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

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2 The *Bacillus subtilis* conjugative transposon ICEBs1 mobilizes plasmids
3 lacking dedicated mobilization functions
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25 **Abstract**

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

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48 Introduction

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

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

62 *ICEBs1* (Fig. 1) is a conjugative transposon found integrated at the gene for tRNA-
63 Leu2 (*trnS-leu2*) in *Bacillus subtilis* (5, 10). *ICEBs1* is particularly useful for the study of
64 conjugation. Several of the *ICEBs1* genes are similar to genes in other ICEs, including
65 Tn916 (8, 10, 41), the first conjugative transposon identified (17). *ICEBs1* gene
66 expression is induced during the RecA-mediated SOS response or when the cell sensory
67 protein RapI is expressed and active (5). Analyses of *ICEBs1* functions are facilitated by
68 the ability to induce *ICEBs1* gene expression simply by overproduction of RapI from an
69 exogenous promoter, leading to excision of *ICEBs1* from the chromosome of >90% of
70 the cells in a population (5, 32). Induction of gene expression also leads to nicking of
71 *ICEBs1* by its relaxase (encoded by *nick*) at a site in *oriT* (34). Nicking and subsequent
72 unwinding by a host-encoded helicase (PcrA) are required for conjugative transfer and

73 for autonomous plasmid-like replication of ICEBs1 (33). Several ICEBs1 genes encode
74 proteins required for transfer from donor to recipient, and high mating efficiencies of 1-
75 10% transconjugants per donor (7, 32, 34) are obtained with *B. subtilis* recipients that do
76 not contain ICEBs1 (4). ICEBs1 can also transfer to other Gram-positive bacterial species
77 (5).

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

90 We found that *B. subtilis* ICEBs1 donors are capable of efficiently mobilizing three
91 different plasmids, pC194, pBS42, and pHP13 (Fig. 2) that replicate by rolling circle
92 replication (RCR) and are typically described as non-mobilizable (30, 43, 44, 46),
93 although very low efficiencies of mobilization of pC194 and a relative of pBS42 by the
94 conjugative transposon Tn916 have been reported (38, 45). The three plasmids used
95 here contain an origin of replication, but are not known to contain an origin of transfer
96 or mobilization functions. For pBS42, we found that the plasmid replicative relaxase
97 was required for plasmid mobilization. Our results indicate that, similar to the ICEBs1

98 relaxase (33), the plasmid replicative relaxases may function in both replication and
99 DNA transfer. The plasmid relaxase may facilitate DNA transfer by interacting with the
100 conjugation machinery of *ICEBs1*. These findings indicate that many more plasmids
101 than previously thought might be readily mobilized and disseminated by conjugative
102 elements.

103

104

105 **Materials and Methods**

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

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

116 *ICEBs1* Δ (*conG-yddM*)_{319::kan} (Fig 1C) is an insertion-deletion that removes several
117 essential conjugation genes and replaces them with the *kan* cassette. The mutation
118 removes 661 bp from the the 3' end of *conG* (of the 2,445 bp *conG* open reading frame),
119 782 bp from the 5' end of *yddM* (of the 939 bp *yddM* open reading frame) and everything
120 between the two genes (34).

121 *ICEBs1* Δ *nick306* (Fig. 1D) is a 519 bp deletion that disrupts the *ICEBs1* conjugative
122 relaxase encoded by *nick*, but leaves a functional *oriT* (34).

123 For this study, a 1,113 bp unmarked, in-frame ICEBs1 deletion $\Delta conQ848$ (Fig. 1E)
 124 was constructed that fuses the first two codons to the last 107 codons of *conQ* (of the
 125 1440 bp *conQ* open reading frame), using the same method described for construction of
 126 $\Delta nicK306$ (34). The $\Delta conQ848$ allele does not appear to affect the function of *oriT*, which
 127 likely overlaps the 3' end of *conQ* and the 5' end of *nicK* (34) (Fig. 1A).

128 Four truncated derivatives of ICEBs1 were inserted at *thrC* to test for
 129 complementation of the $\Delta conQ848$ mutant (Fig. 1F-I). *thrC229::*{ICEBs1-303 $\Delta(conQ-$
 130 *attR)::tet*} *mls*} (Fig. 1I) and *thrC229::*{ICEBs1-1637 $\Delta(conQ-attR)::cat$ } *mls*} (Fig. 1H) did
 131 not complement the $\Delta conQ848$ deletion as they only contain ICEBs1 genes upstream of
 132 *conQ*, from *int* to *ydcP* (Fig. 1H-I). In contrast, *thrC229::*{ICEBs1-304 $\Delta(ydcS-attR)::tet$ }
 133 *mls*} (Fig. 1F) and *thrC229::*{ICEBs1-337 $\Delta(nicK-attR)::cat$ } *mls*} (Fig. 1G) both contain
 134 wild type *conQ* and complement the $\Delta conQ848$ mutant. These ICEBs1 derivatives are
 135 integrated at *thrC* and are unable to excise due to loss of *attR*. $\Delta(conQ-attR)::tet$ and
 136 $\Delta(conQ-attR)::cat$ remove sequences starting with the 109th codon of *conQ*. $\Delta nicK-attR::cat$
 137 removes sequences starting immediately downstream of the *conQ* stop codon. $\Delta(ydcS-$
 138 *attR)::tet* removes sequences starting immediately downstream of the *nicK* stop codon.
 139 *tet* was from pDG1513 (22). *cat* was from pGEM-cat (50). All four alleles at *thrC* were
 140 derived from *thrC229::*{ICEBs1 $\Delta(rapI-phrI)342::kan$ } *mls*} (34) and were constructed
 141 using the long-flanking homology PCR method (48) or one-step isothermal DNA
 142 assembly (20). Introduction of the $\Delta conQ-attR$, $\Delta nicK-attR$ and $\Delta ydcS-attR$ alleles into
 143 *thrC229::*{ICEBs1 $\Delta rapI-phrI)342::kan$ } *mls*} yielded tetracycline-resistant or
 144 chloramphenicol-resistant, kanamycin-sensitive transformants due to replacement of
 145 the $\Delta rapI-phrI::kan$ insertion.

