Critical components of the conjugation machinery of the integrative and conjugative element ICEBs1 of *Bacillus subtilis*

Running title: Conjugation machinery of ICEBs1

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Conjugation, or mating, plays a profound role in bacterial evolution by spreading genes that allow bacteria to adapt to and colonize new niches. ICEBs1, an integrative and conjugative element of Bacillus subtilis, can transfer itself and mobilize resident plasmids. DNA transfer is mediated by a Type IV secretion system (T4SS). Characterized components of the ICEBs1 T4SS include the conserved VirB4-like ATPase ConE, a bifunctional cell wall hydrolase CwlT, and the presumed VirD4-like coupling protein ConQ. Previously, we showed that a fusion of ConE to green fluorescent protein (GFP) localizes to the membrane preferentially at the cell poles. One or more ICEBs1 proteins are required for ConE’s localization at the membrane, as ConE lacks predicted transmembrane segments and ConE-GFP was found dispersed throughout the cytoplasm in cells lacking ICEBs1. Here we analyzed five ICEBs1 genes to determine if they are required for DNA transfer and/or ConE-GFP localization. We found that conB, conC, conD, and conG, but not yddF, are required for both ICEBs1 transfer and plasmid mobilization. All four required genes encode predicted integral membrane proteins. conB, and to some extent conD, were required for localization of ConE-GFP to the membrane. Using an adenylate cyclase-based bacterial two-hybrid system, we found that ConE interacts with ConB. We propose a model in which the ICEBs1 conjugation machinery is composed of ConB, ConC, ConD, ConE, ConG, CwlT, ConQ, and possibly other ICEBs1 proteins, and that ConB interacts with ConE, helping to recruit and/or maintain ConE at the membrane.

Conjugation is a major form of horizontal gene transfer and has played a profound role in bacterial evolution by moving genes, including those involved in antibiotic resistance,
metabolism, symbiosis, and infectious disease. During conjugation, DNA is transferred from cell to cell through the conjugation machinery, a type of secretion system. Relatively little is known about the conjugation machinery of Gram-positive bacteria. Here we analyzed five genes of the integrative and conjugative element ICEBs1 of Bacillus subtilis. Our research identifies four new components of the ICEBs1 conjugation machinery (ConB, ConC, ConD and ConG), and shows an interaction between ConB and ConE that is required for ConE to associate with the cell membrane.

INTRODUCTION

Conjugation is a major form of horizontal gene transfer and plays a profound role in bacterial evolution and the acquisition of new traits (1-3). Conjugation can spread antibiotic resistance and disseminate genes involved in symbiosis, degradation of pollutants, metabolism, and pathogenesis. Conjugative elements encode specialized DNA translocation channels classified as Type IV secretion systems (T4SSs) (4-7). T4SSs are composed of many interacting proteins that span the envelope of the donor cell. In addition to transferring the conjugative DNA element, the conjugation machinery can also mobilize resident plasmids or other DNA elements that do not encode their own machinery.

There is a rich body of mechanistic and structural information on the T4SSs of Gram-negative bacteria (4, 7, 8). The Gram-negative T4SS is generally composed of 11 conserved mating-pair formation proteins (VirB1-VirB11, using the nomenclature of the Agrobacterium tumefaciens pTi plasmid) that form the DNA translocation channel along with a so-called “coupling protein” ATPase (VirD4) that delivers the relaxase-conjugative DNA nucleoprotein complex to the channel.
In comparison, much less is known about the “minimized” T4SSs of Gram-positive bacteria, which seem to be composed of a subset of the Gram-negative T4SS components (6-8).

Conjugative elements from Gram-positive organisms generally encode homologs to three T4SS proteins from Gram-negative bacteria: a VirD4-like coupling protein, a VirB1-like cell wall hydrolase, and a VirB4-like ATPase. In addition, elements from Gram-positive bacteria encode proteins with similar structures and/or predicted membrane topologies and sizes to VirB3, VirB6, and VirB8. Many Gram-positive elements encode additional proteins that might also form part of the machinery.

The T4SSs of Gram-positive bacteria feature several significant differences from those found in Gram-negative bacteria, which is not surprising given the differences in the cell envelopes and sequences of the component proteins (6, 7). For example, Gram-positive elements do not encode homologs of any components that comprise the Gram-negative outer membrane “core complex,” which plays crucial roles in T4SS assembly and gating. In addition, conjugative cell wall hydrolases are critical for conjugation in Gram-positive organisms (9-12), whereas they are generally dispensable for most Gram-negatives (13-15).

**ICE**}_{Bs1} is an integrative and conjugative element (ICE) found in the chromosome of *Bacillus subtilis* (reviewed in (16)). The genes needed for transfer of ICE}_{Bs1} are related to those from Tn916 and ICE}_{St1} and other conjugative elements. ICE}_{Bs1} contains approximately two dozen open reading frames, many of which have been previously characterized for their roles in regulation, DNA processing, DNA replication, and conjugation (Fig. 1A) (9, 17-28). ICE}_{Bs1} normally resides stably integrated in *trnS-leu2*, the gene for a leucine tRNA, unless its major operon is derepressed (20). Derepression, and subsequent excision and mating, are induced upon DNA damage or when cells are crowded by potential recipients that do not have ICE}_{Bs1}. Upon
induction, ICEBs1 can transfer itself and mobilize resident plasmids, such as pBS42, that lack dedicated mobilization functions (26). Although plasmid mobilization requires ICEBs1’s putative coupling protein ConQ, it does not require the ICEBs1 conjugative relaxase, ICEBs1 excision, or co-transfer with ICEBs1. Thus, ICEBs1 is required to build the conjugation machinery allowing for mobilization to occur.

