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Identification of the Origin of Transfer (*oriT*) and DNA Relaxase Required for Conjugation of the Integrative and Conjugative Element ICE*Bs1* of *Bacillus subtilis*▽

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Integrative and conjugative elements (ICEs), also known as conjugative transposons, are mobile genetic elements that can transfer from one bacterial cell to another by conjugation. ICE*Bs1* is integrated into the *trnS-leu2* gene of *Bacillus subtilis* and is regulated by the SOS response and the RapI-PhrI cell-cell peptide signaling system. When *B. subtilis* senses DNA damage or high concentrations of potential mating partners that lack the element, ICE*Bs1* excises from the chromosome and can transfer to recipients. Bacterial conjugation usually requires a DNA relaxase that nicks an origin of transfer (*oriT*) on the conjugative element and initiates the 5′-to-3′ transfer of one strand of the element into recipient cells. The ICE*Bs1 ydcR* (*nicK*) gene product is homologous to the pT181 family of plasmid DNA relaxases. We found that transfer of ICE*Bs1* requires *nicK* and identified a *cis*-acting *oriT* that is also required for transfer. Expression of *nicK* leads to nicking of ICE*Bs1* between a GC-rich inverted repeat in *oriT*, and NicK was the only ICE*Bs1* gene product needed for nicking. NicK likely mediates conjugation of ICE*Bs1* by nicking at *oriT* and facilitating the translocation of a single strand of ICE*Bs1* DNA through a transmembrane conjugation pore.

Mobile genetic elements are ubiquitous in bacteria and can contain genes for antibiotic resistance, symbiosis, and virulence; their dissemination contributes to bacterial evolution by conferring new genes and phenotypes to their recipients (reviewed in references 8 and 21). The most common mobile genetic elements are phages, plasmids, and integrative and conjugative elements (ICEs), also known as conjugative transposons. Conjugative plasmids and ICEs are transferred directly from cell to cell and generally encode their own conjugation systems (6, 25).

ICE*Bs1* is an ICE that is found integrated into the *trnS-leu2* genes of some *Bacillus subtilis* strains (Fig. 1A) (3, 7). Detailed analyses of ICE*Bs1* have been aided by its efficient transfer, its site-specific integration, and the ease of genetic manipulations in *B. subtilis* (2, 3; C. A. Lee, J. M. Auchtung, R. E. Monson, and A. D. Grossman, submitted for publication). When induced, ICE*Bs1* excises from the chromosome and can transfer to recipient cells. ICE*Bs1* gene expression and excision are induced by the SOS response or when cells are at high density surrounded by neighbors that do not contain a copy of ICE*Bs1* (3). Regulation by population density and recognition of self are mediated by the regulator RapI and the pentapeptide PhrI (3).

Both DNA damage and RapI-PhrI regulation affect the activity of the ICE*Bs1* immunity repressor ImmR (2), and inactivation of ImmR causes increased ICE*Bs1* gene expression, production of the excisionase Xis, and excision of ICE*Bs1* (2, 3; Lee et al., submitted). Integration into and excision from the chromosome by site-specific recombination is mediated by a lambda-like integrase, Int, encoded in ICE*Bs1* (Lee et al., submitted). Excision requires both Xis and Int, whereas integration requires only Int (Lee et al., submitted).

Once excised from the chromosome, some ICEs transfer to other cells by using mechanisms similar to those of conjugative plasmids (reviewed in references 6, 11, 20, 25, 34, 36, and 50). Transfer of conjugative plasmids typically initiates from a specific site in the plasmid, the origin of transfer, *oriT*. *oriT* functions in *cis* and is required for efficient transfer. A relaxase, usually encoded by the plasmid, recognizes *oriT*, makes a single-strand DNA break (a nick) in *oriT*, and covalently attaches to the 5′ end of the nicked DNA strand via a phosphotyrosyl linkage (9, 34). Some conjugal relaxases have a helicase domain, which unwinds the single strand of DNA for transfer from the donor into the recipient (39, 45). In the absence of a cognate helicase activity, conjugative plasmids can use leading-strand DNA synthesis (rolling-circle replication) from the nicked 3′ end to promote strand displacement and single-strand DNA transfer (9, 34). In either case, the covalently attached relaxase interacts with a coupling protein in the bacterial membrane that targets the single strand of plasmid DNA to a transmembrane conjugation pore (26, 37, 57, 58). The attached relaxase may transfer into the recipient cell, while another relaxase monomer may remain bound to the plasmid DNA in the donor cell (17, 22, 37). The DNA relaxase terminates transfer by precisely rejoining the ends of the plasmid and releasing a single-stranded DNA circle into the recipient (37, 48). Synthesis of the complementary strand of the transferred circle initiates primarily at an origin of plasmid replication (53).

In contrast to those of conjugative plasmids, origins of transfer and the cognate relaxases from only a few ICEs have been identified and characterized (1, 12, 56, 62). Where characterized, *oriT* on an ICE is required in *cis* for transfer but usually not for excision, although there are possible exceptions (60).

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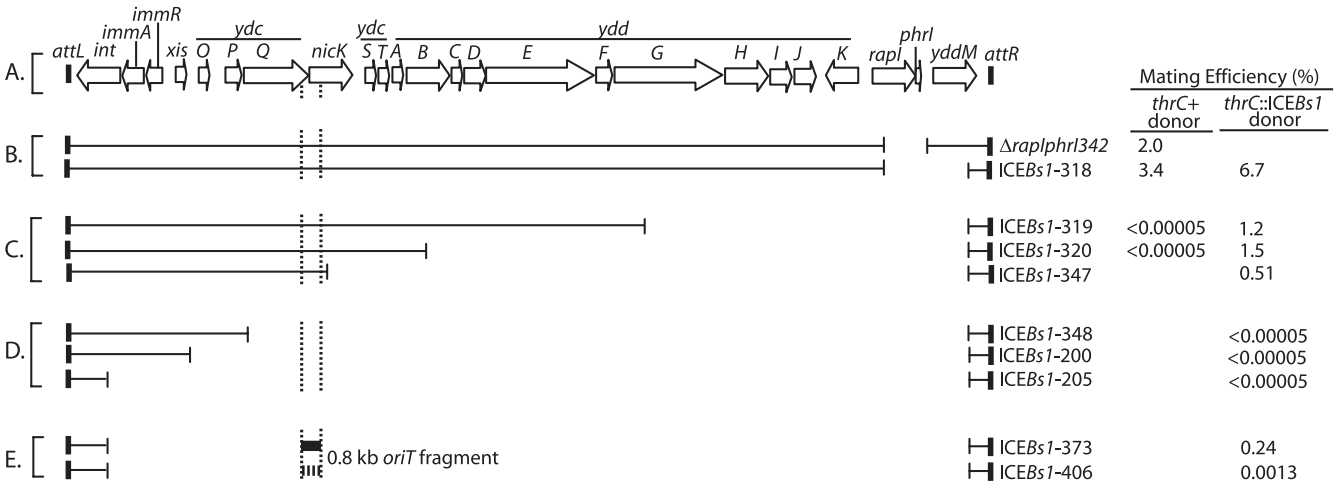

