL1396	Δ(conG-yddM)319::kan amyE::[(Pspank(hy)-rapI) spc]; pBS42 (cat)
CAL1397	ICEBs1 ⁰ amyE::[(Pspank(hy)-rapI) spc]; pHP13 (cat mls)
CAL1398	Δ(rapI-phrI)342::kan amyE::[(Pspank(hy)-rapI) spc]; pHP13 (cat mls)
CAL1400	ΔnicK306 Δ(rapI-phrI)342::kan amyE::[(Pspank(hy)-rapI) spc]; pHP13 (cat mls)
CAL1532	ICEBs1 ⁰ amyE::[(Pspank(hy)-rapI) spc]; pC194 (cat)
CAL1533	Δ(rapI-phrI)342::kan amyE::[(Pspank(hy)-rapI) spc]; pC194 (cat)
CAL1535	ΔnicK306 Δ(rapI-phrI)342::kan amyE::[(Pspank(hy)-rapI) spc]; pC194 (cat)
CAL1548	Δ(conG-yddM)319::kan amyE::[(Pspank(hy)-rapI) spc]; pC194 (cat)
CAL1590	ΔconQ848 thrC229::[(ICEBs1-303 ΔconQ-attR::tet) mls] Δ(rapI-phrI)342::kan amyE::[(Pspank(hy)-rapI) spc]
CAL1591	ΔconQ848 Δ(rapI-phrI)342::kan amyE::[(Pspank(hy)-rapI) spc]; pBS42 (cat)
CAL1592	ΔconQ848 Δ(rapI-phrI)342::kan amyE::[(Pspank(hy)-rapI) spc]; pC194 (cat)
CAL1593	ΔconQ848 thrC229::[(ICEBs1-304 ΔydcS-attR::tet) mls] Δ(rapI-phrI)342::kan amyE::[(Pspank(hy)-rapI) spc]; pBS42 (cat)
CAL1594	ΔconQ848 thrC229::[(ICEBs1-304 ΔydcS-attR::tet) mls] Δ(rapI-phrI)342::kan amyE::[(Pspank(hy)-rapI) spc]; pC194 (cat)
CAL1595	ΔconQ848 thrC229::[(ICEBs1-303 ΔconQ-attR::tet) mls] Δ(rapI-phrI)342::kan amyE::[(Pspank(hy)-rapI) spc]; pBS42 (cat)
CAL1596	ΔconQ848 thrC229::[(ICEBs1-303 ΔconQ-attR::tet) mls] Δ(rapI-phrI)342::kan amyE::[(Pspank(hy)-rapI) spc]; pC194 (cat)
CAL1597	ΔconQ848 Δ(rapI-phrI)342::kan amyE::[(Pspank(hy)-rapI) spc]; pHP13 (cat mls)
CAL1598	ΔconQ848 thrC229::[(ICEBs1-304 ΔydcS-attR::tet) mls] Δ(rapI-phrI)342::kan amyE::[(Pspank(hy)-rapI) spc]; pHP13 (cat mls)
CAL1599	ΔconQ848 thrC229::[(ICEBs1-303 ΔconQ-attR::tet) mls] Δ(rapI-phrI)342::kan amyE::[(Pspank(hy)-rapI) spc]; pHP13 (cat mls)
CAL1749	Δ(rapI-phrI)342::kan amyE::[(Pspank(hy)-rapI) spc]; pCAL1737 {pBS42 cat 'mob::(oriN-repN)}
CAL1751	Δ(rapI-phrI)342::kan amyE::[(Pspank(hy)-rapI) spc]; pCAL1738 {pBS42 cat repU::(oriN-repN)}
JMA168	Δ(rapI-phrI)342::kan amyE::[(Pspank(hy)-rapI) spc] (5)
JT298	ΔconQ848 thrC229::[(ICEBs1-304 ΔydcS-attR::tet) mls] Δ(rapI-phrI)342::kan amyE::[(Pspank(hy)-rapI) spc]
JT338	ΔconQ848 thrC229::[(ICEBs1-1637 ΔconQ-attR::cat) mls] Δ(rapI-phrI)342::kan amyE::[(Pspank(hy)-rapI) spc]
JT339	ΔconQ848 thrC229::[(ICEBs1-337 ΔnicK-attR::cat) mls] Δ(rapI-phrI)342::kan amyE::[(Pspank(hy)-rapI) spc]

^a Strains are derived from laboratory strain JH642 and contain *trpC* and *pheA* mutations.