By analogy to other conjugation systems, the ICEBs1 conjugation machinery is likely composed of several interacting proteins. So far, the putative coupling protein ConQ (26), a VirB1-like cell wall hydrolase CwlT (9) and a VirB4-like ATPase ConE (27) are characterized components (Fig. 1A). Previously, we found that a fusion of ConE to a monomeric green fluorescent protein (GFP) localizes to the periphery of the cell membrane, with a large concentration found at the cell poles (27). One or more ICEBs1 proteins appear to be required for ConE to associate with the membrane, as ConE lacks predicted transmembrane segments and is found dispersed throughout the cytoplasm in cells lacking ICEBs1.

We were interested in identifying other ICEBs1 genes needed for conjugation and determining which ICEBs1 proteins were needed for ConE to localize to the membrane. We constructed deletions in five ICEBs1 genes: conB, conC, conD, conG (previously yddB, yddC, yddD, yddG, respectively) and yddF (Fig. 1A). We then characterized the effects of the deletions on ICEBs1 transfer, plasmid mobilization, and localization of ConE-GFP. Together, our results are consistent with a model in which the ICEBs1 conjugation machinery is composed of putative transmembrane proteins ConB, ConC, ConD, ConG, along with the previously described ATPase ConE, cell wall hydrolase CwlT, and coupling protein ConQ. Furthermore, our results indicate that the peripheral membrane protein ConE is recruited to or kept at the membrane, at least in part, through direct interaction with ConB.
MATERIALS AND METHODS

Media and growth conditions

For *B. subtilis* and *Escherichia coli* strains, routine growth and strain constructions were done in LB medium. For all reported experiments with *B. subtilis*, cells were grown first in liquid LB at 37°C to an optical density (OD) 600nm of ~1.0. Cells were then diluted ~50-fold into LB or S7 defined minimal medium (29) with MOPS buffer at 50 mM rather than 100 mM, containing 1% glucose, 0.1% glutamate, and supplemented with auxotrophic requirements (40 μg/ml tryptophan; 40 μg/ml phenylalanine; 200 μg/ml threonine) as needed. Antibiotics were used at standard concentrations (30). For induction of ICE*Bs1*, 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added for 1-2 hours to overexpress *rapI* from *Pspank(hy)* in single copy on the chromosome at *amyE* (*amyE::[(Pspank(hy)-rapI) spc]*) as described previously (20).

Strains, alleles, and plasmids

*E. coli* strains used for routine cloning were NEB 5a (New England Biolabs), AG115 (MC1061 F' *lacI* *lacZ*::Tn5), and AG1111 (MC1061 F' *lacI* *lacZ* M15 Tn10). *B. subtilis* strains and relevant genotypes are listed in Table 1; all are derivatives of JH642 containing the *trpC2* and *pheA1* mutations (31). *B. subtilis* strains were constructed by natural transformation (30). Strains cured of ICE*Bs1* (ICE*Bs1*), the spontaneous streptomycin resistance allele (*str-84*), the *amyE::[Pspank(hy)-rapI] spc* allele, and Δ(*rapI phrI*)342::kan were described previously (20). The unmarked *conE(K476E)* allele, the complementation construct thrC::*[(Pxis-(conD conE-*lacZ*)) mls], and lacA::*[(Pxis-(conD conE-mgfpmut2) ter] allele expressing conD and conE-gfp from the ectopic locus lacA were described previously (27). The *conQ848* deletion was described...
previously (26). The thrC325::[(ICEBs1-311 (ΔattR::tet)) mls] allele, containing ICEBs1 inserted at thrC, is incapable of excision due to deletion of the right attachment site attR as described previously (25). The truncation alleles ICEBs1-319 (Fig. 1B) and ICEBs1-320 (Fig. 1C) at attB have been described previously (25). pBS42 uses rolling circle replication, expresses chloramphenicol resistance in B. subtilis, lacks mob/oriT, and can be mobilized by ICEBs1 (26).

All inserts in newly cloned plasmids were verified by sequencing.

(i) Construction of unmarked in-frame gene deletions

The basic strategy for constructing unmarked gene deletions was similar to that previously described for construction of nicK306 (25). All gene deletions are unmarked, in-frame, and keep the upstream and downstream genes intact. conBΔ(9-350) deletes codons 9 through 350 of conB, resulting in the fusion of codons 1 through 8 to codons 351 through 357. conCΔ(5-81) deletes codons 5 through 81 of conC, resulting in the fusion of codons 1 through 4 to the last codon, 82. conDΔ(5-131) deletes codons 5 through 131 of conD, resulting in the fusion of codons 1 through 4 to codons 132 through 174. yddFΔ(5-103) deletes codons 5 through 103 of yddF, resulting in the fusion of codons 1 through 4 to codons 104 through 108. conGΔ(5-805) deletes codons 5 through 805 of conG, resulting in the fusion of codons 1 through 4 to codons 806 through 815. The splice-overlap-extension (SOE) PCR method (32) was used to generate DNA fragments containing the alleles. These fragments were cloned into the chloramphenicol resistance vector pEX44 (33) upstream of lacZ. The resulting plasmids were pMMB1257 for conBΔ(9-350), pMMB1251 for conCΔ(5-81), pMMB1253 for conDΔ(5-131), pMMB1252 for yddFΔ(5-103), and pMMB1254 for conGΔ(5-805). To replace the wild-type allele with the deleted gene, each plasmid was first integrated onto the chromosome of strain JMA168 by single crossover. The resulting strain was grown without selection for at least 20
generations, and loss of the plasmid was screened for by loss of both lacZ and chloramphenicol resistance. Detection of the desired unmarked deletion was confirmed by PCR.

