Polar Positioning of a Conjugation Protein from the Integrative and Conjugative Element ICEBs1 of Bacillus subtilis

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ICEBs1 is an integrative and conjugative element found in the chromosome of Bacillus subtilis. ICEBs1 encodes functions needed for its excision and transfer to recipient cells. We found that the ICEBs1 gene conE (formerly yddE) is required for conjugation and that conjugal transfer of ICEBs1 requires a conserved ATPase motif of ConE. ConE belongs to the HerA/FtsK superfamily of ATPases, which includes the well-characterized proteins FtsK, SpoIIIE, VirB4, and VirD4. We found that a ConE-GFP (green fluorescent protein) fusion associated with the membrane predominantly at the cell poles in ICEBs1 donor cells. At least one ICEBs1 product likely interacts with ConE to target it to the membrane and cell poles, as ConE-GFP was dispersed throughout the cytoplasm in a strain lacking ICEBs1. We also visualized the subcellular location of ICEBs1. When integrated in the chromosome, ICEBs1 was located near midcell along the length of the cell, a position characteristic of that chromosomal region. Following excision, ICEBs1 was more frequently found near a cell pole. Excision of ICEBs1 also caused altered positioning of at least one component of the replisome. Taken together, our findings indicate that ConE is a critical component of the ICEBs1 conjugation machinery, that conjugal transfer of ICEBs1 from B. subtilis likely initiates at a donor cell pole, and that ICEBs1 affects the subcellular position of the replisome.

Integrative and conjugative elements (also known as conjugative transposons) and conjugative plasmids are key elements in horizontal gene transfer and are capable of mediating their own transfer from donor to recipient cells. ICEBs1 is an integrative and conjugative element found in some Bacillus subtilis strains. Where found, ICEBs1 is integrated into the leucine tRNA gene trnS-leu2 (Fig. 1) (7, 14, 21).

ICEBs1 gene expression, excision, and potential mating are induced by activation of RecA during the SOS response following DNA damage (7). In addition, ICEBs1 is induced by increased production or activation of the ICEBs1-encoded regulatory protein RapI. Production and activity of RapI are indicative of the presence of potential mating partners that do not contain a copy of ICEBs1 (7). Under inducing conditions, the ICEBs1 repressor ImmR (6) is inactivated by proteolytic cleavage mediated by the anti-terminator and protease ImmA (12). Most ICEBs1 genes then become highly expressed (7). One of these genes (xis) encodes an excisionase, which in combination with the element’s integrase causes efficient excision and formation of a double-stranded circle (7, 38). The circular form is nicked at the origin of transfer, oriT, by a DNA relaxase, the product of nickK (39). Under appropriate conditions, ICEBs1 can then be transferred by mating into B. subtilis and other species, including the pathogens Listeria monocytogenes and Bacillus anthracis (7). Once transferred to a recipient, ICEBs1 can be stably integrated into the genome at its attachment site in trnS-leu2 by the ICEBs1-encoded integrase (38).

In contrast to what is known about ICEBs1 genes and proteins involved in excision, integration, and gene regulation, less is known about the components that make up gram-positive organisms’ mating machinery, defined as the conjugation proteins involved in DNA transfer (18, 24). The well-characterized mating machinery of gram-negative organisms can serve as a preliminary model (15, 16, 37, 48). Gram-negative organisms’ mating machinery is a type IV secretion system composed of at least eight conserved proteins that span the cell envelope. For example, the conjugation apparatus of the Agrobacterium tumefaciens Ti plasmid (pTi) is composed of 11 proteins (VirB1 through VirB11), including the ATPase VirB4 (16). VirB4 family members interact with several components of their cognate secretion systems and may energize machine assembly and/or substrate transfer (16, 48). The secretion substrate is targeted to the conjugation machinery by a coupling protein. Coupling proteins, such as VirD4 of pTi, interact with a protein attached to the end of the DNA substrate and couple the substrate to other components of the conjugation machinery. Coupling proteins might also energize the translocation of DNA through the machinery. Both VirB4 and VirD4 belong to the large HerA/FtsK superfamily of ATPases (29). Two other characterized members of this superfamily are the chromosome-partitioning proteins FtsK and SpoIIIE (29), which are ATP-dependent DNA pumps (reviewed in reference 2).

Some of the proteins encoded by the conjugative elements of gram-positive organisms are homologous to components of the conjugation machinery from gram-negative organisms (1, 9, 14, 29), indicating that some aspects of conjugative DNA transfer may be similar in gram-positive and gram-negative organisms. For example, ConE (formerly YddE) of ICEBs1 has sequence

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similarities to VirB4 (29). YdcQ may be the ICEBs1-encoded coupling protein, as it is phylogenetically related to other coupling proteins (29, 44). Despite some similarities, the cell envelopes and many of the genes encoding the conjugation machinery are different between gram-positive and gram-negative organisms, indicating that there are likely to be significant structural and mechanistic differences as well.