FIG 2 Plasmid maps. Schematic diagrams of plasmids pC194 (A), pBS42 (B), and pHP13 (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 nonfunctional 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 pHP13 (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 locations of two NsiI restriction sites are indicated with a line and an "N." The three plasmids have a 0.98-kb sequence, containing *cat*, in common. Other DNA sequences common to all three plasmids are ≤12 bp.

(Fig. 2B) is a nonfunctional 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). The *oriN-repN* genes support bidirectional 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 to 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 cooriented with the disrupted '*mob*' and *repU* read-

TABLE 2 Mobilization of plasmids by ICEBs1

ICEBs1 in donor ^a	% plasmid mobilization ^b		
	pBS42	pC194	pHP13
Δ(<i>rapI-phrI</i>)::kan	2.8 ± 1.6	2.7 ± 1.9	0.068 ± 0.04
None	<0.00002	<0.00002	<0.00002
Δ(<i>conG-yddM</i>)::kan	<0.00002	<0.00002	<0.00002

^a Donor strains contained the indicated plasmid (chloramphenicol resistant) and *amyE*::[P_{spank}(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), or ICEBs1 Δ(*conG-yddM*)::kan (CAL1396, CAL1548, and CAL1034, respectively).

^b The recipient strain in the mating experiments was CAL89 (ICEBs1⁰ *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^r Str^r transconjugant CFU per donor CFU in the original mating mixture plus or minus the standard deviation.

ing 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 h. Mixtures of donor and recipient cells were filtered onto nitrocellulose membranes and incubated on agar containing minimal salts as described previously (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 the recipient). Mating efficiencies were calculated as percentages of transconjugant 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 nonmobilizable appeared to be mobilized by ICEBs1. Based on these preliminary findings, we characterized the mobilization of three plasmids, pC194, pBS42, and pHP13, 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 pHP13 are typically described as nonmobilizable.

Mobilization of plasmids by ICEBs1. We found that all three plasmids were mobilized by ICEBs1 (Table 2, line 1, and Fig. 1B). In these experiments, donor strains containing ICEBs1 marked with a gene conferring resistance to kanamycin (*kan*), with or without the indicated plasmid (all conferring 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 [P_{spank}(hy)-*rapI*] for 1 h. Production of active RapI induces ICEBs1 gene expression, excision, and conjugation ability (5). The recipients did not contain ICEBs1 (ICEBs1⁰) and were defective in the development of genetic competence (*comK*::*spc*) and hence nontransformable. Activated donors were mixed with recipient cells at a ratio of ~1:1, and mating efficiencies were determined (see Materials and Methods).

TABLE 3 Transfer of ICEBs1 is not affected by the presence of plasmids

Plasmid (strain) ^a	% transfer of ICEBs1 ^b
None (JMA168)	6.3 ± 3.5
pBS42 (CAL1393)	9.9 ± 5.1
pC194 (CAL1533)	7.0 ± 3.6
pHP13 (CAL1398)	7.3 ± 3.1

^a Donor strains contained the indicated plasmid, ICEBs1 with the Δ(*rapI-phrI*)342::kan allele, and *amyE*::[P_{spank}(hy)-*rapI* *spc*] for overproduction of RapI to induce ICEBs1.

^b The recipient strain in the mating experiments was CAL89 (ICEBs1⁰ *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^r Str^r CFU per donor CFU in the original mating mixture plus or minus the standard deviation.

pBS42 and pC194 were transferred with frequencies of ~3% plasmid-containing transconjugants per donor, and pHP13 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* [Δ(*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, and Fig. 1C). Together, these results indicate that ICEBs1 can mobilize the three plasmids (pHP13, 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 to 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, and pHP13, 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 cotransfer 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 cotransfer 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 to 60% (of 100 transconjugants tested for each plasmid) also acquired ICEBs1. Furthermore, of the transconjugants that acquired ICEBs1, 2 to 20% also acquired

TABLE 4 Plasmid mobilization does not require ICEBsI nickase NickK but does require the putative coupling protein ConQ