(ii) Construction of ICEBs1 single gene complementation alleles

Complemented genes were cloned downstream of the IPTG-inducible promoter Pspank(hy). The thrC::[(Pspank(hy)-conC) mls] allele was constructed to express conC. conC was cloned into pCAL838, downstream of Pspank(hy), creating plasmid pMMB1338. pCAL838 allows for double crossover at the thrC locus in B. subtilis, and contains Pspank(hy), lacI, and mls. pMMB1338 was transformed into B. subtilis to create the thrC::[(Pspank(hy)-conC) mls] allele. A similar strategy was used to produce thrC::[(Pspank(hy)-conB) mls] from plasmid pMMB1695, thrC::[(Pspank(hy)-conD) mls] from plasmid pMMB1339, thrC::[(Pspank(hy)-yddF) mls] from plasmid pMMB1340, and thrC::[(Pspank(hy)-conG) mls] from plasmid pMMB1341. For pMMB1695, an ATG start codon was used to replace the TTG native conB start codon, improving complementation (data not shown). For several genes tested, complementation was similar when genes were expressed from either Pspank(hy) or the ICE Bs1 promoter Pxis, but was inferior when expressed from Pspank (data not shown).

(iii) Construction of truncated ICEBs1 complementation alleles

Complementation of ConE-GFP localization in the ΔconB strain was tested using truncated ICEBs1 complementation constructs that cannot excise or transfer, similar to the strategy described previously (26). The truncated ICEBs1 derivative integrated at thrC, thrC1755::[ICEBs1 (ΔconC-attR::cat) mls], contains all of the ICEBs1 genes upstream of and including conB (Fig. 1D). The construct thrC1756::[ICEBs1 (ΔconB-attR::cat) mls] is essentially the same ICEBs1 insertion at thrC, but is missing conB (Fig. 1E). These alleles were constructed by transforming CAL1496, harboring thrC325::[(ICEBs1-311 (ΔattR::tet)) mls], with SOE PCR.
products that deleted the desired downstream region of ICEBs1, replacing the tetracycline
resistance gene with a chloramphenicol resistance gene.

(iv) Conversion of Δ(rapI phrI)342::kan to Δ(rapI phrI)::cat

The Δ(rapI phrI)342::kan allele (20) was altered to confer chloramphenicol resistance,
creating the Δ(rapI phrI)::cat allele. JMA168 was transformed with linearized pMMB1487,
selecting for resistance to chloramphenicol and screening for kanamycin sensitivity. pMMB1487
was constructed through isothermal assembly (34) so that it contains the cat gene flanked by
yddK/rapI and yddM/yddN.

(v) Construction of conE-gfp expressed from the conE locus in ICEBs1

Strains MMB1547-1550 have the conE gene fused in frame to monomeric gfpmut2
(mgfpmut2) at its native locus in ICEBs1. These strains were made by integrating pMMB1530
into the B. subtilis chromosome via a single crossover into conE. pMMB1530 contains the 3’ end
of conE fused to DNA sequence encoding a 23 amino acid linker and mgfpmut2. The plasmid
was introduced into B. subtilis by transformation and selection for kanamycin resistance.
pMMB1530 was constructed by ligating the XhoI and SphI-cut vector from pMMB1445 with the
XhoI and SphI-cut fragment encoding the 23 amino acid linker and mgfpmut2 from pLS31 (35).
pMMB1445 was constructed by inserting a PCR fragment containing the 3’ end of conE into
pKL168 (36) digested with EcoRI and XhoI.

(vi) Construction of Pspank(hy) conB conC conD conE-gfp at thrC

pMMB1702 was transformed into B. subtilis to create the thrC::[(Pspank(hy)-(conB conC
conD conE-mgfpmut2) mls] allele that expresses conB, conC, conD and conE-mgfpmut2.
pMMB1702 was constructed via isothermal assembly (34) of the PCR inserts (conB conC conD)
and $(conE-mgfpmut2)$ into pCAL838 downstream of Pspank(hy). An ATG start codon was used to replace the native $conB$ start codon TTG.

**Conclusions**

*(vii) Construction of bacterial two-hybrid protein fusions plasmids*

ICEBs1 genes were cloned in-frame into vectors carrying either the N-terminal (T25) or C-terminal (T18) portions of the *Bordetella pertussis cyaA* gene for adenylate cyclase as previously described (37). The plasmids encoding N- and C-terminal fusions of T18 to ConE were constructed by cloning into the BamHI/PstI sites of the vectors. pMMB1457 and pMMB1458 encode ampicillin resistance along with ConE-T18 and T18-ConE, respectively. Plasmids encoding N- and C-terminal fusions of T25 to ConB or ConD were constructed via isothermal assembly (34). pMMB1603, pMMB1604, pMMB1605, and pMMB1626 encode kanamycin resistance along with T25-ConD, ConB-T25, ConD-T25, and T25-ConB, respectively.