FIG. 1. Effect of deletions in ICE*Bs1* on transfer and mobilization. (A) The genetic map of ICE*Bs1*, indicating genes as open arrows and the flanking 60-bp repeats at *attL* and *attR* as thin black rectangles. The vertical dotted lines indicate the region of ICE*Bs1 oriT*. (B to E) Thin lines below the map of ICE*Bs1* indicate the regions of ICE*Bs1* between *attL* and *attR* that are present in the various ΔICE*Bs1* mutations. Open spaces represent regions that are missing. Mating efficiencies are indicated to the right. Donor cells were induced with MMC and mixed with recipient strain CAL264, an ICE*Bs1*^{0} recipient strain that expresses *int* from the P*spank* promoter. Donor strains either contained the indicated ΔICE*Bs1* allele alone (*thrC*$^+$) or also carried an immobilized ICE*Bs1* at *thrC* {*thrC325*::[ICE*Bs1*(Δ*attR*::*tet*)]} that supplied all of the ICE*Bs1* excision and conjugation functions in *trans* but is unable to excise due to the deletion of *attR*. Mating efficiency was calculated as the percentage of transconjugant CFU per donor cell. The mean from at least two independent assays is reported. Mating efficiencies for the Δ(*rapI-phrI*)342::*kan* donor strain ranged from 0.81% to 3.7% in six independent assays and gave a mean of 2.0% with a standard deviation of 1.2%. Except for donor strains that gave no detectable transconjugants, mating efficiencies for other donor strains had similar amounts of variability. (E) Thick lines indicate that two derivatives of ΔICE*Bs1*-205::*kan* carry an ~0.8-kb *oriT* fragment from the *ydcQ-nicK* region (wild-type fragment, solid; mutant fragment, dashed).

Like plasmids, ICEs typically encode a relaxase that binds to the cognate *oriT*, nicks the DNA, and becomes covalently attached. In some cases, there appears to be an additional protein providing specificity to the relaxase. For example, Tn*916*, an ICE from *Enterococcus faecalis*, contains a *cis*-acting origin of transfer, *oriT* (31), and encodes a DNA nuclease, the *orf20* gene product (56). In vitro, Orf20 protein from Tn*916* requires the transposon integrase for strand and site specificity. In the absence of the integrase, the Orf20 protein functions as an endonuclease cleaving both strands of Tn*916 oriT* DNA "at several distinct sites favoring GT dinucleotides" (56).

We have identified and characterized the origin of transfer, *oriT*, of ICE*Bs1*. We found that induction of ICE*Bs1* gene expression leads to nicking in a GC-rich inverted repeat in *oriT*. We also found that *ydcR* (renamed *nicK*) is required for nicking and transfer of ICE*Bs1* and that NicK is the only ICE*Bs1* gene product needed for specific nicking at *oriT*. The *oriT* nicking site is actually located within the *nicK* open reading frame (ORF). Nicking of *oriT* by NicK likely facilitates the transfer of one strand of ICE*Bs1* into recipient cells.

MATERIALS AND METHODS

Media and growth conditions. *B. subtilis* was grown in LB or defined minimal glucose medium at 37°C (27). The following antibiotics and other chemicals were used: isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM), mitomycin C (MMC) (1 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) (80 μg/ml), chloramphenicol (5 μg/ml), kanamycin (5 μg/ml), spectinomycin (100 μg/ml), streptomycin (100 μg/ml), and erythromycin (0.5 μg/ml) and lincomycin (12.5 μg/ml) together to select for macrolide-lincosamide-streptogramin B resistance (*mls* or *erm*).

***B. subtilis* strains and alleles.** *B. subtilis* strains are listed in Table 1. *B. subtilis* strains were constructed by natural transformation or conjugation (2, 3, 27; Lee et al., submitted). *comK*::*cat* is an insertion of mini-Tn*10* (*cat*) and prevents competence development (41). The spontaneous streptomycin resistance allele (*str-84*, most likely in *rpsL*) was from strain CAL84 and is often used as a counterselective marker in mating experiments (2, 3). ICE*Bs1*^{0} indicates that the strain is cured of ICE*Bs1*. *rapI* was overexpressed from P*spank*(*hy*)-*rapI* integrated into *amyE*, *amyE*::{[P*spank*(*hy*)-*rapI*] *spc*}, to induce ICE*Bs1* gene expression and excision. *int* was expressed from *amyE*::[(P*spank-int*) *spc*] to provide integrase when needed (2; Lee et al., submitted). Δ(*rapI-phrI*)*342*::*kan* is a deletion-insertion (3).