146 Strain CAL89 is streptomycin-resistant (*str-84*) and cured of ICEBs1 (ICEBs1⁰) and
 147 was used as the recipient in mating experiments. It also contains a *comK::spc* null

148 mutation that prevents acquisition of DNA by transformation (natural genetic
149 competence). Results from mobilization experiments with different alleles of *ICEBs1*
150 are summarized in Fig. 1.

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

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

172 replicons, *oriU-repU* and *oriN-repN*, on pCAL1737 may affect plasmid stability and cell
173 growth.

174 pCAL1737 and pCAL1738 were constructed using one-step isothermal DNA
175 assembly (20) to piece together three DNA fragments: the 3.13 kb *NsiI-NsiI* fragment of
176 pBS42; a PCR product with the 1.64 kb *NsiI-NsiI* fragment of pBS42; and a PCR product
177 with the 1.22 kb *oriN-repN* region. The assembly reactions were designed to yield
178 plasmids identical to those generated by ligation of the *oriN-repN* fragment into full
179 length pBS42, linearized at the *NsiI* restriction site in *repU* or in '*mob* (Fig. 2B). The *oriN-*
180 *repN* sequence was obtained from pDL110 (35) and transcription of *repN* was co-
181 oriented with the disrupted '*mob* and *repU* reading frames. The 1.22 kb insert includes
182 251 bp upstream and 112 bp downstream of the 861 bp *repN* open reading frame.

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

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

196

197 Results

198 In the course of defining the *ICEBs1 oriT* (34), we found that plasmids previously
199 described as non-mobilizable appeared to be mobilized by *ICEBs1*. Based on these
200 preliminary findings, we characterized the mobilization of three plasmids, pC194,
201 pBS42, and pHP13, by *ICEBs1*. All three plasmids (Fig. 2) use rolling circle replication
202 and express chloramphenicol-resistance in *B. subtilis*. These plasmids do not have a
203 known *oriT* and none contain an intact *mob* gene (21, 30, 44, 46). Thus, pC194, pBS42 and
204 pHP13 are typically described as non-mobilizable.

205 Mobilization of plasmids by *ICEBs1*

206 We found that all three plasmids were mobilized by *ICEBs1* (Table 2, line 1; Fig. 1B).
207 In these experiments, donor strains containing *ICEBs1* marked with a gene encoding
208 resistance to kanamycin (*kan*), with or without the indicated plasmid (all encoding
209 chloramphenicol resistance), were grown in rich medium (LB) and *ICEBs1* gene
210 expression was induced by ectopic expression of *rapI* from a fusion to a LacI-
211 repressible-IPTG-inducible promoter (*Pspank(hy)-rapI*) for one hour. Production of
212 active RapI induces *ICEBs1* gene expression, excision, and conjugation ability (5). The
213 recipients did not contain *ICEBs1* (*ICEBs1*⁰) and were defective in the development of
214 genetic competence (*comK::spc*) and hence non-transformable. Activated donors were
215 mixed with recipient cells at a ratio of ~1:1 and mating efficiencies were determined
216 (Materials and Methods).

217 pBS42 and pC194 were transferred with frequencies of ~3% plasmid-containing
218 transconjugants per donor and pHP13 was transferred with a frequency of ~0.07%
219 plasmid-containing transconjugants per donor. Plasmid transfer was dependent on the
220 presence of *ICEBs1* in the donor as there was no detectable acquisition of
221 chloramphenicol-resistance from cells that did not contain *ICEBs1* (Table 2, line 2).

222 Plasmid transfer required components of the *ICEBs1* mating machinery. An *ICEBs1*
223 mutant that is missing genes from *conG* to *yddM* $\{\Delta(\textit{conG-yddM})319::kan\}$ (Fig. 1C) is
224 defective in *ICEBs1* conjugation (34). This mutant was incapable of mobilizing all three
225 plasmids tested (Table 2, line 3; Fig. 1C). Together, these results indicate that *ICEBs1*
226 can mobilize the three plasmids pHP13, pBS42, and pC194, and that mobilization
227 requires at least some of the *ICEBs1* mating components.

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

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

234 We analyzed transconjugants that acquired a plasmid to determine the frequency
235 that they also acquired *ICEBs1*. In experiments analogous to those described above
236 (Table 2), single colonies of plasmid-containing transconjugants (chloramphenicol-
237 resistant) were picked and tested for resistance to kanamycin, indicative of acquisition
238 of *ICEBs1*. Of the transconjugants acquiring pBS42, pC194, or pHP13, 19%, 45%, and
239 35%, respectively (of ≥ 200 transconjugants tested for each plasmid), also acquired
240 *ICEBs1*. If transconjugants that acquired both the plasmid and *ICEBs1* received the
241 elements from a single donor, then these relatively high frequencies of co-transfer
242 indicate that once a mating pair is formed, it is likely that both elements will be
243 transferred.