**Bacterial two hybrid interaction assays**

The bacterial two hybrid assays were performed similarly to prior reports (37). Two plasmids (one containing a T18-fusion and another containing a T25-fusion) were co-transformed into BTH101 [F-, cya-99, araD139, gal15, galK16, rpsL1 (StrR), hsdR2, mcrA1, mcrB1] *E. coli* competent cells. For negative controls, an empty vector was co-transformed with a T18/25-fusion protein or another empty vector. For qualitative analysis, the transformations were plated on MacConkey Base Agar supplemented with 0.1 % maltose, 0.1 mg/ml ampicillin, 0.05 mg/ml kanamycin, and 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The plates were incubated at 37°C overnight and then room temperature (∼20°C) for an additional 24 hours for visual inspection of colony color. For quantitative assays, cells were grown shaking in LB.
containing 0.1 mg/ml ampicillin, 0.05 mg/ml kanamycin and 0.5 mM IPTG at 30°C overnight for 14-16 hours. β-galactosidase activity was quantified as previously described (38). The reported results are averages of at least four independent experiments.

Mating assays
ICEBs1 mating was assayed as described previously (20). Briefly, donor cells contained a kanamycin resistance gene in ICEBs1. Recipient cells (strain CAL419) lacked ICEBs1 (ICEBs1<sup>−</sup>) and were distinguishable from donors by being streptomycin resistant (<i>str-84</i>). Recipients were comK null to prevent acquisition of DNA via transformation. Cells were grown at least four generations to mid-exponential phase (OD600 to ~0.35) in minimal medium. ICEBs1 was induced in donors by addition of IPTG (1 mM) to induce expression of <i>rapI</i> from Pspank<sup>(hy)</sup>. Donors and recipients were mixed 1:1 and filtered onto sterile cellulose nitrate membrane filters (0.2 μm pore size). Filters were placed in Petri dishes containing Spizizen’s minimal salts (30) with 1.5% agar and incubated at 37°C for 3 hours. Cells were washed off the filter and the number of transconjugants was measured by determining the number of kanamycin- and streptomycin-resistant colony forming units (CFUs) after the mating. The number of donors was measured by determining the number of kanamycin-resistant CFUs after the mating. Percent mating is the (number of transconjugant CFUs per donor CFUs) x 100%. The reported results are averages of at least three independent experiments.

Plasmid mobilization assays
Mobilization of plasmid pBS42 by ICEBs1 was assayed essentially as described (26), similar to the mating assay described above. In addition to containing ICEBs1, donor cells...
contained the plasmid pBS42 and were grown with chloramphenicol (2.5 µg/mL) and kanamycin
(2.5 µg/mL) to maintain selection of the plasmid and ICEBs1, respectively. The recipient strain
(CAL89) was ICEBs1<sup>0</sup>, streptomycin resistant (str-84) and comK null. Cells were grown at least
four generations to mid-exponential phase in LB medium before ICEBs1 was induced in donors
by addition of IPTG (1 mM) to induce expression of rapI from Pspank(hy). Mating was
performed on filters as described above. The number of pBS42 transconjugants was measured by
determining the number of chloramphenicol- and streptomycin-resistant CFUs. The number of
donors was measured by determining the number of chloramphenicol-resistant CFUs after the
mating. Plasmid mobilization efficiencies were calculated as the (number of pBS42
transconjugant CFUs per donor CFUs) x 100%. Transconjugants receiving ICEBs1 were also
monitored as described above for mating assays. The reported results are averages of at least
three independent experiments.

Live-cell fluorescence microscopy

Microscopy was performed as described (39). Cells were grown at least four generations
to mid-exponential phase in minimal medium. ICEBs1 was induced by addition of IPTG (1 mM)
to induce expression of rapI from Pspank(hy). Experiments using ConE-GFP were done with
strains that also contained a wild-type version of conE in ICEBs1, except where noted. Live cells
were immobilized on pads of 1% agarose containing Spizizen’s minimal salts and visualized at
room temperature. Some images were captured with a Nikon E800 microscope equipped with a
100x differential interference contrast objective, a Hamamatsu digital camera, and Chroma filter
set 41012 (for GFP). Improvision Openlabs 4.0 Software was used to process these images. The
remaining images were captured with a Nikon H550L microscope equipped with a 100x Plan
Fluor Phase Contrast Objective, a high resolution monochrome cooled CCD Andor digital camera, and Chroma filter set 96362 (for GFP). NIS Nikon Elements 4.0 Software was used to process these images. Each strain was examined in at least three independent experiments.

RESULTS

conB, conC, conD, and conG (but not yddF) are required for the conjugative transfer of ICEBs1

We analyzed five ICEBs1 genes (conB, conC, conD, yddF, and conG; Fig. 1A) as each had characteristics that suggested it might encode a component of the ICEBs1 T4SS. ConB is a putative bitopic membrane protein homologous to conjugation proteins that are structurally similar, but not phylogenetically related, to VirB8 (40, 41). ConC is a putative integral membrane protein with two predicted transmembrane helices (8). Although ConC homologs are not widespread, some conjugative elements in Gram-positive organisms encode proteins of similar size and predicted topology. ConD contains two transmembrane helices and resembles VirB3 in terms of size and predicted topology (7, 8). yddF encodes a putative DNA-binding protein that is found mainly in crenarchaeal viruses (42, 43). It was analyzed here given its proximity to other predicted ICEBs1 T4SS genes (Fig. 1A) and the modular nature of ICEs where genes of shared function are often linked (44, 45). Lastly, conG encodes a conserved putative polytopic membrane protein analogous to VirB6 and was previously shown to be involved in ICEBs1 conjugation (7).