TABLE 1. *Bacillus subtilis* strains used

Strain	Genotype[a]
CAL223	ΔICE*Bs1*-200::*kan*
CAL224	ΔICE*Bs1*-205::*kan*
CAL264	ICE*Bs1*^{0} *str-84 amyE*::[(P*spank-int*) *spc*] *comK*::*cat*
CAL306	Δ*nicK306* Δ(*rapI-phrI*)342::*kan amyE*::{[P*spank*(*hy*)-*rapI*] *spc*}
CAL321	ΔICE*Bs1*-318::*kan*
CAL322	ΔICE*Bs1*-319::*kan*
CAL323	ΔICE*Bs1*-320::*kan*
CAL326	ΔICE*Bs1*-318::*kan thrC325*::[(ICE*Bs1*-311 Δ*attR*::*tet*) *mls*]
CAL327	ΔICE*Bs1*-319::*kan thrC325*::[(ICE*Bs1*-311 Δ*attR*::*tet*) *mls*]
CAL328	ΔICE*Bs1*-320::*kan thrC325*::[(ICE*Bs1*-311 Δ*attR*::*tet*) *mls*]
CAL332	ΔICE*Bs1*-200::*kan thrC325*::[(ICE*Bs1*-311 Δ*attR*::*tet*) *mls*]
CAL346	Δ*nicK306* Δ(*rapI-phrI*)342::*kan amyE*::{[P*spank*(*hy*)-*rapI*] *spc*} *thrC329*::[(P*xis-nicK-lacZ*) *mls*]
CAL347	ΔICE*Bs1*-347::*kan*
CAL348	ΔICE*Bs1*-348::*kan*
CAL349	ΔICE*Bs1*-205::*kan thrC325*::[(ICE*Bs1*-311 Δ*attR*::*tet*) *mls*]
CAL350	ΔICE*Bs1*-347::*kan thrC325*::[(ICE*Bs1*-311 Δ*attR*::*tet*) *mls*]
CAL351	ΔICE*Bs1*-348::*kan thrC325*::[(ICE*Bs1*-311 Δ*attR*::*tet*) *mls*]
CAL381	ΔICE*Bs1*-373::*kan*
CAL386	ΔICE*Bs1*-373::*kan thrC325*::[(ICE*Bs1*-311 Δ*attR*::*tet*) *mls*]
CAL413	ΔICE*Bs1*-406::*kan*
CAL417	ΔICE*Bs1*-406::*kan thrC325*::[(ICE*Bs1*-311 Δ*attR*::*tet*) *mls*]
CAL419	ICE*Bs1*^{0} *str-84 comK*::*cat*
CAL501	ICE*Bs1*^{0} *amyE501*::{[P*spank*(*hy*)-*nicK477*] *spc*}
CAL502	ICE*Bs1*^{0} *amyE502*::{[P*spank*(*hy*)-*nicK488*] *spc*}
IRN342	Δ(*rapI-phrI*)342::*kan*
JMA168	Δ(*rapI-phrI*)342::*kan amyE*::{[P*spank*(*hy*)-*rapI*] *spc*}

[a] All strains are derived from JH642 (55) and contain the *pheA1* and *trpC2* mutations.

Construction of two large deletions at the endogenous ICE*Bs1* locus, ΔICE*Bs1*-205::*kan* and ΔICE*Bs1*-200::*kan* (Fig. 1D), was described previously (Lee et al., submitted). The deletion in ΔICE*Bs1*-205::*kan* disrupts every ORF in ICE*Bs1*, replacing all but 651 bp of *int* and 157 bp of *yddM* with the kanamycin resistance gene from pGK67 (35). The deletion in ΔICE*Bs1*-200::*kan* starts 86 bp downstream of the *xis* ORF. Five additional deletions in ICE*Bs1* (Fig. 1B to D), each extending to the same 3′ endpoint in the *yddM* ORF, were constructed as described previously (Lee et al., submitted). The deletion in ΔICE*Bs1*-348::*kan* starts in *ydcQ*, leaving 222 bp of the 1,440-bp *ydcQ* ORF. ΔICE*Bs1*-347::*kan* starts in *nicK*, leaving 367 bp of the 1,050-bp *nicK* ORF. ΔICE*Bs1*-320::*kan* starts in *yddB*, leaving 491 bp of the 1,062-bp *yddB* ORF. ΔICE*Bs1*-319::*kan* starts in *yddG*, leaving 1,784 bp of the 2,445-bp *yddG* ORF. ΔICE*Bs1*-318::*kan* starts in *rapI*, leaving 586 bp of the 1,173-bp *rapI* ORF. The deletion in ΔICE*Bs1*-318::*kan* starts at almost the same position as that in the Δ(*rapI-phrI*)342::*kan* allele, which leaves 587 bp of *rapI* (3).

Hybrid derivatives of the ΔICE*Bs1*-205::*kan* element that contain a 802-bp *oriT* fragment at the PstI site between the *int* and *kan* gene sequences were constructed. The *oriT* fragment is in its native orientation, relative to *attL* and *attR*. The 802-bp fragment includes 378 bp upstream and 400 bp downstream of the 24-bp sequence ACCCCCCCACGCTAACAGGGGGGT, which is located 17 bp downstream of the start of the *nicK* ORF. ΔICE*Bs1*-373::*kan* contains the wild-type fragment, while ΔICE*Bs1*-406::*kan* contains a mutant fragment, which was generated by the splice-overlap-extension PCR method (29).

An ICE*Bs1* element was immobilized at *thrC*, which allowed us to stably express all of the ICE*Bs1* gene products in *trans* to ICE*Bs1* derivatives located at the endogenous chromosomal locus. *thrC325*::[(ICE*Bs1*-311 Δ*attR100*::*tet*) *mls*] contains the entire ICE*Bs1* element except for 161 bp at the right-hand end, which were removed by the Δ*attR100*::*tet* mutation. This *attR* mutation prevents excision (Lee et al., submitted). The *thrC325*::ICE*Bs1*-311 allele also includes sequences that usually flank the 60-bp direct repeats that mark the left and right ends of ICE*Bs1* in its normal attachment site in the chromosome. Thus, 206 bp of chromosomal DNA upstream of the left direct repeat and 768 bp of chromosomal DNA downstream of the Δ*attR100*::*tet* mutation are included.

Construction of *thrC325*::[(ICE*Bs1*-311 Δ*attR100*::*tet*) *mls*] involved many steps. First, we inserted the ICE*Bs1 attB* site at *thrC*. This was accomplished by replacing all of the ICE*Bs1* genes, except for *immR*, *immA*, and *int*, with the *cat* gene. The ΔICE*Bs1*-117::*cat* element, including 206 bp upstream and 823 bp downstream of the flanking 60-bp direct repeats, was cloned and inserted into the *thrC* locus (pDG795 vector, a gift of P. Stragier). Excision of the ΔICE*Bs1*-117::*cat* element at *thrC* was induced by expressing *xis* from *amyE168*::[(P*spank-xis*) *spc*] (Lee et al., submitted). By screening for those cells that had lost chloramphenicol resistance, we obtained *thrC213*::(*attB*-117 *mls*), in which the ICE*Bs1 attB* region is inserted at *thrC*. A functional kanamycin-resistant ICE*Bs1* Δ(*rapI-phrI*)342::*kan* was integrated into *attB* at *thrC* by mating JMA168 donors with *thrC213*::(*attB*-117 *mls*) recipients that lacked the native *attB* region (Δ*attB*::*cat*) (Lee et al., submitted). Finally, the conjugation-proficient ICE*Bs1* in *thrC229*::{[ICE*Bs1* Δ(*rapI-phrI*)342::*kan*] *mls*} was converted to an excision-defective *rapI-phrI*$^+$ derivative by recombination with a DNA fragment containing *rapI-phrI*$^+$ and the Δ*attR100*::*tet* allele (Lee et al., submitted), yielding the desired tetracycline-resistant, kanamycin-sensitive *thrC325*::[(ICE*Bs1*-311 Δ*attR100*::*tet*) *mls*] allele.