244 In these mating experiments, the ratio of donor to recipient was approximately 1:1,
245 and it seemed possible a single transconjugant could have acquired *ICEBs1* from one
246 donor and a plasmid from another. If so, then the frequency of co-transfer should drop

247 if the ratio of donor to recipient is reduced. We repeated the mating experiments
248 described above using a donor to recipient ratio of 1:100, rather than 1:1. Of the
249 transconjugants that acquired pBS42, pC194, or pHP13, 20-60% (of 100 transconjugants
250 tested for each plasmid) also acquired *ICEBs1*. Furthermore, of the transconjugants that
251 acquired *ICEBs1*, between 2-20% also acquired pBS42, pC194, or pHP13 (of ≥ 100
252 transconjugants tested for each plasmid). Together, these results indicate that a single
253 donor is capable of transferring both *ICEBs1* and a plasmid and that the relatively high
254 frequency of co-transfer indicates that once a mating pair is formed, it is likely that both
255 elements will be transferred.

256 **Plasmid mobilization does not require the *ICEBs1* relaxase *NicK* or transfer of**
257 ***ICEBs1***

258 There are several mechanisms by which plasmids and transposons can be mobilized.
259 Mobilizable elements typically contain an *oriT* and a gene (*mob*) that encodes a
260 conjugative relaxase (40, 46). The *mob* gene product nicks a site in *oriT* and is required
261 for mobilization. Plasmids (or transposons) lacking *mob* functions can sometimes be
262 mobilized by cross-recognition of an *oriT* site on the mobilizable element {e.g., (13)}.
263 Plasmids (or transposons) lacking both *mob* and *oriT* functions can sometimes be
264 mobilized when the plasmid integrates into a conjugative element and is transferred in
265 cis as a co-integrate with the conjugative element {e.g., (12)}. In these situations,
266 mutations in the relaxase gene of the conjugative element prevent transfer of the
267 conjugative element and also prevent mobilization of the plasmid (or transposon). Since
268 the plasmids used here do not contain *mob* and a cognate *oriT*, we tested whether the
269 *ICEBs1* relaxase was required for mobilization of these plasmids.

270 Using an *ICEBs1 nick* null mutant, we found that plasmid mobilization was
271 independent of the *ICEBs1* relaxase and of *ICEBs1* transfer. Although the *ICEBs1*

272 relaxase encoded by *nicK* is essential for ICEBs1 transfer (34), it was not required for
273 plasmid mobilization. There was no detectable decrease in mobilization of pHP13,
274 pBS42, and pC194 from ICEBs1 donors lacking *nicK* (Table 4, lines 1, 2; Fig. 1D). In the
275 same experiment, there was no detectable transfer of ICEBs1 ($<0.00002\%$
276 transconjugants per donor), as previously reported (34). These results indicate that the
277 ICEBs1 relaxase Nick is not needed for plasmid mobilization. Thus, transfer is not
278 occurring by cross-recognition of an *oriT* on the mobilized plasmids by the ICEBs1
279 relaxase. Furthermore, since the relaxase mutant is incapable of transferring ICEBs1,
280 these results demonstrate that plasmid mobilization does not require co-transfer with
281 ICEBs1. Instead, plasmid mobilization by ICEBs1 is likely mediated by direct transfer of
282 the plasmid DNA through the ICEBs1 conjugation machinery.

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

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

293 We found that the replicative relaxase of pBS42 is needed for ICEBs1-mediated
294 mobilization of pBS42. The plasmid with *repU* disrupted, pCAL1738 {pBS42 *repU::(oriN-*
295 *repN)*} was not detectably mobilized by ICEBs1 ($<10^{-5}\%$ plasmid-containing
296 transconjugants per donor). Transfer of ICEBs1 was normal from these donors ($\sim 6\% \pm$

297 2% transconjugants/donor) indicating that the ICEBs1 transfer machinery was
298 functional. In contrast, the control plasmid pCAL1737 {pBS42 'mob::(*oriN-repN*)} in
299 which *repU* is not disrupted was still mobilized ($\sim 0.7\% \pm 0.1\%$ plasmid-containing
300 transconjugants/donor), indicating that the presence of *oriN-repN* did not prevent
301 mobilization. Based on these results, we conclude that the plasmid replicative relaxase
302 RepU is required for pBS42 mobilization and is most likely functioning as both a
303 replicative and a conjugative relaxase.

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

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

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

317 We also found that the *conQ* deletion does not significantly affect *oriT* function nor
318 was it polar on the downstream genes needed for conjugation. The inability of the *conQ*
319 mutant to transfer was largely relieved when *conQ*, along with all the ICEBs1 genes
320 upstream of *conQ* were expressed in trans (Fig. 1G) (mating efficiency of $\sim 1\%$
321 transconjugants per donor, JT339). The upstream genes were provided in addition to

322 *conQ* because we commonly find that complementation (and presumably protein
323 production) is more efficient when upstream genes are included {e.g., (7)}. The control
324 that provided all the upstream genes, but not a functional *conQ*, was unable to restore
325 conjugation to the *ICEBs1* $\Delta conQ$ mutant ($<10^{-5}$ % transconjugants per donor, JT338; Fig.
326 1H). We conclude that *conQ* is required for *ICEBs1* conjugation, that the $\Delta conQ$
327 mutation is not polar on downstream conjugation genes, and that it does not affect *oriT*
328 function or nicking of *oriT* by the *ICEBs1* relaxase.