We constructed in-frame unmarked deletions of conB, conC, conD, yddF, and conG in ICEBs1 to determine whether these genes were required for mating. We compared mating efficiencies of ICEBs1 from donor strains containing the various deletions into recipient B.
subtilis cells lacking the conjugative element. The donor ICEBs1 contained a kanamycin resistance marker that had been inserted to allow selection and monitoring of ICEBs1 acquisition (20). We found that an ICEBs1+ donor strain transferred with an average mating frequency of ~3% (percent transconjugant CFU per donor CFU; Fig. 2, row a), as seen previously (20).

Conjugation was undetectable from ΔconB, ΔconC, ΔconD, or ΔconG donor strains (Fig. 2, rows b, d, f, i). Given our limit of detection, we estimate that mating is down at least 300,000-fold for each. The ΔyddF donor strain mated similarly to wild type B. subtilis cells (Fig. 2, row h).

The defect in mating caused by each gene deletion was complemented at least partially when the appropriate gene was reinserted at thrC, outside of ICEBs1 (Fig. 2). Only the conG complementation construct restored mating to near wild type efficiency (Fig 2, row j). Mating was restored at least 500-fold for conB, 1000-fold for conC, and 5000-fold for conD (Fig 2, rows c, e, g). Attempts to improve complementation using alternative promoters to drive expression of the complemented gene were not successful (See Materials and Methods). The mating frequency of each strain was restored to near wild-type levels when a largely intact transfer-defective ICEBs1 was inserted elsewhere on the chromosome (data not shown), indicating that the in-frame deletions do not remove a cis-acting site required for conjugation. Partial complementation is commonly observed for conjugative systems (27, 46) and could be due to unexpected effects of the deletion on other ICEBs1 genes and/or insufficient expression of the complemented gene.

Based on prior work (27), we suspect that the conjugation proteins are not efficiently translated and/or assembled into an active complex when expressed in trans to other ICEBs1 proteins. Nevertheless, we conclude that conB, conC, conD, and conG are critical for the conjugative transfer of ICEBs1, while yddF appears to be dispensable under the conditions tested.
ICEBs1 genes required for the mobilization of plasmid pBS42

We predicted that conB, conC, conD, and conG are required for the conjugative transfer of ICEBs1 because they encode critical components of the ICEBs1 DNA translocation channel. Alternatively, these genes could be important for other aspects of ICEBs1 biology. For example, they could be critical for excision, replication or integration of ICEBs1. To distinguish these possibilities, we tested whether these genes were required for plasmid mobilization. Mobilization would require the ICEBs1 conjugation machinery, but would not require genes involved in ICEBs1 DNA processing events (26).

We found that conB, conC, conD, conE, and conG were required for the ICEBs1-mediated mobilization of plasmid pBS42 (Fig. 3). The same mating procedure was used as above, except that the ICEBs1+ donor strains also contained pBS42, a plasmid that confers chloramphenicol resistance allowing measurement of its acquisition. As seen previously (26), a wild type donor strain (ICEBs1+) transferred pBS42 with an average mobilization frequency of ~2% (Fig. 3, row a). Mobilization of pBS42 was not detectable from donor strains containing deletions in conB, conC, conD, or conG or a donor strain containing a missense mutation (K476E) in the ATP-binding motif (Walker A box) of ConE (Fig. 3, rows b, d, f, h, l). We estimate that plasmid mobilization is down at least 10,000-fold for each of these mutants. The ΔyddF donor strain mobilized pBS42 at a frequency of 0.07%, ~30-fold lower than wild type (Fig. 3, row j), indicating that yddF contributes, at least modestly, to efficient pBS42 transfer. As observed for the mating assays, mobilization was partially complemented when the genes were expressed in trans (Fig. 3, rows c, e, g, i, k, m). Taken together with their predicted membrane locations and sequence conservation, these results indicate that ConB, ConC, ConD, and ConG are likely components of the ICEBs1 DNA translocation channel since they are required for both...
conjugation of ICEBs1 and plasmid mobilization. YddF does not seem to be a critical component of the channel since it is not conserved in other conjugative elements, was not required for ICEBs1 transfer, and made only a minor contribution to plasmid mobilization.

*conB,* and to some extent *conD,* are required for ConE-GFP localization at the cell membrane

ConE-GFP localizes to the membrane, predominantly at the cell poles, when ICEBs1 gene expression is induced (27). In contrast, ConE-GFP mislocalizes to the cytoplasm in cells lacking ICEBs1. These results indicate that at least one ICEBs1 gene product may recruit and/or retain ConE at the membrane. The four ICEBs1 proteins shown above to be required for both mating and mobilization (ConB, ConC, ConD and ConG) all contain at least one predicted transmembrane helix (Fig. 1A). To test whether any of these proteins might be required for ConE membrane localization, we examined the subcellular localization of ConE-GFP in strains containing large deletions of ICEBs1. We used a construct in which *conE-gfp* is expressed from the ICEBs1 promoter Pxis, together with the upstream gene *conD,* at a heterologous locus (*lacA*) as previously described (27). *conD* was included upstream of *conE-gfp* as ConE-GFP is not detectable in cells in the absence of *conD* in this context (data not shown). We observed ConE-GFP at the membrane preferentially at the cell poles in cells containing an intact ICEBs1 (Fig. 4a), as seen previously (27). Furthermore, we found that ConE-GFP localized properly in a strain that contained a large deletion starting midway through *conG* through to *yddM* (Fig. 1B; Fig. 4b). Thus, ConE localization does not require the C-terminus of ConG, nor the seven ICEBs1 proteins encoded downstream (CwlT, YdlI, YddJ, YddK, RapI, PhrI, and YddM). In contrast, we found that ConE-GFP mislocalized to the cytoplasm in a strain containing a larger deletion spanning...
from conB to yddM (Fig. 1C; Fig. 4c). Previous experiments demonstrated that ConE-GFP expressed from lacA does not require wild type conE in ICEBs1 for proper localization (27). Therefore, we conclude that ConB, ConC, ConD, YddF, and/or a part of ConG are required for localization of ConE-GFP.