Δ*nicK306* is an unmarked, in-frame 519-bp deletion, which fuses the first 125 codons of *nicK* to the last 54 codons. Δ*nicK306* deletes most of the NicK-coding region that corresponds to the conserved pfam02486 Rep_trans domain, but it appears to leave the *cis*-acting *oriT* region of ICE*Bs1* intact. A 2.2-kb DNA fragment containing the Δ*nicK306* allele was obtained by the splice-overlap-extension PCR method (29) and cloned into the EcoRI and BamHI sites of the chloramphenicol-resistant vector pEX44 (a gift from E. Küster-Schöck) (15) with the promoterless *spoVG-lacZ* ORF in pEX44 placed downstream of the ICE*Bs1* ORFs on the 2.2-kb insert. The resulting plasmid, pCAL285, was used to replace the *nicK* gene with the Δ*nicK306* allele in the chromosome of JMA168, as described previously (Lee et al., submitted).

The *amyE501*::{[P*spank*(*hy*)-*nicK477*] *spc*} and *amyE502*::{[P*spank*(*hy*)-*nicK488*] *spc*} alleles were designed to express *nicK* from the IPTG-inducible P*spank*(*hy*) promoter (pDR111 vector, a gift of D. Rudner) (5). The *amyE501*::{[P*spank*(*hy*)-*nicK477*] *spc*} and *amyE502*::{[P*spank*(*hy*)-*nicK488*] *spc*} alleles contain 87 bp and 393 bp from the region upstream of the *nicK* ORF and include 92 bp and 398 bp of the 1,440-bp *ydcQ* ORF, respectively.

The *thrC*329::[P*xis*-(*nicK-lacZ*) *mls*] allele was designed to express *nicK* from the *xis* promoter (P*xis*) of ICE*Bs1*. *nicK* was cloned into the BamHI site between P*xis* and *lacZ* in pKG1, which had previously been used to construct *thrC*::[(P*xis-lacZ*Ω343) *mls*] (2). The resultant plasmid, pCAL178, was linearized and used to introduce *thrC*329::[P*xis*-(*nicK-lacZ*) *mls*] into the *B. subtilis* chromosome.

ICE*Bs1* excision assays. Excision of ICE*Bs1* in RapI-induced or MMC-induced cells was assayed by detecting the excised circular intermediate and repaired chromosomal junctions, as described previously (3; Lee et al., submitted).

ICE*Bs1* mating assays. Matings were done essentially as described previously (3; Lee et al., submitted). Equal numbers of donor (Kan^r) and recipient (Str^r) cells were mixed and filtered onto cellulose nitrate filters. The filters were placed on plates comprised of Spizizen's minimal salts (27) and 1.5% agar for 3 h at 37°C. The mean of mating efficiencies from at least two independent experiments is reported. Mating efficiencies for RapI-induced donors were calculated as the percentage of Kan^r Str^r transconjugant CFU per Kan^r donor CFU recovered postmating. Since MMC treatment reduced the recovery of donor cells postmating, mating efficiencies for MMC-induced donors were calculated as the percentage of Kan^r Str^r transconjugant CFU recovered postmating per donor cell present in the initial mating mixture. In this case, the number of donor cells was determined using a value of 1.65×10^8 cells per ml for cultures grown to an optical density at 600 nm of 1.

Identification of the site of nicking within ICE*Bs1*. *B. subtilis* genomic DNA was purified on QIAGEN DNeasy minicolumns from cell lysates treated with RNase A and proteinase K in the optional lysis buffer for gram-positive bacteria (QIAGEN). The DNA was digested with HindIII, bound to QIAGEN PCR purification minicolumns, and washed three times with PB buffer and once with PE buffer, before elution with EB buffer (all buffers from QIAGEN). Five hundred nanograms of digested DNA was used as a template with *Taq* polymerase (Roche) and 2 pmol ^{32}P-labeled primer in 50-μl primer extension reaction mixtures incubated for 20 cycles of 94°C for 30 s, 54°C for 2 min, and 72°C for 3 min. Primers (50 pmol) were end labeled with T4 polynucleotide kinase (New England Biolabs), as per the manufacturer's instructions, with 150 μCi [γ-^{32}P]ATP (6,000 Ci/mmol; Perkin-Elmer) and then purified on QIAGEN nucleotide removal columns. ^{32}P-labeled primers were also used in dideoxy-DNA sequencing reactions (Promega fmol sequencing system), which were run with primer extension products on 8% polyacrylamide–Tris-borate-EDTA–urea gels. Primers CLO75 and CLO76 were designed to detect breaks in the *oriT* region by hybridizing on opposite strands in the *ydcQ-nicK* region, 61 bp upstream and 72 bp downstream of the 24-bp GC-rich inverted repeat sequence, respectively. Controls showed that each primer could detect cleaved templates generated by restriction enzyme digestion.

RESULTS

Rationale and experimental design. We set out to identify the origin of transfer, *oriT*, of ICE*Bs1* and the gene encoding the ICE*Bs1* relaxase. Our expectation was that ICE*Bs1* contains a single *oriT* that is required, in *cis*, for transfer and is nicked in induced donor cells. We started by making a series of deletions of ICE*Bs1* starting from near the right end and extending to different left endpoints (Fig. 1B to D). We had previously shown that the only ICE*Bs1* genes needed for excision were *int* and *xis*. *int* and *xis* are necessary and sufficient for excision, and *int* is necessary for integration (Lee et al., submitted). Also, we found that DNA near the ends of the integrated ICE*Bs1* was sufficient for excision (Lee et al., submitted). Since *int* is at the far left end and *xis* is the fourth gene from the left, we expected that nested deletions starting from the right end (leaving *attR* intact) might affect conjugation but not excision. We tested these nested deletions for mating and the ability to be mobilized by complementation in *trans*. Since *oriT* should be needed in *cis*, inactivation of *oriT* should render ICE*Bs1* unable to transfer to recipients even though the element excises and all other ICE*Bs1* functions are provided in *trans* by complementation.