To begin to define the conjugation machinery of ICEBs1 and to understand spatial aspects of conjugation, we examined the function and subcellular location of ConE of ICEBs1. Our results indicate that ConE is likely a crucial ATPase component of the ICEBs1 conjugation machinery. We found that ConE and excised ICEBs1 DNA were located at or near the cell poles. We propose that the conjugation machinery is likely located at the cell poles and that mating might occur from a donor cell pole.

**TABLE 1. B. subtilis strains used**

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<sup>a</sup> All strains are derived from JH642 (45) and contain pheA1 and trpC2.
RESULTS

cone is required for mating. Conjunctive transfer of ICEB1 is a multistep process. Previous work indicated that cone is not required for ICEB1 gene expression, excision, integration, circularization, or nicking (6, 7, 12, 38, 39). Since ConE is a putative ATPase and distantly related to other ATPases known to be required for conjugation, we tested the effects of cone mutations on mating of ICEB1.

We constructed three different cone alleles: (i) an in-frame deletion [coneΔ(88–808)] removing codons 88 through 808 (of 831), (ii) a missense mutation in the Walker A box [coneE(K476E)] that is predicted to eliminate nucleotide binding, and (iii) a double missense mutation in the Walker B box [coneE(D703A/E7044)] that is predicted to eliminate ATPase activity (reviewed in reference 26). Each cone mutant allele was introduced unmarked into ICEB1, replacing the wild-type allele (see Materials and Methods).

We found that cone is required for ICEB1 conjugative transfer. We compared mating efficiencies of ICEB1 from donor strains containing the various cone alleles into recipient B. subtilis cells lacking ICEB1 (Fig. 2). ICEB1 was induced by overproduction of RapI from a heterologous promoter, and potential donor cells were mixed with potential recipients that lacked ICEB1, essentially as described previously (7). The donor ICEB1-containing cell contained an antibiotic resistance marker that had been inserted to allow selection and monitoring of ICEB1 acquisition (7). A donor strain with an intact cone (coneE) transferred ICEB1 with an average mating frequency of ~7% (percent transconjugant CFU per donor CFU [Fig. 2, row a]). In contrast, there were no detectable transconjugants from the iceB1 cone mutants (Fig. 2, rows b to d).

Cometant with previous results indicating that cone is not involved in ICEB1 gene expression, excision, or circularization (6, 7, 12, 38, 39), we found that neither coneE(K476E) nor coneEΔ(88–808) mutant alleles had any detectable effect on these processes (data not shown).
FIG. 2. conE is required for mating of ICEBs1. Cells were grown in minimal glucose medium. Mating was performed 1 h after induction of *rapI* with 1 mM IPTG from the indicated donor cells into ICEBs1<sup>5</sup> recipient cells (CAL419). Percent mating is calculated as (number of transconjugant CFU per donor CFU) × 100%. Data are averages from at least two experiments. Error bars indicate one standard deviation. The asterisk indicates that no transconjugants were observed. Given our limit of detection, we estimate that the percent mating for these strains is <5 × 10<sup>−3</sup>%.

Complementation tests with conE. We used complementation tests to determine if the defect in mating caused by the conE<sup>(K476E)</sup> mutation was due to loss of ConE function and/or an unintended effect on some other gene. The defect in mating caused by the conE<sup>(K476E)</sup> mutation was complemented partially when wild-type conE was provided in the donor in trans under the control of the ICEBs1 promoter *pxs* (Fig. 2, row e). Measurements of mRNA levels using DNA microarrays indicated that the partial complementation is not due to unexpected defects in expression of other ICEBs1 genes or of *pxs-conE* (data not shown).

The partial complementation of the conE<sup>(K476E)</sup> mutation is probably due, in part, to inefficient translation of wild-type ConE expressed from P<sub>pxs-conE</sub>, *yddD*, the gene immediately upstream of conE, is predicted to overlap with the first 37 codons of conE, and thus, the two are likely to be translationally coupled. Complementation of the conE<sup>(K476E)</sup> mutant was significantly higher when *yddD* and conE were expressed together (*pxs-yddD conE*) than when conE was expressed alone (*pxs-conE*) (Fig. 2, rows e and f). Neither expression of *yddD* alone nor expression of *yddD* and conE<sup>(K476E)</sup> together improved the efficiency of transfer of the ICEBs1 conE mutant (Fig. 2, rows g and h). conE<sup>(K476E)</sup> was complemented fully if an additional copy of ICEBs1 was placed at the ectopic locus *thc* (Fig. 2, row i). These results and results from additional mating experiments with conE expressed in recipients indicate that conE function is needed in the donor and not the recipient (data not shown). Based on these findings, we suspect that ConE is not efficiently translated and assembled into an active complex when expressed in trans to YddD and other ICEBs1 proteins.