To narrow down the requirements, we visualized ConE-GFP in strains containing single in-frame unmarked gene deletions. We found that ConE-GFP was dispersed throughout the cytoplasm in ΔconB cells (Fig. 4d). Localization was restored in ΔconB cells with addition of an ICEBs1 at an ectopic locus that contained the genes up to and including conB (Fig. 1D; data not shown), but localization was not restored with addition of an ICEBs1 that contained the genes up to but not including conB (Fig. 1E; data not shown), indicating that the defect in localization of ConE-GFP in the absence of conB was not due to polarity on downstream genes. ConE-GFP targeted normally to the membrane in strains containing single gene deletions of conC, yddF, or conG (Fig. 4e, g, h). These results indicate that proper localization of ConE requires conB, but not conC or any of the genes from conE-yddM.

Since expression of ConE-GFP at lacA required the presence of the upstream gene conD, we were unable to use this construct to test whether conD is required for ConE-GFP localization. Therefore, we fused gfp downstream of conE in its native position in ICEBs1. To confirm that this new construct recapitulates ConE-GFP localization, we first examined ConE-GFP in this strain and in strains deleted for conB or conC. As expected, ConE-GFP localized properly in the parental and ΔconC strains, but mislocalized in ΔconB cells (data not shown). We next examined ConE-GFP in ΔconD cells. We observed a partial defect in localization in the ΔconD strain (Fig. 4f). ConE-GFP localized to the membrane preferentially at the cell poles in most cells, however, a large proportion of ConE-GFP was also found dispersed throughout the cytoplasm.
Localization of ConE-GFP in a ΔconD strain was restored when conD was added back in trans (data not shown).

We also tested ConE-GFP localization in a ΔconQ strain, as ConQ is the only ICEBs1 transmembrane protein that had not been tested (Fig. 1A). We found that ConE-GFP localized normally in the absence of the presumed coupling protein ConQ (Fig. 4i). We conclude that ConB, and to some extent ConD, are required for recruiting and/or maintaining ConE at the membrane. Furthermore, our results show that conQ, conC, and the ten genes from conE to yddM (Fig. 1A) are not required for ConE-localization. Previously, we showed that xis, which encodes the excisionase, was also not required for ConE localization (27).

To determine whether ConB and ConD are sufficient for ConE’s localization, we placed conB, conC, conD, and conE-gfp at an ectopic locus under control of an inducible promoter. The genes were cloned in tandem, as arranged in ICEBs1, and placed on the chromosome in a strain lacking ICEBs1. We found that ConE-GFP largely mislocalized to the cytoplasm in these cells (Fig. 4j). Most cells contained several foci or clusters of GFP fluorescence, oftentimes localized near the membrane and cell poles. We hypothesize that the foci of ConE-GFP are formed due to formation of subsets of the conjugation machinery proteins. When ConE-GFP is expressed in the presence of ConD alone, it mislocalizes to the cytoplasm uniformly and does not form small foci (27), indicating that ConB and/or ConC might be involved in formation of the clusters.

Furthermore, the result indicates that while ConB and ConD are required for the localization of ConE to the membrane, they may not be sufficient. Ten ICEBs1 proteins encoded upstream of conB (excluding ConQ and Xis) have not been tested and may also play a role. The best candidates include uncharacterized ICEBs1 proteins such as YdzL, YdcO, YdcS, YdcT, and YddA, although none of these are predicted membrane proteins. Alternatively, ConB and ConD
may be sufficient, but they are not produced in the correct stoichiometry and/or targeted correctly when expressed from an ectopic locus. This second interpretation is consistent with our complementation data (Fig. 2 and 3) that indicate mating proteins do not function optimally when expressed in trans.

ConE and ConB interact in a bacterial two hybrid assay

Our results indicate that ConB, and to some extent ConD, are required for localization of ConE to the membrane. To test whether ConE directly interacts with either protein, we used a bacterial two hybrid assay that is based on the interaction between the T18 and T25 domains of the *Bordetella pertussis* enzyme adenylate cyclase (37). If the two domains are fused to interacting proteins, cAMP is produced, resulting in increased expression of a *lacZ* reporter gene. Because the enzyme is cytoplasmic and does not rely on interactions with DNA, it has been useful for detecting interactions between membrane proteins, including components of T4SSs (40, 47-49). As we were uncertain as to whether attachment of T18 or T25 at the N- or C-terminus of a protein would interfere with interaction or targeting, we made both types of fusions. Therefore, we constructed plasmids that encode ConE-T18 and T18-ConE, along with ConB-T25, T25-ConB, ConD-T25, and T25-ConD.

We co-transformed plasmids encoding T18 and T25 fusion proteins and assayed their interactions by measuring β-galactosidase activity. Negative controls were cells co-transformed with one plasmid encoding a fusion protein (e.g., ConE-T18) and one empty vector. Of the four predator-prey ConE-ConB combinations tested, three showed statistically higher levels of β-galactosidase activity (P-value <0.05) than the corresponding negative controls (~80 Miller units; Fig. 5). No statistically significant interactions were detected between ConE and ConD (Fig. 5).
These data indicate that ConE and ConB may directly interact in vivo.