In complementation experiments, *trans*-acting functions of ICE*Bs1* were provided by ICE*Bs1* located at *thrC* (see Materials and Methods). The element at *thrC* was unable to excise (was "locked in") due to loss of the right end of ICE*Bs1* (Δ*attR*), but it was able to mobilize an otherwise defective

ICE*Bs1* located at the normal attachment site in the chromosome. We used recipients that expressed *int*, encoding integrase, because some of the donor ICE*Bs1* mutants did not contain their own *int*. Expression of *int* in the recipient is sufficient to complement loss of *int* on the donor element (2; Lee et al., submitted).

ICE*Bs1* gene expression and excision were induced by adding MMC to induce the SOS response. Induced ICE*Bs1*-containing strains were mixed with recipients cured of ICE*Bs1* (ICE*Bs1*^{0}) but that expressed ICE*Bs1 int* from a heterologous promoter. After mixing potential donors and recipients, cells were filtered, incubated for 3 h to allow mating, and then plated selectively to detect transconjugants.

Induction of ICE*Bs1* with MMC is less efficient and a bit more variable than induction by overproduction of RapI (3; Lee et al., submitted). However, we used MMC and not overproduction of RapI because strains containing ICE*Bs1* at the normal attachment site, the "locked-in" ICE*Bs1* at *thrC*, and the P*spank*(*hy*)-*rapI* construct were unstable, even without IPTG, likely because low-level expression of genes from the "locked-in" ICE*Bs1* and nicking of *oriT* at *thrC* cause defects in cell viability and ICE*Bs1* maintenance at the normal attachment site (2; Lee et al., submitted).

Genes at the right end of ICE*Bs1* that are not required for mating. Previously, we found that *rapI* and *phrI* are not needed for mating (3). The ICE*Bs1* deletion ΔICE*Bs1*-318 removes *yddM* in addition to *rapI* and *phrI* (Fig. 1B). The mating frequency of ΔICE*Bs1*-318 was normal (Fig. 1B, *thrC*$^+$ donor), indicating that *yddM* is not required for mating. The function of *yddM* is unknown, and YddM does not yet appear to be homologous to any other protein.

Deletions from the right end of ICE*Bs1* that are defective in mating. ICE*Bs1* deletions ΔICE*Bs1*-319 and ΔICE*Bs1*-320, which remove additional *ydd* genes (Fig. 1C), were defective for mating (Fig. 1C, *thrC*$^+$ donors). These two ICE*Bs1* deletion mutants and even larger deletions are capable of normal excision (data not shown) (Lee et al., submitted). Despite this, we were unable to detect any transconjugants when these mutants were used as donors without complementation.

The results with ΔICE*Bs1*-319 and ΔICE*Bs1*-320 indicate that at least one gene in the *yddGHIJK* region is required for transfer of ICE*Bs1* and may encode a component of the conjugation apparatus. YddG and YddH are similar to proteins encoded by other ICEs and are predicted to be membrane proteins with eight transmembrane spanning domains and one transmembrane spanning domain, respectively (3, 7) (TopPred http://bioweb.pasteur.fr/seqanal/interfaces/toppr.html [14, 63]). YddH contains a domain (cd00254 LT_GEWL [42]) that is found in murein hydrolases (61) and may facilitate ICE*Bs1* transfer by degrading the peptidoglycan barrier.

When ICE*Bs1* functions were provided in *trans*, the defects in mating of ΔICE*Bs1*-319 and ΔICE*Bs1*-320 were largely complemented (Fig. 1C, *thrC*::ICE*Bs1* donor), indicating that the ICE*Bs1* at *thrC*, although not capable of excising due to the loss of *attR*, was capable of mobilizing the defective ICE*Bs1* at the chromosomal attachment site. In addition, a larger deletion that extends into *nicK* (*ydcR*), ΔICE*Bs1*-347, and leaves intact only seven ORFs at the left end of ICE*Bs1* was mobilized when ICE*Bs1* functions were provided in *trans* (Fig. 1C, ICE*Bs1*-347). These results indicate that *oriT* lies somewhere to the left of the endpoint in this deletion.

The ability of the "locked-in" ICE*Bs1* at *thrC* to complement these ICE*Bs1* mutants indicates that excision and circularization are not required for ICE*Bs1* gene expression and production of a functional conjugation apparatus. However, the mating efficiencies of the three ICE*Bs1* derivatives (ΔICE*Bs1*-319, -320, and -347) were consistently lower than the mating efficiencies of those that did not require complementation for mating (Fig. 1B and 1C). We suspect that this is due to a combination of effects; perhaps expression of the ICE*Bs1* genes from the excision-defective construct at *thrC* is not completely normal. Also, it is possible that having two copies of some of the ICE*Bs1* genes in the merodiploid alters the stoichiometry and assembly of a functional conjugation apparatus. In addition, perhaps some of the ICE*Bs1* proteins function better in *cis* than in *trans* (e.g., the relaxase).

Identification of a *cis*-acting region of ICE*Bs1* required for its mobilization. We tested three additional deletions in ICE*Bs1* for their ability to be mobilized by functions provided in *trans*. These deletions, ΔICE*Bs1*-348, ΔICE*Bs1*-200, and ΔICE*Bs1*-205, all extend past *nicK* and into or past *ydcQ* (Fig. 1D). Despite the presence of ICE*Bs1* Δ*attR* at *thrC*, these deletions could not be mobilized (Fig. 1D, *thrC*::ICE*Bs1* donor). Combined with the finding that the deletion mutant ΔICE*Bs1*-347 can be mobilized, these results indicate that there is a *cis*-acting element needed for transfer in the 1.5-kb region that is present in ΔICE*Bs1*-347 and absent in ΔICE*Bs1*-348 (Fig. 1C and D).