Taken together, our results indicate that ConE and its ATPase domain are required in the donor for mating of ICEBs1 but are not required for induction of ICEBs1, excision, circularization, nicking, or integration. Based on these results and the homology of ConE to VirB4-like conjugative ATPases, the simplest interpretation is that ConE is a component of the ICEBs1 conjugation machinery and that ATP binding and hydrolysis are required for ConE function in ICEBs1 DNA transfer.

ConE-GFP localizes to the cell poles, in close association with the membrane. We found that ConE is located predominantly at the cell poles, in close association with the membrane. We visualized the subcellular location of ConE in live cells by visualizing a fusion of GFP to the C terminus of ConE. conE-gfp was expressed from its presumed native promoter (*pxs*), together with *yddD*, at the heterologous locus (*lacA*) outside of ICEBs1. This fusion was partially functional and did not interfere with transfer of conE<sup>+</sup> ICEBs1 (see Materials and Methods). Most experiments using ConE-GFP were done with strains that also contained a wild-type version of conE in ICEBs1.

We monitored ConE-GFP prior to and after induction of ICEBs1 gene expression. Little or no fluorescence was observed in cells in which ICEBs1 gene expression was not induced (data not shown). This was expected, since the *pxs* promoter driving expression of conE-gfp is not active without induction (6, 7, 12). When ICEBs1 gene expression was induced by overproduction of RapI, ConE-GFP was found predominantly at the cell poles in most cells (Fig. 3A). This is most evident with simultaneous visualization of ConE-GFP and the cell membrane stained with the dye FM4-64 (Fig. 3B). ConE-GFP appeared to form a “polar cap” along the entire pole near the membrane. ConE-GFP was most often found at both cell poles but was also commonly observed at only one pole. A lower level of fluorescence was also detected throughout the cell and sometimes along the lateral sides of the cells.

Positioning of ConE-GFP at the cell poles requires at least one other ICEBs1 gene. The polar positioning of ConE-GFP did not depend on the wild-type conE in ICEBs1. We visualized ConE-GFP in cells deleted for conE<sup>(conEΔ[88–808])</sup> at its native locus in ICEBs1 and found that its subcellular position was indistinguishable from that in cells expressing wild-type conE<sup>(Fig. 3C)</sup>. These results indicate that the positioning of ConE-GFP at the poles does not depend on expression of wild-type conE in ICEBs1. In addition, we fused conE<sup>Δ(88–808)</sup> to *gfp* and expressed this from *pxs* (along with *yddD*) as described above. The ConE<sup>Δ(88–808)</sup>–GFP fusion was found throughout the cytoplasm, in both the presence and absence of functional conE in ICEBs1 (Fig. 3D; also data not shown). These results indicate that ConE<sup>Δ(88–808)</sup>–GFP is not capable of localizing at the cell poles.

We found that positioning of ConE-GFP to the membrane and cell poles required at least one other ICEBs1 gene. In cells missing ICEBs1 entirely (ICEBs1<sup>5</sup>), ConE-GFP was dispersed throughout the cytoplasm (Fig. 3E). In these experiments, ConE-GFP was produced constitutively from P<sub>pxs</sub> in combination with *yddD* (*pxs-yddD conE-gfp*). These results indicate that proper positioning of ConE-GFP at the poles and near the membrane requires an ICEBs1 gene product and that YddD is not sufficient to recruit ConE-GFP to the membrane. Alternat-
membrane association of ConE, but we favor a model in which at least one of these ICEBs1 proteins interacts with ConE and targets it to the polar membrane.

**Positioning of ConE-GFP at the cell poles does not require a functional conE.** We found that positioning of ConE at the poles did not require that ConE be functional for mating. We fused the mating-deficient allele conE(k476e) to gfp and expressed this fusion from P\(_\text{xis}\) (along with yddD) as described above. Following induction of ICEBs1, ConE(K476E)-GFP was found at the cell poles near the membrane (Fig. 3F) similar to the location of wild-type ConE-GFP (Fig. 3A and B). This polar localization of ConE(K476E)-GFP did not depend on a functional copy of conE in ICEBs1 (data not shown). Since ConE(K476E) localized properly but did not support mating, these results indicate that positioning of ConE at the cell poles is not sufficient for its function in mating. Furthermore, assuming that the ConE(K476E) mutant protein is defective in nucleotide binding, as predicted, these results indicate that neither binding nor hydrolysis of ATP by ConE is required for its proper subcellular positioning.