DISCUSSION

We found that conB, conC, conD, and conG are required for conjugative transfer of ICEBs1 and mobilization of PBS42. Homologs of these genes are found in other conjugative elements in Gram-positive bacteria and encode putative integral membrane proteins. We propose a model in which the ICEBs1 T4SS is composed of ConB, ConC, ConD, ConG, along with the previously described ConE ATPase, presumed coupling protein ConQ, and cell wall hydrolase CwlT (Fig. 6). Similar models have recently been proposed for the conjugative plasmid pCW3 of Clostridium perfringens (10, 40, 46, 50, 51), the broad-host range conjugative plasmid pIP501(12, 41, 52), and pCF10 of Enterococcus faecalis (11, 53, 54), indicating that a general consensus is building as to the composition of the Gram-positive T4SS.

ConG and ConB may form a major portion of the ICEBs1 DNA translocation channel within the membrane. ConG is large (815 amino acids long) and the N-terminal half is predicted to have seven transmembrane segments (Fig. 6). ConG likely forms higher-order oligomers as seen for the Gram-positive homolog TcpH (47). ConB is shorter (354 amino acids long) and bitopic with two tandem NTF2-like domains outside the cell membrane (40, 41). The extracytoplasmic domains of homologs of ConB crystallize as trimers. Given ConE’s cytoplasmic location, we propose that ConE directly interacts with the short intracellular N-terminal tail of ConB (Fig. 6).

Recently, a low resolution 3 megadalton structure of a Gram-negative T4SS was determined using electron microscopy (55). Biochemical analysis of the complex indicates that most of the inner membrane components form very large oligomers within the T4SS. Notably,
VirB3 and VirB8 appear to be 12-mers, VirB6 a 24-mer, and VirB4 is associated with the T4SS as two separate hexameric rings on the cytoplasmic face of the complex. It will be interesting to determine whether the Gram-positive counterparts form a complex with similarly large oligomeric proportions.

In our model, the peripheral membrane protein ConE likely associates with the ICEBs1 T4SS through direct interaction with ConB. This interaction was supported by bacterial two hybrid data (Fig. 5) and the observation that ConE-GFP’s localization to the membrane depended upon conB (Fig. 4d). Two lines of evidence indicate a potential interaction between ConE and ConD. First, a large proportion of ConE-GFP mislocalizes to the cytoplasm in ΔconD cells (Fig. 4f). Second, conD and conE (and their homologs) are linked genetically. conD is encoded directly upstream of and translationally overlaps with conE in ICEBs1. In many organisms, the virB3-like gene is directly fused to the virB4 gene (8). Fusion of the T18 or T25 domains to ConE and ConD may have prevented their interaction in bacterial two hybrid assays. More studies are necessary to address whether ConE and ConD indeed directly interact.

VirB4 proteins like ConE localize to the membrane, but the precise localization patterns are specific to each conjugative element. For example, TcpF of pCW3 localizes to the cell poles (47) and VirB4 of the B. subtilis plasmid pLS20 localizes at a single pole and at several sites along the membrane (56). In contrast, both VirB4 of A. tumefaciens and TrhC of the E. coli R27 conjugative plasmid localize to several foci along the periphery of the cell membrane (57, 58). While ConE requires only two of the seven other known ICEBs1 T4SS proteins for its localization, TrhC requires 12 different R27 T4SS proteins (58). All 12 proteins likely do not interact directly with TrhC; rather, this group of proteins may form an ordered network of
interacting proteins whereby disruption of initializing components could result in incomplete assembly and mislocalization of TrhC.

The ICEBs1 protein YddF was not required for conjugation and is not conserved in other conjugative elements. While the function of YddF is still unclear, our results indicate that this putative DNA-binding protein may, in part, facilitate plasmid mobilization (Fig. 3). This effect could be indirect, such as yddF increasing pBS42 copy number. While YddF was dispensable for transfer of ICEBs1 under our conditions, YddF could be required when mating into other types of bacteria or under different conditions. Nevertheless, YddF is not a critical component of the ICEBs1 T4SS.

Together, our results provide a first model for the T4SS of ICEBs1 (Fig. 6). Future experiments will be required to verify the predicted topologies of the protein components and determine their protein-protein interactions and functions.

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25. Lee CA, Grossman AD. 2007. Identification of the origin of transfer (oriT) and DNA relaxase required for conjugation of the integrative and conjugative element ICEBs1 of


Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods 6:343-345.


**FIGURE LEGENDS**

**FIG 1.** Genetic structure of ICEBs1 and its derivatives. (A). Schematic of ICEBs1 integrated at the normal attachment site, attB. Each ORF (arrowed box oriented in the direction of its transcription) and attL and attR (black boxes) are indicated. Previously characterized genes that encode ICEBs1 conjugation machinery are shaded in black. Genes shown here to be required for conjugation are shaded in grey. The number of predicted transmembrane helices (TMH) for each protein is indicated below each gene. Predictions were obtained from Polyphobius, a HMM topology prediction program that uses homology information (59), guided by the bacterial subcellular location and secretion prediction program LocateP (60). Other topology programs yield similar but not identical predictions. Genes that encode proteins with homology or predicted structural similarity or membrane topology to *A. tumefaciens* pTi VirB/D T4SS components are designated below. An antibiotic resistance marker (*kan* or *cam*) is inserted in *rapI-phrI* in most strains, but is not shown. (B-E). Diagram of truncated ICEBs1 derivatives that were used to analyze ConE-GFP localization. (B-C) Derivatives of ICEBs1, ICEBs1-319 (*ΔconG-yddM*) and ICEBs1-320 (*ΔconB-yddM*), at attB have the genes indicated by the horizontal line from attL to the middle of conG (B) or conB (C). (D-E) Truncated ICEBs1 derivatives integrated at thrC, *thrC1755::ICEBs1 (ΔconC-attR)* and *thrC, thrC1756::ICEBs1 (ΔconB-attR)*, have the genes indicated by the horizontal line from attL and up to and including conB (D) or yddA (E).