A 0.8-kb fragment of ICE*Bs1* contains *oriT* and an essential GC-rich inverted repeat. Since the *oriT*s of conjugative and mobilizable plasmids often contain inverted repeats (19, 34), we searched for an inverted repeat in the 1.5-kb *ydcQ-nicK* region and found a 24-bp sequence, ACCCCCCCACGCTAACAGGGGGGGT, comprised of a perfect 7-bp GC-rich inverted repeat (underlined) and an intervening 10 bp. This 24-bp sequence seemed particularly noteworthy since *oriT* sequences are often in close proximity to the genes encoding their cognate DNA relaxases (19, 20, 36, 49), and this 24-bp sequence is located in the 5′ end of *nicK*, which is predicted to encode a DNA relaxase. In addition, a nearly identical sequence, ACCCCCCgtatCTAACAGGGGGGT (four mismatches are in lowercase), is located in the *oriT* region of Tn*916*, 330 bp upstream of *orf20*, which encodes an enzyme with endonuclease activity in vitro (56).

We found that a 0.8-kb fragment of ICE*Bs1* containing this 24-bp sequence confers mobility to the nonmobilizable mutant ΔICE*Bs1*-205. We cloned this 0.8-kb fragment into ΔICE*Bs1*-205, generating ΔICE*Bs1*-373 (Fig. 1E). In contrast to ΔICE*Bs1*-205, this element (ΔICE*Bs1*-373) could be mobilized when ICE*Bs1* functions were provided in *trans* (Fig. 1D and E, *thrC*::ICE*Bs1* donor).

We also constructed ΔICE*Bs1*-406, which is identical to the mobilizable ΔICE*Bs1*-373 but contains four point mutations in the 24-bp sequence (ACCaCCaCACGCTAACAGaGGaGT) (mutations are in lowercase). We found that these mutations reduced mating activity conferred by the 0.8-kb fragment by greater than 100-fold (Fig. 1E, *thrC*::ICE*Bs1* donor). These results narrow down the location of the *oriT* of ICE*Bs1* to a

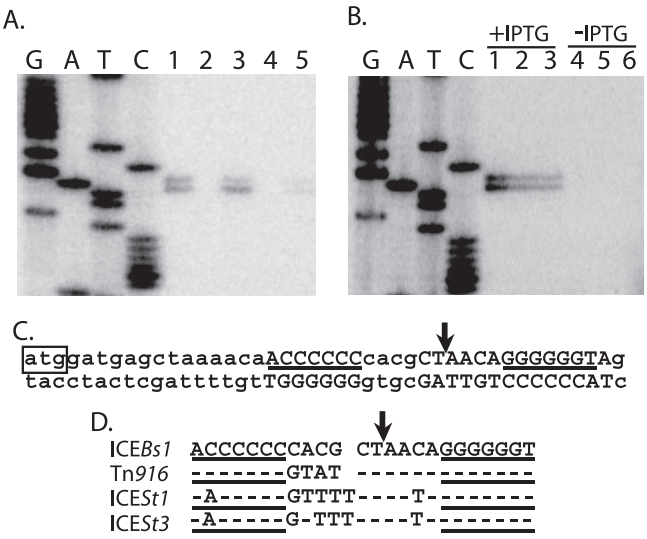

FIG. 2. NicK-dependent nicking of ICE*Bs1* between the GC-rich inverted repeat in *oriT*. (A and B) Primer extension products generated using end-labeled CLO76 and *B. subtilis* genomic DNA are shown along with DNA sequencing reactions (GATC). (A) Lanes 1 to 3, *nicK*$^+$ (JMA168); lane 4, Δ*nicK306* (CAL306); lane 5, Δ*nicK306* P*xis-nicK* (CAL346). Strains contained the IPTG-inducible P*spank*(*hy*)-*rapI* and were grown without IPTG (lane 2) or with IPTG for 1 h (lanes 1 and 3 to 5). (B) Lanes 1 and 4, control ICE*Bs1*$^+$ P*spank*(*hy*)-*rapI* (JMA168); lanes 2 and 5, ICE*Bs1*^{0} P*spank*(*hy*)-*nicK488* (CAL502); lanes 3 and 6, ICE*Bs1*^{0} P*spank*(*hy*)-*nicK477* (CAL501). Strains were grown for 1 h with (lanes 1 to 3) or without (lanes 4 to 6) IPTG. (C) Diagram of the double-stranded DNA sequence showing the *nicK* start codon (ATG in box), inverted repeats (horizontal lines), location of the *nic* site (vertical arrow), and base pairs conserved in the Tn*916* *oriT* region (uppercase). (D) Alignment of the top strands of the conserved sequence in ICE*Bs1*, Tn*916*, ICE*St1*, and ICE*St3*. A gap in the top two sequences indicates that ICE*Bs1* and Tn*916* have one less base pair than ICE*St1* and ICE*St3* in the intervening region. Dashes indicate identity with ICE*Bs1* sequence. The lines and arrow are as in panel C.

0.8-kb fragment, which contains a GC-rich inverted repeat that is important for *oriT* function.

***oriT* is nicked within the GC-rich inverted repeat after activation of ICE*Bs1*.** Transfer of conjugative plasmids requires nicking of one strand of their *oriT*, often several base pairs downstream of an inverted repeat (34, 38). We used primer extension assays designed to detect nicks near the 24-bp sequence. Primers CLO75 and CLO76 are complementary to opposite strands in the *ydcQ-nicK* region, 61 bp upstream and 72 bp downstream of the 24-bp inverted repeat, respectively. Controls showed that each primer could detect cleaved templates generated by restriction enzyme digestion in vitro (data not shown).

We identified a nick in the top strand of ICE*Bs1* in primer extension reactions using CLO76 as a primer and *B. subtilis* DNA as the template. ICE*Bs1* was induced by overexpression of RapI, and DNA was purified and subjected to primer extension analysis. Two primer extension products were detected in reactions using end-labeled primer CLO76 (Fig. 2A, lane 1). The lower band likely corresponds to the primer extension product terminated at the nick in ICE*Bs1*, whereas the upper band likely corresponds to the same extension product with an extra base added by the *Taq* polymerase terminal transferase activity (13). By running the primer extension reactions in the same lanes as the DNA sequencing ladder (data not shown), the *nic* site was found to be located between the repeated elements in the inverted repeat in *nicK*, a sequence that is also conserved in Tn*916* (Fig. 2C).