**Following induction, ICEBs1 DNA is found more frequently at the cell poles.** We determined the subcellular location of ICEBs1 DNA in live cells and compared this with the location of nearby chromosomal DNA (Fig. 4). These comparisons were done in cells with ICEBs1 integrated into the genome at its normal attachment site at 47° and in cells in which ICEBs1 was induced to excise (through overproduction of RapI). We inserted an array of lac operators (lacO) in the right end of ICEBs1, adjacent to yddM (47°), or outside of ICEBs1, adjacent to ydeD, at 48° in the chromosome (Materials and Methods). We visualized the location of the lacO array using a fusion of Lac repressor to the cyan fluorescent protein (LacI-CFP). The position of LacI-CFP is indicative of the subcellular position of either double stranded ICEBs1 DNA or chromosomal DNA, depending on the location of the lacO array.

We examined cells growing slowly, when most cells were generally engaged in no more than one round of replication. Under these conditions, most cells contain one incompletely replicated chromosome and therefore contain one or two copies of each chromosomal region. Without induction, ICEBs1 DNA is integrated into the chromosome near 47° (7, 38). As expected, we found that most uninduced cells (88% of 1,535 cells) contained one or two foci of double-stranded ICEBs1 DNA (Fig. 4A). In cells with a single focus, the ICEBs1 DNA was generally located near midcell (Fig. 4A). Approximately 94% of these cells (of 246 cells with a single focus) had the focus in the middle 50% of cell length. Only 6% of cells (of 246) had the focus of ICEBs1 DNA in a polar quarter of the cell. These findings are consistent with expectations for this region of the chromosome based on previously published findings (11, 40, 50, 56).

In contrast to the position of ICEBs1 when integrated in the chromosome, significantly more cells had a focus of ICEBs1 DNA in a polar quarter after induction and excision. Overproduction of RapI causes efficient induction of ICEBs1 gene expression, excision from the chromosome, and formation of a double-stranded circle (7, 38, 39). Under these conditions, most cells (87% of 1,804 cells) contained one or two foci of double-stranded ICEBs1 DNA (Fig. 4B), similar to that in uninduced cells (Fig. 4A). However, following induction, 41%
Polar positioning of ICEBs1 following induction depends on excision. We found that excision of ICEBs1 from the chromosome was required for the increase in ICEBs1 foci that were in the polar quarters of the cell. We induced ICEBs1 gene expression in an xis-null mutant incapable of excision. ICEBs1 gene expression is induced normally in excision-defective mutants (J. Auchtung, C. A. Lee, and A. D. Grossman, unpublished results). After induction of ICEBs1 gene expression in the xis mutant, we found that only 13% of 276 cells with a single focus of ICEBs1 had the focus in a polar quarter (Fig. 4D). This is in contrast to the 41% of xis+ cells with ICEBs1 in a polar quarter (Fig. 4B). Thus, the change in position of ICEBs1 DNA upon induction likely requires its excision from the chromosome. This result is consistent with ICEBs1 DNA appearing at the poles either due to direct association with the conjugation machinery or due to its random positioning in the cell once it is released from the chromosome.

In contrast to the requirement for xis for the high frequency of ICEBs1 DNA found near the cell poles, xis was not required for polar positioning of ConE-GFP. Following induction of ICEBs1 carrying a xis deletion, ConE-GFP localization was indistinguishable from that of xis+ ICEBs1 (Fig. 3G). Together, these results indicate that excisionase is required for the change in position of ICEBs1 DNA upon induction and that polar positioning of ConE-GFP is most likely not due to association with ICEBs1 DNA at the poles.

The position of the replication machinery is altered following induction of ICEBs1. Excision of ICEBs1 generates an extrachromosomal circle, analogous to a circular plasmid. Previous work indicated that the subcellular position of replisome proteins was altered in cells containing a multicopy plasmid (55). We therefore wished to determine if excision of ICEBs1 caused altered subcellular positioning of the replisome. We visualized the location of one component of the replication machinery using a functional fusion of the Tau subunit of DNA polymerase to YFP (42). Components of the replisome (the complex of replication proteins associated with a replication fork) normally form discrete foci at regular positions (41, 43). During slow growth, when most cells are engaged in no more than one round of replication at a time, most cells have one focus or two closely spaced foci of the replisome located near midcell along the length of the rod-shaped cell (10, 41).