329 *conQ* was also required for mobilization of pHP13, pBS42 and pC194, none of which
330 encode their own dedicated coupling protein. When the *conQ* null mutant was used as
331 donor, there was no detectable transfer of any of the three plasmids to recipient cells
332 (Table 4, line 3; Fig. 1E). The inability of the *conQ* mutant to mobilize the plasmids was
333 largely relieved when *conQ*, along with all the *ICEBs1* genes upstream of and one gene
334 (*nicK*) downstream of *conQ* were expressed from an ectopic locus (Table 4, line 4; Fig.
335 1F). The control that provided all the upstream genes, but not a functional *conQ*, was
336 unable to restore mobilization to the *conQ* mutant (Table 4, line 5; Fig. 1I). Since *nicK* is
337 not needed for plasmid mobilization (Table 4, line 2), these results indicate that the
338 defect in mobilization was due to loss of *conQ* and not a polar effect on downstream
339 genes, and that the putative coupling protein of *ICEBs1* is likely needed to recruit a
340 plasmid-associated relaxosome complex to the *ICEBs1* mating machinery.

341

342

343 Discussion

344 Experiments described here indicate that *ICEBs1* of *B. subtilis* is capable of
345 mobilizing at least three different plasmids, pC194, pBS42, and pHP13. Mobilizable
346 plasmids are thought to require dedicated mobilization functions; a conjugative

347 relaxase (Mob) and a cognate *oriT*, that are separate from the replication functions.
348 None of the plasmids used here have dedicated mobilization functions. Mobilization
349 by *ICEBs1* requires the *ICEBs1* conjugation machinery and the putative coupling
350 protein, ConQ. In characterized systems, the coupling protein is needed to link the
351 relaxosome complex, which contains a conjugative relaxase attached to the 5' end of a
352 single strand of DNA, to the conjugation machinery (14, 36, 46). Plasmid mobilization
353 by *ICEBs1* did not require the *ICEBs1* relaxase encoded by *nicK* indicating that
354 mobilization was not due to cross-recognition of a cryptic *oriT* on the plasmids or co-
355 transfer of *ICEBs1* and plasmid DNA. Mobilization of pBS42 required the replicative
356 relaxase RepU of pBS42, indicating that this replicative relaxase can also function as a
357 conjugative relaxase. These findings have practical applications for the characterization
358 of *ICEBs1* genes and the genetic manipulation of heterologous bacteria. In addition, our
359 findings indicate that there is more potential for horizontal gene transfer of non-
360 conjugative plasmids than previously recognized.

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

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

370 Plasmid mobilization by *ICEBs1* could also be a useful and efficient way to
371 introduce DNA to strains that are difficult to transform. *ICEBs1* can be transferred to

372 other organisms (5), and at least some of the plasmids used here are capable of
373 replicating in other organisms. Cloning and genetic manipulations could be done with
374 plasmids in *E. coli* or *B. subtilis* and then the desired plasmids could be mobilized from
375 *B. subtilis* by ICEBs1 into other Gram-positive organisms that are suitable recipients for
376 conjugation. Other conjugative elements have been used for plasmid mobilization (16),
377 but the high efficiencies of mobilization by ICEBs1 and the ability to easily manipulate
378 *B. subtilis* make mobilization by ICEBs1 an attractive system for use with Gram positive
379 bacteria.

380 **Functional relationship between replicative and conjugative relaxases**

381 Duplication of pC194, pBS42, and pHP13 requires a plasmid origin of replication
382 (*ori*) and a cognate replicative relaxase (Rep) that enables rolling circle replication (29).
383 The first steps in production of a substrate for conjugation and for rolling circle
384 replication are similar. Both require a relaxase (a conjugative or replicative relaxase)
385 that nicks a site in an origin (origin of transfer or origin of replication). The relaxase
386 becomes covalently attached to the cognate origin and serves to mark the site for
387 transfer or replication. Following nicking, a helicase is required for unwinding the
388 double-stranded DNA substrate for either conjugation or replication. Many rolling
389 circle replicating plasmids in Gram positive organisms use the host-encoded helicase
390 PcrA for replication (29). Similarly, ICEBs1 uses PcrA both for replication and for
391 conjugation, although replication is not required for conjugation (33).

392 The conjugative relaxases are similar to the replicative relaxases, although they are
393 generally thought to belong to different sub-types of the relaxase family (19, 27, 31).
394 However, recent work demonstrated that the ICEBs1 conjugative relaxase NicK also
395 functions as a replicative relaxase using a single origin for both conjugative transfer and
396 replication (33). Results presented here indicate that at least three different replicative

397 relaxases, from pC194, pBS42 (pUB110), and pHP13 (pTA1060), likely also function as
398 conjugative relaxases. This is in contrast to the prevalent view that mobilizable
399 plasmids have separate replication (Rep/*ori*) and mobilization (Mob/*oriT*) functions
400 (21, 46).

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

412 **Likely mechanism of plasmid mobilization in the absence of dedicated** 413 **mobilization functions**

414 Plasmid mobilization mediated by ICEBs1 probably occurs by a mechanism similar
415 to transfer of ICEBs1. We propose that the plasmid replicative relaxasome, consisting of
416 the replicative relaxase attached to plasmid DNA, and perhaps associated with the
417 helicase PcrA, interacts with the putative coupling protein from ICEBs1, ConQ. This
418 interaction might be analogous to the interactions between coupling proteins and the
419 cognate relaxasomes from ICEs and conjugative plasmids (14, 15, 36). The coupling
420 protein ConQ would then recruit the plasmid relaxasome to the ICEBs1 conjugation
421 machinery at the cell membrane. Interactions between the coupling protein and the