Induction of ICE*Bs1* was necessary for efficient nicking. We did not detect any nicking in the absence of induction of RapI expression with IPTG (Fig. 2A, lane 2). Nicking did not require excision of ICE*Bs1*; we detected RapI-dependent nicking of *oriT* in a nonexcisable Δ*attR100*::*tet* derivative of ICE*Bs1* (data not shown). We did not find any nicks on the bottom strand of ICE*Bs1* using primer CLO75 (data not shown). These results indicate that activation of ICE*Bs1* gene expression leads to nicking within an inverted repeat that is important for *oriT* activity. Analogous to conjugative and mobilizable plasmids that are nicked on one strand of their *oriT*s, only one strand of the excised ICE*Bs1* may be transferred to recipient cells, (52). Furthermore, our results indicate that nicking of ICE*Bs1* does not require excision and circularization of the element.

***nicK* is necessary for nicking and transfer of ICE*Bs1*.** NicK is homologous to Orf20 of Tn*916*, which nicks the *oriT* of Tn*916* in vitro and may facilitate the transfer of a single strand of Tn*916* to recipient cells (56). NicK and Orf20 are also homologous to DNA relaxases involved in rolling-circle replication of the pT181 family of plasmids (32, 46).

We found that *nicK* is necessary for cleavage within the ICE*Bs1* *oriT*, located within the *nicK* ORF. We constructed a deletion of *nicK* (Δ*nicK306*) that starts 332 bp downstream of the 24-bp inverted repeat and leaves almost the entire 0.8-kb sequence that contains *oriT* intact. This mutation abolished detectable nicking at *oriT* (Fig. 2A, lane 4).

Expression of *nicK* in *trans* restored nicking (Fig. 2A, lane 5). However, since the nicking assay was based on primer extension with a primer that detects both the *oriT* associated with the *nicK306* allele and the *oriT* associated with the ectopic *nicK*$^+$ allele, we could not distinguish whether nicking was restored at the Δ*nicK306* locus or was just occurring within *oriT* in *nicK*$^+$.

To test whether nicking was restored in the ICE*Bs1* Δ*nicK306* mutant, we measured mating efficiencies. After induction of wild-type ICE*Bs1* by overproduction of RapI (donor strain JMA168), the mating efficiency (into recipient CAL419) was ~7%. In contrast, when the ICE*Bs1* Δ*nicK306* mutant was used as the donor, mating was undetectable (<0.0002%). This defect in mating was not due to a defect in excision (data not shown), consistent with previous results showing that the only ICE*Bs1* genes necessary and sufficient for excision are *int* and *xis* (Lee et al., submitted).

The mating defect caused by the Δ*nicK306* mutation was partially complemented by providing relaxase in *trans*. We fused *nicK* to the promoter that drives *xis* (P*xis-nicK*). This promoter is normally repressed by the ICE*Bs1* immunity repressor ImmR and induced when ICE*Bs1* is activated by RapI or DNA damage (2). When the ICE*Bs1* Δ*nicK306* mutant was used as a donor and relaxase was provided from P*xis-nicK* (donor CAL346), the mating efficiency was significantly restored, to ~1% from undetectable levels (<0.0002% in the absence of the P*xis-nicK* fusion).

Whereas expression of *nicK* in *trans* significantly restored mating, the efficiency was not up to levels seen with *nicK*$^+$

ICE*Bs1*. This significant but partial complementation might be due to poor expression of *nicK* from the ectopic P*xis-nicK* fusion. Alternatively, it might indicate that, while not destroying *oriT*, the Δ*nicK306* might delete part of *oriT*. A third possibility is that relaxase might function preferentially in *cis*. Nonetheless, taken together, our results indicate that *nicK* is required for nicking and that *oriT* in the ICE*Bs1* Δ*nicK306* mutant is mostly or completely functional.

***nicK* is the only ICE*Bs1* gene product needed for nicking at *oriT*.** We found that expression of *nicK* is sufficient to cause nicking within *oriT*. We made two fusions of *nicK* to the IPTG-inducible promoter P*spank(hy)*, P*spank(hy)-nicK477* and P*spank(hy)-nicK488*, which extend 87 and 393 bp upstream of the *nicK* ORF, respectively. The P*spank*(*hy*)*-nicK488* construct contains the entire 0.8-kb sequence that confers mobility to ΔICE*Bs1*-205, and the P*spank*(*hy*)*-nicK477* construct is missing 274 bp at the 5′ end of the 0.8-kb sequence. In strains cured of ICE*Bs1* (ICE*Bs1*^{0}), nicking occurred in both constructs (Fig. 2B, lanes 2 and 3) and was not observed in the absence of induction with IPTG (Fig. 2B, lanes 5 and 6). These results indicate that NicK is the only ICE*Bs1* gene product needed for specific nicking within the GC-rich inverted repeat in *nicK* and that the same site is nicked in the intact ICE*Bs1*, the "locked-in" element, and the isolated *nicK*.

DISCUSSION

We found that *oriT* of ICE*Bs1* is contained on a 0.8-kb DNA fragment overlapping *ydcQ* and *nicK*. This fragment was sufficient to allow mobilization of a mutant derivative of ICE*Bs1* that contains DNA only from the ends of the element. Furthermore, this fragment contains a GC-rich inverted repeat internal to *nicK* that is necessary for full *oriT* function. When ICE*Bs1* was activated, the top strand of the element was nicked between the arms of the repeat. Nicking required NicK and no other ICE*Bs1* gene products and did not require excision of ICE*Bs1* from the chromosome. We propose that increased expression of NicK, induced when ICE*Bs1* is activated by RapI or DNA damage, leads to nicking of ICE*Bs1* at *oriT* and covalent attachment of NicK to one strand of ICE*Bs1*, analogous to homologous relaxases. This form of ICE*Bs1* is likely the substrate for transfer of a single strand of ICE*Bs1* DNA to mating partners.