ICEBsI in donor ^a	% plasmid mobilization (<i>n</i>) ^b		
	pBS42	pC194	pHP13
$\Delta(\text{rapI-phrI})::\text{kan}$	2.8 $\pm$ 1.6 (9)	2.7 $\pm$ 1.9 (4)	0.068 $\pm$ 0.04 (5)
$\Delta\text{nickK306}$	3.6 $\pm$ 2.0 (4)	3.2 $\pm$ 0.64 (2)	0.15 $\pm$ 0.12 (4)
$\Delta\text{conQ848}$	<0.00005 (4)	<0.00005 (3)	<0.00005 (2)
$\Delta\text{conQ848}/\text{thrC}::\text{conQ}^+$	2.7 $\pm$ 1.8 (2)	8.8 $\pm$ 5.1 (2)	0.094 $\pm$ 0.02 (2)
$\Delta\text{conQ848}/\text{thrC}::\text{conQ}\Delta$	<0.00005 (2)	<0.00005 (2)	<0.00005 (2)

^a Donor strains contained the indicated plasmid (chloramphenicol-resistant), *amyE*::[(P_{spank}(hy)-*rapI*) *spc*], the ICEBsI $\Delta(\text{rapI-phrI})342::\text{kan}$ allele (present in all donors but not indicated in lines 2 to 5), mutations in *nickK* or *conQ* (lines 2 to 5), and *trans*-acting ICEBsI genes at *thrC* (lines 4 and 5). Donor strains contained pBS42, pC194, and pHP13 and the following ICEBsI genotypes (strains listed in parentheses): $\Delta(\text{rapI-phrI})342::\text{kan}$ (CAL1393, CAL1533, and CAL1398, respectively, as in Table 2), $\Delta\text{nickK306}$ $\Delta(\text{rapI-phrI})342::\text{kan}$ (CAL1395, CAL1535, and CAL1400, respectively), $\Delta\text{conQ848}$ $\Delta(\text{rapI-phrI})342::\text{kan}$ (CAL1591, CAL1592, and CAL1597, respectively), $\Delta\text{conQ848}$ $\Delta(\text{rapI-phrI})342::\text{kan}$ *thrC*::*conQ*⁺ (CAL1593, CAL1594, and CAL1598, respectively), and $\Delta\text{conQ848}$ $\Delta(\text{rapI-phrI})342::\text{kan}$ *thrC*::*conQ* Δ (CAL1595, CAL1596, and CAL1599, respectively). Although plasmid mobilization did not require *nickK*, *nickK* is required for transfer of ICEBsI from plasmid-containing donors. There was no transfer of ICEBsI from plasmid-containing $\Delta\text{nickK306}$ donors (data not shown). The *thrC*::*conQ*⁺ variant (line 4) used for plasmid mobilization contains the ICEBsI genes upstream from and including *conQ* and *nickK* (Fig. 1). Since *nickK* is not needed for mobilization, the defect in the $\Delta\text{conQ848}$ mutant is due to loss of *conQ* and not a polar effect on downstream genes. The *thrC*::*conQ* Δ [*thrC*::[ICEBsI ΔconQ -*attR*::*tet*]] variant (line 5) contains all ICEBsI 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 means from ≥ 2 independent experiments $\pm$ standard deviations. The number of independent experiments for each strain (*n*) is indicated in parentheses.

pBS42, pC194, or pHP13 (of ≥ 100 transconjugants tested for each plasmid). Together, these results indicate that a single donor is capable of transferring both ICEBsI and a plasmid, and the relatively high frequency of cotransfer indicates that once a mating pair is formed, it is likely that both elements will be transferred.

Plasmid mobilization does not require the ICEBsI relaxase NickK or transfer of ICEBsI. 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 (see, e.g., reference 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 cointegrate with the conjugative element (see, e.g., reference 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 ICEBsI relaxase was required for mobilization of these plasmids.

Using an ICEBsI *nickK* null mutant, we found that plasmid mobilization was independent of the ICEBsI relaxase and of ICEBsI transfer. Although the ICEBsI relaxase encoded by *nickK* is essential for ICEBsI transfer (34), it was not required for plasmid mobilization. There was no detectable decrease in mobilization of pHP13, pBS42, and pC194 from ICEBsI donors lacking *nickK* (Table 4, lines 1 and 2, and Fig. 1D). In the same experiment, there was no detectable transfer of ICEBsI (<0.00002% transconjugants per donor), as previously reported (34). These results indicate that the ICEBsI relaxase NickK is not needed for plasmid mobilization. Thus, transfer is not occurring by cross-recognition of an *oriT* on the mobilized plasmids by the ICEBsI relaxase. Furthermore, since the relaxase mutant is incapable of transferring ICEBsI, these results demonstrate that plasmid mobilization does not require cotransfer with ICEBsI. Instead, plasmid mobilization by ICEBsI is likely mediated by direct transfer of the plasmid DNA through the ICEBsI conjugation machinery.