422 helicase PcrA and/or the target DNA could also be involved, either in addition to or
423 instead of interactions with the replicative relaxase. However, the lower efficiency of
424 pHP13 mobilization by *ICEBs1* as compared to pC194 and pBS42 mobilization argues
425 against mobilization primarily occurring through interactions between the coupling
426 protein and the helicase PcrA. We postulate that the specificity comes from protein-
427 protein interaction between the relaxase and the coupling protein. In this case, the
428 lower efficiency of pHP13 mobilization may be due to a lower affinity of the pHP13
429 replicative relaxase for the *ICEBs1* coupling protein and for components of the *ICEBs1*
430 conjugation machinery

431 **Evolutionary implications**

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

445 The persistence of plasmids in bacterial populations is likely due to benefits they
446 confer on the host cell or to their efficient dissemination to new hosts by horizontal

447 transfer. Otherwise, the burden placed on the host by the plasmid is thought to result
448 in loss of the plasmid. Based on the lack of a *mob* gene, approximately 60% of 1,730
449 sequenced plasmids are inferred to be non-mobilizable (46). Because of this inference, it
450 was proposed that persistence of many of these "non-mobilizable" plasmids is due to
451 unknown benefits conferred upon the host (46). The ability of ICEBs1 to mobilize three
452 plasmids lacking dedicated *mob* functions indicates that many "non-mobilizable"
453 plasmids may in fact be mobilizable. This could account for the persistence of so many
454 "non-mobilizable" plasmids, indicating that the impact of conjugation on plasmid
455 mobilization and persistence may be much greater than previously thought.

456

457

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462

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601
602
603

603 **Table 1. *B. subtilis* strains^a**

604

Strain	Relevant genotype (reference)
CAL13	ICEBs1 ⁰ <i>amyE</i> ::{(Pspank(hy)- <i>rapI</i>) <i>spc</i> } (34)
CAL89	ICEBs1 ⁰ <i>comK</i> :: <i>spc str-84</i> (5)
CAL306	Δ <i>nicK306</i> Δ (<i>rapI-phrI</i>)342:: <i>kan amyE</i> ::{(Pspank(hy)- <i>rapI</i>) <i>spc</i> } (34)
CAL421	Δ (<i>conG-yddM</i>)319:: <i>kan amyE</i> ::{(Pspank(hy)- <i>rapI</i>) <i>spc</i> } (34)
CAL848	Δ <i>conQ848</i> Δ (<i>rapI-phrI</i>)342:: <i>kan amyE</i> ::{(Pspank(hy)- <i>rapI</i>) <i>spc</i> }
CAL1034	Δ (<i>conG-yddM</i>)319:: <i>kan amyE</i> ::{(Pspank(hy)- <i>rapI</i>) <i>spc</i> }; pHP13 (<i>cat mls</i>)
CAL1392	ICEBs1 ⁰ <i>amyE</i> ::{(Pspank(hy)- <i>rapI</i>) <i>spc</i> }; pBS42 (<i>cat</i>)
CAL1393	Δ (<i>rapI-phrI</i>)342:: <i>kan amyE</i> ::{(Pspank(hy)- <i>rapI</i>) <i>spc</i> }; pBS42 (<i>cat</i>)
CAL1395	Δ <i>nicK306</i> Δ (<i>rapI-phrI</i>)342:: <i>kan amyE</i> ::{(Pspank(hy)- <i>rapI</i>) <i>spc</i> }; pBS42 (<i>cat</i>)
CAL1396	Δ (<i>conG-yddM</i>)319:: <i>kan amyE</i> ::{(Pspank(hy)- <i>rapI</i>) <i>spc</i> }; pBS42 (<i>cat</i>)
CAL1397	ICEBs1 ⁰ <i>amyE</i> ::{(Pspank(hy)- <i>rapI</i>) <i>spc</i> }; pHP13 (<i>cat mls</i>)
CAL1398	Δ (<i>rapI-phrI</i>)342:: <i>kan amyE</i> ::{(Pspank(hy)- <i>rapI</i>) <i>spc</i> }; pHP13 (<i>cat mls</i>)
CAL1400	Δ <i>nicK306</i> Δ (<i>rapI-phrI</i>)342:: <i>kan amyE</i> ::{(Pspank(hy)- <i>rapI</i>) <i>spc</i> }; pHP13 (<i>cat mls</i>)
CAL1532	ICEBs1 ⁰ <i>amyE</i> ::{(Pspank(hy)- <i>rapI</i>) <i>spc</i> }; pC194 (<i>cat</i>)
CAL1533	Δ (<i>rapI-phrI</i>)342:: <i>kan amyE</i> ::{(Pspank(hy)- <i>rapI</i>) <i>spc</i> }; pC194 (<i>cat</i>)
CAL1535	Δ <i>nicK306</i> Δ (<i>rapI-phrI</i>)342:: <i>kan amyE</i> ::{(Pspank(hy)- <i>rapI</i>) <i>spc</i> }; pC194 (<i>cat</i>)
CAL1548	Δ (<i>conG-yddM</i>)319:: <i>kan amyE</i> ::{(Pspank(hy)- <i>rapI</i>) <i>spc</i> }; pC194 (<i>cat</i>)
CAL1590	Δ <i>conQ848 thrC229</i> ::{(ICEBs1-303 Δ <i>conQ-attR</i> :: <i>tet</i>) <i>mls</i> } Δ (<i>rapI-phrI</i>)342:: <i>kan amyE</i> ::{(Pspank(hy)- <i>rapI</i>) <i>spc</i> }
CAL1591	Δ <i>conQ848</i> Δ (<i>rapI-phrI</i>)342:: <i>kan amyE</i> ::{(Pspank(hy)- <i>rapI</i>) <i>spc</i> }; pBS42 (<i>cat</i>)
CAL1592	Δ <i>conQ848</i> Δ (<i>rapI-phrI</i>)342:: <i>kan amyE</i> ::{(Pspank(hy)- <i>rapI</i>) <i>spc</i> }; pC194 (<i>cat</i>)
CAL1593	Δ <i>conQ848 thrC229</i> ::{(ICEBs1-304 Δ <i>ydcS-attR</i> :: <i>tet</i>) <i>mls</i> } Δ (<i>rapI-phrI</i>)342:: <i>kan amyE</i> ::{(Pspank(hy)- <i>rapI</i>) <i>spc</i> }; pBS42 (<i>cat</i>)
CAL1594	Δ <i>conQ848 thrC229</i> ::{(ICEBs1-304 Δ <i>ydcS-attR</i> :: <i>tet</i>) <i>mls</i> } Δ (<i>rapI-phrI</i>)342:: <i>kan amyE</i> ::{(Pspank(hy)- <i>rapI</i>) <i>spc</i> }; pC194 (<i>cat</i>)
CAL1595	Δ <i>conQ848 thrC229</i> ::{(ICEBs1-303 Δ <i>conQ-attR</i> :: <i>tet</i>) <i>mls</i> } Δ (<i>rapI-phrI</i>)342:: <i>kan amyE</i> ::{(Pspank(hy)- <i>rapI</i>) <i>spc</i> }; pBS42 (<i>cat</i>)