Consistent with previous results, we found that during slow growth, only a small fraction of cells with ICEBs1 integrated into the chromosome (uninduced) had a focus of Tau-YFP in a polar quarter. Of 250 cells with a single focus of Tau-YFP, only 4% had the focus in a polar quarter (Fig. 4E). In contrast, following excision of ICEBs1, induced by overproduction of RapI, the replication machinery was much more frequently observed in the polar quarters. Of 212 cells observed with a single focus of Tau-YFP, 32% had the focus in a polar quarter (Fig. 4F). We suspect that the replisome foci were associated with ICEBs1 DNA, although we have not been able to test this directly. Due to photobleaching, we were unable to capture high-quality micrographs of both Tau-YFP and ICEBs1 DNA (LacI-CFP) foci in the same cells to determine if the foci colocalize. Nonetheless, our data indicate that the subcellular position of at least one component of the replication machinery is altered following induction of ICEBs1. These results

![Image](https://example.com/image.png)
might indicate that ICEBs1 DNA is replicated autonomously after excision. We are currently investigating this possibility.

**DISCUSSION**

We found that ConE (formerly YddE) and its ATPase motifs are required for conjugation of the integrative and conjugative element ICEBs1 of *B. subtilis*. We found that a ConE-GFP fusion was positioned predominantly at the cell poles, apparently associated with the membrane, and that this positioning required at least one other ICEBs1 gene product. In addition, after excision from the chromosome, ICEBs1 DNA was found more frequently near the cell poles. Our results indicate that ConE is most likely part of the ICEBs1 conjugation machinery. If its subcellular location is indicative of where the protein functions, then mating of ICEBs1 from *B. subtilis* likely occurs from a donor cell pole. Attempts to test this by directly visualizing mating pairs have so far been unsuccessful.

**VirB4-like proteins.** ConE belongs to the VirB4 clade of the HerA/FtsK superfamily of ATPases (29). Characterized members of this clade are required for substrate secretion, form membrane-associated oligomers, and interact with several components of their cognate secretion machineries (16, 48). Analysis of virB4 Walker A box mutants indicates that ATP binding and/or hydrolysis is required for DNA transfer through the secretion machinery but not for association of VirB4 with itself or other machinery components (4, 57).

Results with the few VirB4 homologs from gram-positive organisms that have been studied indicate that these proteins likely operate analogously to *A. tumefaciens* VirB4. The VirB4-like TcpF protein of the *Clostridium perfringens* plasmid pCW3 is required for DNA transfer and localizes to the cell poles (9, 51). Another VirB4 homolog, Orf5pIP501, of the broad host-range plasmid pIP501, interacts with itself and several putative components of its cognate conjugation machinery (1).

**Subcellular location of conjugation proteins.** ConE-GFP appears associated with the cell membrane and predominantly at both cell poles, indicating that mating may occur at either end of a *B. subtilis* donor cell. Mating pairs of live *E. coli* cells have been observed using fluorescence microscopy (8, 36). Transfer of the conjugative plasmid R751 in *E. coli* can occur along any orientation between donors and recipients that are in direct contact, suggesting that the conjugative machinery of R751 may assemble along both the lateral and polar sides of the cell (36). This type of lateral and polar localization of the mating machinery has been directly observed for the R27 conjugative plasmid in *E. coli* (22, 25). R27’s VirB4-like TrhC and coupling protein TrhG were distributed at multiple sites along all sides of the cell.

In other systems, the mating machinery is seen at one or both cell poles. For example, the conjugative apparatus of the gram-positive *Clostridium perfringens* plasmid pCW3 likely localizes at both cell poles, as evidenced by immunofluorescence microscopy of the VirB4-like TcpF protein (51). Components of the gram-negative *Agrobacterium* pTi conjugative apparatus are typically located at a single cell pole (3, 5, 30, 33, 34).

For ConE, our results indicate that ATP-binding and hydrolysis are not required for targeting but that at least one other ICEBs1 gene is required. The R27 VirB4-like protein TrhC also does not require a functional ATPase domain for localization but requires 12 of the other 18 R27 transfer proteins (23). VirB4 also does not require a functional ATPase domain for localization but, unlike TrhC or ConE, is able to target itself independently of other conjugation proteins (30).

It is not yet known where other ICEBs1 conjugation proteins are positioned in the cell or how they interact. Recent studies indicate that the gram-positive conjugation apparatus may be as structurally complex as its gram-negative counterpart (1, 9, 51). Since many ICEBs1 genes are conserved between diverse conjugative elements found in a wide range of gram-positive bacteria, we suspect that an understanding of ICEBs1 will likely shed light on other conjugative systems as well.

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