The plasmid replicative relaxase RepU is required for pBS42 plasmid mobilization. Since ICEBsI-mediated plasmid mobilization did not require the ICEBsI 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 NsiI 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. The *oriN*-*repN* genes support bidirectional 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 ICEBsI-mediated mobilization of pBS42. The plasmid with *repU* disrupted, pCAL1738 {pBS42 *repU*::(*oriN*-*repN*)} was not detectably mobilized by ICEBsI (<10⁻⁵% plasmid-containing transconjugants per donor). Transfer of ICEBsI was normal from these donors ($\sim 6\% \pm 2\%$ transconjugants/donor), indicating that the ICEBsI transfer machinery was functional. In contrast, the control plasmid pCAL1737 [pBS42 '*mob*::(*oriN*-*repN*)'], in which *repU* is not disrupted, was still mobilized ($\sim 0.7\% \pm 0.1\%$ plasmid-containing transconjugants/donor), indicating that the presence of *oriN*-*repN* did not prevent mobilization. Based on these results, we conclude that the plasmid replicative relaxase RepU is required for pBS42 mobilization and is most likely functioning as both a replicative relaxase and a conjugative relaxase.

The putative coupling protein of ICEBsI 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). *conQ* (previously called *ycdQ*) of ICEBsI (Fig. 1) encodes the putative coupling protein (28).

We found that *conQ* was required for transfer of ICEBsI. We made an in-frame deletion in *conQ* and integrated this into

ICEBs1 (Fig. 1E). Following overproduction of RapI and induction of ICEBs1, the *conQ* null mutant was unable to transfer ICEBs1 to recipients ($<10^{-5}$ % transconjugants per donor; CAL848) (Fig. 1E).

We also found that the *conQ* deletion does not significantly affect *oriT* function, and it was not polar on the downstream genes needed for conjugation. The inability of the *conQ* mutant to transfer was largely relieved when *conQ*, along with all the ICEBs1 genes upstream of *conQ*, were expressed in *trans* (Fig. 1G) (mating efficiency of $\sim 1\%$ 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 (see, e.g., reference 7). The control that provided all the upstream genes, but not a functional *conQ*, was unable to restore conjugation to the ICEBs1 $\Delta conQ$ mutant ($<10^{-5}$ % transconjugants per donor; JT338) (Fig. 1H). We conclude that *conQ* is required for ICEBs1 conjugation, that the $\Delta conQ$ mutation is not polar on downstream conjugation genes, and that it does not affect *oriT* function or nicking of *oriT* by the ICEBs1 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 a donor, there was no detectable transfer of any of the three plasmids to recipient cells (Table 4, line 3, and Fig. 1E). The inability of the *conQ* mutant to mobilize the plasmids was largely relieved when *conQ*, along with all the ICEBs1 genes upstream of and one gene (*nicK*) downstream of *conQ* were expressed from an ectopic locus (Table 4, line 4, and 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, and 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 ICEBs1 is likely needed to recruit a plasmid-associated relaxosome complex to the ICEBs1 mating machinery.

DISCUSSION

The experiments described here indicate that ICEBs1 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, corresponding to 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 relaxosome 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 cotransfer 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 nonconjugative 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 pHP13 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 subtypes 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 pHP13 (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 *Bacillus 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 relaxosome, 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 relaxosomes from ICEs and conjugative plasmids (14, 15, 36). The coupling protein ConQ would then recruit the plasmid relaxosome 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 efficiency of pHP13 mobilization by ICEBs1 being lower than that of 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 pHP13 mobilization may be due to a lower affinity of the pHP13 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. First, at least three plasmids that are mobilized by the ICEBs1 conjugation machinery encode a single relaxase that may mediate both replication and mobilization. Second, 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 nonconjugative 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 nonmobilizable (46). Because of this inference, it was proposed that persistence of many of these “nonmobilizable” 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 “nonmobilizable” plasmids may in fact be mobilizable. This could account for the persistence of so many “nonmobilizable” plasmids, indicating that the impact of conju-

gation on plasmid mobilization and persistence may be much greater than previously thought.

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