CAL1596	$\Delta conQ848 thrC229::\{ICEBs1-303 \Delta conQ-attR::tet\} mls\} \Delta(rapI-phrI)342::kan amyE::\{(Pspank(hy)-rapI) spc\}; pC194 (cat)$
CAL1597	$\Delta conQ848 \Delta(rapI-phrI)342::kan amyE::\{(Pspank(hy)-rapI) spc\}; pHP13 (cat mls)$
CAL1598	$\Delta conQ848 thrC229::\{ICEBs1-304 \Delta ydcS-attR::tet\} mls\} \Delta(rapI-phrI)342::kan amyE::\{(Pspank(hy)-rapI) spc\}; pHP13 (cat mls)$
CAL1599	$\Delta conQ848 thrC229::\{ICEBs1-303 \Delta conQ-attR::tet\} mls \Delta(rapI-phrI)342::kan amyE::\{(Pspank(hy)-rapI) spc\} \}; pHP13 (cat mls)$
CAL1749	$\Delta(rapI-phrI)342::kan amyE::\{(Pspank(hy)-rapI) spc\}; pCAL1737 \{pBS42 cat 'mob::(oriN-repN)\}$
CAL1751	$\Delta(rapI-phrI)342::kan amyE::\{(Pspank(hy)-rapI) spc\}; pCAL1738 \{pBS42 cat repU::(oriN-repN)\}$
JMA168	$\Delta(rapI-phrI)342::kan amyE::\{(Pspank(hy)-rapI) spc\} (5)$
JT298	$\Delta conQ848 thrC229::\{ICEBs1-304 \Delta ydcS-attR::tet\} mls\} \Delta(rapI-phrI)342::kan amyE::\{(Pspank(hy)-rapI) spc\}$
JT338	$\Delta conQ848 thrC229::\{ICEBs1-1637 \Delta conQ-attR::cat\} mls\} \Delta(rapI-phrI)342::kan amyE::\{(Pspank(hy)-rapI) spc\}$
JT339	$\Delta conQ848 thrC229::\{ICEBs1-337 \Delta nicK-attR::cat\} mls\} \Delta(rapI-phrI)342::kan amyE::\{(Pspank(hy)-rapI) spc\}$

605

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

607

608

608 **Table 2. Mobilization of plasmids by ICEBs1.**

609

	<u>ICEBs1 in donor</u> ^a	<u>Plasmid mobilization (%)</u> ^b		
		<u>pBS42</u>	<u>pC194</u>	<u>pHP13</u>
1	$\Delta(\text{rapI-phrI})::\text{kan}$	2.8 ± 1.6	2.7 ± 1.9	0.068 ± 0.04
2	None	<0.00002	<0.00002	<0.00002
3	$\Delta(\text{conG-yddM})::\text{kan}$	<0.00002	<0.00002	<0.00002

610

611 ^a Donor strains contained the indicated plasmid (chloramphenicol resistant) and
612 *amyE*::{Pspank(hy)-*rapI* *spc*} for overproduction of RapI to induce ICEBs1. Donor strains
613 contained pBS42, pC194, and pHP13 and ICEBs1 $\Delta(\text{rapI-phrI})_{342}::\text{kan}$ (CAL1393,
614 CAL1533, and CAL1398, respectively); no ICEBs1 (CAL1392, CAL1532 and CAL1397,
615 respectively); and ICEBs1 $\Delta(\text{conG-yddM})::\text{kan}$ (CAL1396, CAL1548 and CAL1034,
616 respectively).

617

618 ^b The recipient strain in the mating experiments was CAL89 (ICEBs1⁰ *comK*::*spc* *str*).
619 Values for the $\Delta(\text{rapI-phrI})::\text{kan}$ donors are the means from 9 (pBS42), 4 (pC194), and 5
620 (pHP13) independent mating assays. Mating assays with donors defective for plasmid
621 mobilization yielded no detectable transconjugants (<0.00002%) in at least two
622 independent experiments. Plasmid mobilization was calculated as percent Cm^R Str^R
623 transconjugant CFU per donor CFU in the original mating mixture plus or minus the
624 standard deviation.