**FIG 2.** conB, conC, conD, and conG are required for mating of ICEBs1. Cells were grown in minimal glucose medium. The indicated donor cells [all containing Δ(*rapI*phrI)342::*kan* in ICEBs1 and *amyE::(Pspank(hy)-rapI)*] were mated with ICEBs1<sup>0</sup> *comK::cat str-84* recipient cells (CAL419). Percent mating is the (number of transconjugant CFUs per donor CFU) x 100%. Data
are averages from at least three experiments. Error bars indicate the standard deviation. An asterisk (*) indicates no detectable transconjugants (<1 x10^{-5}%). Donor strains were: JMA168 (a), MMB1275 (b), MMB1735 (c), MMB1271 (d), MMB1390 (e), MMB1274 (f), MMB1397 (g), MMB1273 (h), MMB1283 (i), and MMB1393 (j).

**FIG 3.** *conB, conC, conD, conE, and conG* are required for ICEBs1-mediated mobilization of pBS42. Cells were grown in LB. The indicated donor cells [all containing pBS42 (*cam*), *Δ(raplphrJ)342::kan* in ICEBs1 and *amyE::(Pspank(hy)-rapf)*] were mated with ICEBs1*comK::spc str-84* recipient cells (CAL89). Percent mobilization is the (number of plasmid-bearing transconjugant CFUs per donor CFU) x 100%. Data are averages from at least three experiments. Error bars indicate the standard deviation. An asterisk (*) indicates no detectable plasmid-bearing transconjugants (<4 x10^{-4}%). Donor strains were: MMB1473 (a), MMB1474 (b), MMB1760 (c), MMB1476 (d), MMB1477 (e), MMB1478 (f), MMB1479 (g), MMB1480 (h), MMB1481(i), MMB1482 (j), MMB1483 (k), MMB1484 (l), and MMB1485 (m).

**FIG 4.** *conB*, and to some extent *conD*, are required for localization of ConE-GFP at the cell membrane. Cells were grown in minimal glucose medium. The indicated genotypes (see also Table 1) were analyzed by live fluorescence microscopy. ConE-GFP fluorescence is shown in green. (a) MMB968; (b) MMB1425; (c) MMB1426; (d) MMB1297; (e) MMB1293; (f) MMB1549; (g) MMB1343; (h) MMB1299; (i) MMB1247; (j) MMB1715. All cells were induced with 1 mM IPTG for one hour, except panel h for two hours. ConE-GFP targeted normally in a strain deleted for *conG*, but the targeting required IPTG induction for two hours instead of one hour. We observed no differences in mating, mobilization, and ConE-GFP localization for one hour.
versus two hour induction for wild-type strains.

**FIG 5.** ConE interacts with ConB *in vivo* in bacterial two-hybrid assays. Quantitative β-galactosidase assays were performed on strains with plasmids expressing T18 and T25 fusion proteins. Strains contained plasmids expressing either T18-ConE (T18-E) or ConE-T18(E-T18). Strains also contained a plasmid expressing ConB-T25(B-T25), T25-ConB(T25-B), ConD-T25(D-T25), or T25-ConD (T25-D). The average β-galactosidase activity and standard error of the mean are reported. The average β-galactosidase activity of the negative controls, expressing one fusion protein and one empty vector, is represented by the dotted line. A statistically significant P-value (<0.05), as determined by a one-tailed heteroscedastic Student’s t-test comparing the experimental value versus the appropriate negative control, is indicated by a *.

**FIG 6.** Model of localization and interactions of the ICE*Bs* T4SS components. Cytoplasmic membrane (CM) and cell wall (CW) are indicated. N-termini of proteins are indicated with an N. Topology predictions were obtained as described in Figure 1 legend, and have not been experimentally verified. The thick arrow indicates the interaction between ConE and ConB consistent with ConE-GFP localization data and bacterial two-hybrid studies. A dotted arrow indicates the possible interaction between ConE and ConD consistent with ConE-GFP localization data and their genetic linkage. No other interactions have been demonstrated. In the model, CwIT is drawn as a transmembrane protein and as a secreted protein since it was observed both cell-associated and in culture supernatants (9). The model does not depict oligomerization or interactions that have been demonstrated for other conjugative T4SSs.
Table 1. *B. subtilis* strains used

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a All strains are derived from JH642 (31) and contain pheA1 and trpC2.
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ICE Box 2 (ΔconB-attR)

ICE Box 3 (ΔconG-yddM)

ICE Box 4 (ΔconB-yddM)

ICE Box 5 (ΔconC-yddM)

ICE Box 6 (ΔconB-yddM)

ICE Box 7 (ΔconC-yddM)

ICE Box 8 (ΔconB-yddM)
a) wt  d) ΔconB  e) ΔconC  
f) ΔconD  g) ΔyddF  h) ΔconG  
  i) ΔconQ  
j) conB conC conD conE-gfp