Conserved relaxases and sequences in *oriT* in ICEs from *B. subtilis*, *E. faecalis*, and *Streptococcus thermophilus*. The relaxase and *oriT* from ICE*Bs1* are similar to those from Tn*916*, ICE*St1*, and ICE*St3* (3, 7, 54, 56). *oriT* of Tn*916* from *E. faecalis* is located near *orf21* and *orf20* (31), homologs of ICE*Bs1 ydcQ* and *nicK*, respectively. ICE*St1* and the closely related ICE*St3* from *S. thermophilus* are predicted to contain *oriT* in the intergenic region between *orfK* and *orfJ* (7, 54), which are homologs of *ydcQ* and *nicK*. All four *oriT* regions contain a highly conserved sequence comprised of a GC-rich inverted repeat and an intervening 10 or 11 bp (Fig. 2D). In ICE*Bs1*, the conserved sequence is located within *nicK*. In Tn*916*, ICE*St1*, and ICE*St3*, the conserved sequence is located in the intergenic region upstream of their *nicK* homologs.

The *orf20* gene product from Tn*916* has been purified and characterized in vitro (56). The purified Orf20 has endonuclease activity that is relatively nonspecific. However, addition of the Tn*916* integrase protein results in specific cleavage in the spacer sequences in an AT-rich inverted repeat 55 bp downstream from the GC-rich inverted repeat. DNase I footprinting results indicate that integrase protects part of the conserved GC-rich inverted repeat, and it was proposed that binding of integrase to the Tn*916 oriT* might coordinate excision and conjugation (28).

The in vitro specificity of the ICE*Bs1 nicK* gene product is not known. However, in vivo, ICE*Bs1 oriT* was nicked at the same site in the presence or absence of ICE*Bs1* integrase. Furthermore, nicking occurred at the same site in ICE*Bs1* derivatives that could excise and in constructs containing only *nicK* and *oriT* (in the absence of other parts of ICE*Bs1*). It is not known if the in vivo activity of ICE*Bs1* NicK is indicative of that of Tn*916* Orf20. Orf20 homologs were divided into two groups based on the amount of sequence identity to Orf20 (56). NicK (YdcR) of ICE*Bs1* belongs to the group with less overall identity and similarity. It is possible that relaxases more similar to Orf20 require a specificity factor and that those more similar to ICE*Bs1* NicK do not. It is also possible that the in vivo activity of Orf20 is not identical to that in vitro.

DNA translocases and conjugation. The gene upstream of *nicK*, *ydcQ*, encodes a homolog of FtsK and SpoIIIE (pfam01580 FtsK_SpoIIE [4]). In addition, the genes upstream of *orf20* and *orfK* of Tn*916* and ICE*St1* (and ICE*St3*), respectively, also encode FtsK/SpoIIIE homologs. FtsK and SpoIIIE are DNA translocases that are distantly related to the coupling proteins of plasmid conjugation systems (10, 18, 30). Coupling proteins interact with their cognate conjugal DNA relaxase and bind to both single- and double-stranded DNA (10, 47, 58). Coupling proteins likely form transmembrane pores and may facilitate the translocation of the conjugal DNA relaxase and the attached single strand of DNA through the membrane (17, 23, 24, 36, 40, 58).

It seems likely that, analogous to the case for conjugal relaxases and coupling proteins, NicK directly interacts with the putative coupling protein YdcQ to promote the 5′-to-3′ transfer of a single strand of ICE*Bs1* through a transmembrane conjugation pore and into the recipient cell. Our model predicts that a large 3′ portion of the *nicK* ORF will be transferred first and that *ydcQ* and the 5′ region of *nicK* will be transferred last. The strand- and site-specific nicking of *oriT* of Tn*916* by the Orf20 endonuclease in the presence of Tn*916* Int similarly indicates that the 5′-to-3′ transfer of a single strand of Tn*916* initiates with *orf20* and terminates with *orf21* (56).

DNA relaxases for plasmid conjugation and rolling-circle replication. DNA relaxases involved in plasmid conjugation and rolling-circle replication have common features. These relaxases attach to the 5′ end of the nicked DNA strand via a phosphotyrosyl covalent linkage (9, 46). Their genes and cognate *nic* sites are often in close proximity to each other (19, 33). Rolling-circle replication relaxases often nick between an inverted repeat, while conjugal relaxases often nick between an inverted repeat or several base pairs downstream of an inverted repeat (19, 33, 34, 38, 46). Rolling-circle replication relaxases recruit replication factors to the double-strand origin of plasmid replication so that leading-strand synthesis can proceed from the nicked 3′ end (16, 33). In plasmid conjugation systems, replication factors may also be recruited to the nicked 3′ ends of *oriT*s so that leading-strand synthesis can replace the

transferred strand in the donor and unwind the single strand for transfer (34).

Some of the DNA relaxases involved in conjugation have diverged from those involved in rolling-circle replication by acquiring additional functions specific for DNA transfer (9). For example, the mobilizable plasmid R1162 produces a DNA relaxase that has primase activity, which may facilitate complementary-strand synthesis of the transferred single strand in certain recipients (51). F TraI, required for F plasmid conjugation, has both DNA relaxase and DNA helicase activities, as well as domains that allow it to interact with other conjugation proteins (44, 45, 57).

Complementary-strand synthesis. For both rolling-circle replication and conjugation, the nicking, unwinding, and recircularization of a single strand of DNA is followed by complementary-strand synthesis (32, 53). For rolling-circle replication, complementary-strand synthesis is initiated from a single-strand origin of replication, which, like lagging-strand synthesis, requires RNA priming (16, 32). For conjugative and mobilizable plasmids, complementary-strand synthesis of the transferred circularized single strand occurs in the recipient (53). Some conjugative plasmids encode primases that are transferred into the recipient and are important for complementary-strand synthesis, while others appear to rely on host-encoded primase activities (34, 51). In either case, complementary-strand synthesis is initiated primarily at the normal origin of replication on the transferred plasmid (53).

Unlike conjugative and mobilizable plasmids, ICE*Bs1* resides in the host chromosome. It excises to form a circular intermediate. If ICE*Bs1* is transferred as a single strand, then complementary-strand synthesis in the recipient is likely to be required to generate the double-stranded circular form of ICE*Bs1* for integration. Complementary-strand synthesis is probably also required before Int can even be produced in the recipient, since most promoters are active only in double-stranded DNA (43), and we predict that the transferred single strand corresponds to the nontemplate strand of *int*. It is not clear if or how much replication of the excised ICE*Bs1* circle occurs in the donor, but most characterized ICEs are not known to replicate autonomously.

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