625

626

626 **Table 3. Transfer of ICEBs1 is not affected by the presence of plasmids.**

627

	<u>Plasmid (strain)^a</u>	<u>Percent transfer of ICEBs1^b</u>
1	None (JMA168)	6.3 ± 3.5
2	pBS42 (CAL1393)	9.9 ± 5.1
3	pC194 (CAL1533)	7.0 ± 3.6
4	pHP13 (CAL1398)	7.3 ± 3.1

628

629 ^a Donor strains contained the indicated plasmid, ICEBs1 with the $\Delta(rapI-phrI)342::kan$
630 allele and *amyE::*{Pspank(hy)-*rapI spc*} for overproduction of RapI to induce ICEBs1.

631

632 ^b The recipient strain in the mating experiments was CAL89 (ICEBs1⁰ *comK::spc str*).
633 Transconjugants containing ICEBs1 were selected as resistant to kanamycin (from
634 ICEBs1) and streptomycin (from the recipient). Values for percent transfer of ICEBs1 are
635 the means from 5 independent mating assays (9 for pBS42) and are calculated as the
636 percent Kan^R Str^R colony-forming units (CFU) per donor CFU in the original mating
637 mixture plus or minus the standard deviation.

638

639

640

640 **Table 4. Plasmid mobilization does not require ICEBs1 nickase NickK, but does**
 641 **require the putative coupling protein ConQ.**

	<u>ICEBs1 in donor^a</u>	<u>Plasmid mobilization (%)^b</u>		
		<u>pBS42</u> (n)	<u>pC194</u> (n)	<u>pHP13</u> (n)
1	$\Delta(\text{rapI-phrI})::\text{kan}$	2.8 ± 1.6 (9)	2.7 ± 1.9 (4)	0.068 ± 0.04 (5)
2	$\Delta\text{nickK306}$	3.6 ± 2.0 (4)	3.2 ± 0.64 (2)	0.15 ± 0.12 (4)
3	$\Delta\text{conQ848}$	<0.00005 (4)	<0.00005 (3)	<0.00005 (2)
4	$\Delta\text{conQ848} / \text{thrC}::\text{conQ}^+$	2.7 ± 1.8 (2)	8.8 ± 5.1 (2)	0.094 ± 0.02 (2)
5	$\Delta\text{conQ848} / \text{thrC}::\text{conQ}\Delta$	<0.00005 (2)	<0.00005 (2)	<0.00005 (2)

642
 643 ^a Donor strains contained the indicated plasmid (chloramphenicol-resistant),
 644 *amyE::(Pspank(hy)-rapI) spc*, the ICEBs1 $\Delta(\text{rapI-phrI})_{342}::\text{kan}$ allele (present in all
 645 donors but not indicated in lines 2-5), mutations in *nickK* or *conQ* (lines 2-5), and trans-
 646 acting ICEBs1 genes at *thrC* (lines 4 and 5). Donor strains contained pBS42, pC194, and
 647 pHP13 and: ICEBs1 $\Delta(\text{rapI-phrI})_{342}::\text{kan}$ (CAL1393, CAL1533, and CAL1398,
 648 respectively, as in Table 2); $\Delta\text{nickK306} \Delta(\text{rapI-phrI})_{342}::\text{kan}$ (CAL1395, CAL1535, and
 649 CAL1400, respectively); $\Delta\text{conQ848} \Delta(\text{rapI-phrI})_{342}::\text{kan}$ (CAL1591, CAL1592, and
 650 CAL1597, respectively); $\Delta\text{conQ848} \Delta(\text{rapI-phrI})_{342}::\text{kan} \text{thrC}::\text{conQ}^+$ (CAL1593, CAL1594,
 651 and CAL1598, respectively); and $\Delta\text{conQ848} \Delta(\text{rapI-phrI})_{342}::\text{kan} \text{thrC}::\text{conQ}\Delta$ (CAL1595,
 652 CAL1596, and CAL1599, respectively).

653 Although plasmid mobilization did not require *nickK*, *nickK* is required for transfer of
 654 ICEBs1 from plasmid-containing donors. There was no transfer of ICEBs1 from
 655 plasmid-containing $\Delta\text{nickK306}$ donors (data not shown).

656 The *thrC::conQ*⁺ (line 4) used for plasmid mobilization contains the ICEBs1 genes
 657 upstream from and including *conQ* and *nickK* (Fig. 1). Since *nickK* is not needed for
 658 mobilization, the defect in the $\Delta\text{conQ848}$ mutant is due to loss of *conQ* and not a polar
 659 effect on downstream genes. The *thrC::conQ* Δ (*thrC::(ICEBs1 $\Delta\text{conQ-attR}::\text{tet}$)*) (line 5)
 660 contains all ICEBs1 genes upstream of *conQ*, but not a functional *conQ* (Fig. 1). This was
 661 used as a control for the complementation (line 4).

662 ^bPercent plasmid mobilization was determined as described for Table 2. Values are
 663 the mean from ≥ 2 independent experiments \pm the standard deviation. The number of
 664 independent experiments for each strain (n) is indicated in parentheses.

665

665 **Figure 1. Map of ICEBs1 and various mutants.**

666 **A.** Genetic map of ICEBs1. ICEBs1 is shown in its linear integrated form. Open
 667 arrows indicate open reading frames and the direction of transcription. Gene names are
 668 indicated above the arrows. The origin of transfer (*oriT*) is indicated by a thick black line
 669 overlapping the 3' end of *conQ* and the 5' end of *nick*. *oriT* is needed for ICEBs1 transfer
 670 (34) and replication (33). The small rectangles at the ends of ICEBs1 represent the 60 bp
 671 direct repeats that contain the site-specific recombination sites in the left and right
 672 attachment sites, *attL* and *attR*, that are required for excision of the element from the
 673 chromosome.

674 **B-E.** Schematic diagram of deletion mutations in ICEBs1 in donor strains. Thin
 675 horizontal lines represent the regions of ICEBs1 that are present in the donor strains.
 676 Gaps in the line represent the regions of ICEBs1 that are deleted. Antibiotic-resistance
 677 markers that are inserted in some of the alleles are not shown. $\Delta(\textit{rapIphrI})$ (B, D, E) (5)
 678 and $\Delta(\textit{conG-yddM})$ (C) (34) are deletion-insertion mutations with a kanamycin-resistance
 679 gene inserted (not shown). $\Delta\textit{nick}$ (E) and $\Delta\textit{conQ}$ (F) are unmarked in-frame deletion
 680 mutations that do not disrupt the expression of downstream genes and do not disrupt
 681 *oriT*.

682 **F-I.** Schematic diagram of truncated ICEBs1 derivatives integrated at *thrC*. These
 683 constructs cannot excise due to deletion of *attR* and were used to provide ICEBs1 genes
 684 in trans for complementation tests of the ICEBs1 $\Delta\textit{conQ}$ mutant. Thin horizontal lines
 685 represent the regions of ICEBs1 that are present at *thrC*.

686 **B-I.** The ability of plasmids to be mobilized or of ICEBs1 to be transferred from
 687 various donor strains are summarized as + or -. nd indicates not determined.

688

689

690 **Figure 2. Plasmid maps.**

691 Schematic diagrams of plasmids pC194 (A), pBS42 (B), and pHP13 (C), all mobilized
692 by ICEBs1. The approximate size of each plasmid is indicated under the plasmid name.
693 Circles represent each plasmid and are not shown to scale. Thin black arrows and gene
694 names within each circle represent functional genes or, in the case of 'mobU (B), a non-
695 functional 3' fragment of *mobU* (missing the 5'end). Antibiotic resistance genes include
696 *cat* (chloramphenicol resistance) and *mls* (macrolide, lincosamide, and streptogramin
697 resistance). Small open arrows on each circle represent the origin of replication, which
698 contains the site for nicking by the cognate replicative relaxase (encoded by *rep*, *repU*,
699 and *rep60*). The sequences flanking the *nic* sites in each plasmid *ori* are highly
700 conserved. The orientation of the open arrows indicates the direction of leading strand
701 synthesis for rolling circle replication. pBS42 (B) and pHP13 (C) are composites of
702 segments from several plasmids (6, 23). The junctions and sources of the different
703 segments are indicated by the intersecting lines and plasmid names on the perimeter of
704 each circle. For pBS42 (B) the location of two *NsiI* restriction sites is indicated with a line
705 and an "N". The three plasmids have a 0.98 kb sequence in common that contains *cat*.
706 Other DNA sequences common to all three plasmids are ≤ 12 bp.

707

708

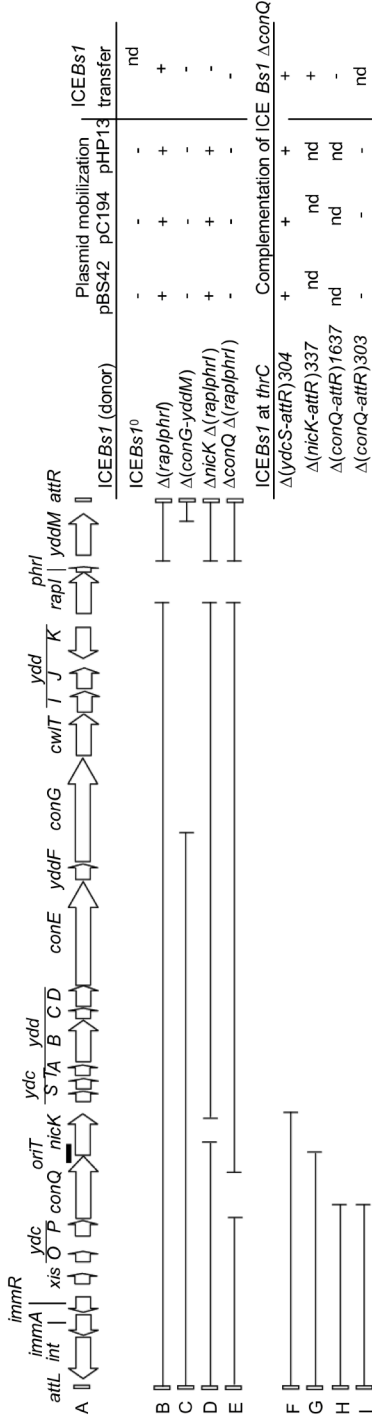


Figure 1

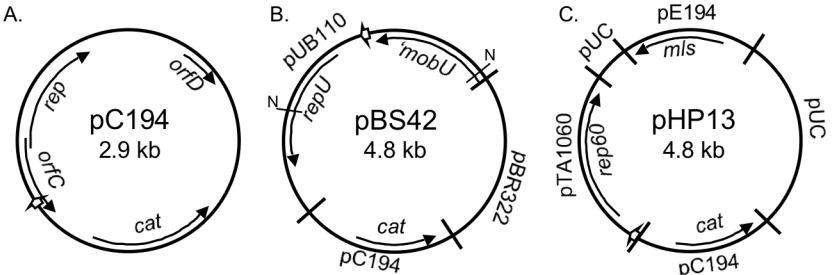


Figure 2