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ABSTRACT  Horizontal gene transfer contributes to evolution and the acquisition of new traits. In bacteria, horizontal gene transfer is often mediated by conjugative genetic elements that transfer directly from cell to cell. Integrative and conjugative elements (ICEs; also known as conjugative transposons) are mobile genetic elements that reside within a host genome but can excise to form a circle and transfer by conjugation to recipient cells. ICEs contribute to the spread of genes involved in pathogenesis, symbiosis, metabolism, and antibiotic resistance. Despite its importance, little is known about the mechanisms of conjugation in Gram-positive bacteria or how quickly or frequently transconjugants become donors. We visualized the transfer of the integrative and conjugative element ICEBs1 from a Bacillus subtilis donor to recipient cells in real time using fluorescence microscopy. We found that transfer of DNA from a donor to a recipient appeared to occur at a cell pole or along the lateral cell surface of either cell. Most importantly, we found that when acquired by 1 cell in a chain, ICEBs1 spread rapidly from cell to cell within the chain by additional sequential conjugation events. This intrachain conjugation is inherently more efficient than conjugation that is due to chance encounters between individual cells. Many bacterial species, including pathogenic, commensal, symbiotic, and nitrogen-fixing organisms, harbor ICEs and grow in chains, often as parts of microbial communities. It is likely that efficient intrachain spreading is a general feature of conjugative DNA transfer and serves to amplify the number of cells that acquire conjugative mobile genetic elements.

IMPORTANCE  Conjugative elements contribute to horizontal gene transfer and the acquisition of new traits. They are largely responsible for spreading antibiotic resistance in bacterial communities. To study the cell biology of conjugation, we visualized conjugative DNA transfer between Bacillus subtilis cells in real time using fluorescence microscopy. In contrast to previous predictions that transfer would occur preferentially from the donor cell pole, we found that transfer of DNA from a donor to a recipient appeared to occur at a cell pole or along the lateral cell surface of either cell. Most importantly, we found that when acquired by 1 cell in a chain, the conjugative DNA spread rapidly from cell to cell within the chain through sequential conjugation events. Since many bacterial species grow naturally in chains, this intrachain transfer is likely a common mechanism for accelerating the spread of conjugative elements within microbial communities.

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Horizontal gene transfer is an important factor in evolution, enabling bacteria to acquire new characteristics (1–4). Conjugative plasmids and integrative and conjugative elements (ICEs) are found in many bacterial species and are key mediators of horizontal gene transfer (4–7). ICEs normally reside integrated in the host genome but can excise to form a double-stranded DNA circle. Some and perhaps most ICEs undergo autonomous plasmid-like replication after excision (8, 9). ICEs can mediate their transfer by conjugation to other cells, where they can then integrate into the recipient genome.

ICEBs1 (Fig. 1) is an ~20-kbp integrative and conjugative element found integrated in the 3’ end of a leucine-tRNA gene in several strains of Bacillus subtilis (10–12). ICEBs1 genes required for excision and mating are derepressed during the RecA-dependent SOS response following DNA damage or when the sensory protein RapI is expressed and active (11, 13, 14). Overproduction of RapI causes ICEBs1 to excise in >90% of cells in a population (11, 13, 15, 16), greatly facilitating the characterization of this mobile genetic element. ICEBs1 can transfer into various Bacillus and Listeria species (11) and perhaps other organisms as well.

Many microbes, including B. subtilis, grow in chains, often in communities of cells, e.g., biofilms (17). The presence of conjugative elements in cells can contribute to the formation of such communities, and conjugation in these communities has been observed (18–20). During conjugation, there are potential donors that harbor a mobile element and potential recipients (here simply referred to as donors and recipients, respectively). A recipient that receives a mobile element is called a transconjugant and has the potential to become a donor. Very little is known about the relative orientation of cells during conjugation or how quickly or frequently transconjugants become donors. Some conjugation proteins localize to the cell periphery, predominantly at the poles, leading to the suggestion that DNA transfer occurs predominantly from a donor pole (21–24). However, in the case of ICEBs1 from B. subtilis, our results indicate otherwise.
We visualized the transfer of ICEBs1 in living cells in real time using fluorescence microscopy. We found that transfer of ICEBs1 from a donor to a recipient appeared to occur at a cell pole or along the lateral cell surface of either cell, in contrast to previous predictions. Furthermore, transconjugants often became donors, and this was especially evident in cell chains. We found that when cells grow in chains, there is efficient and successive transfer to neighboring cells in a chain, likely accelerating the spread of conjugal elements in microbial communities.

RESULTS

lacO/LacI-GFP system to visualize conjugative transfer of ICEBs1. To monitor ICEBs1 DNA transfer, we engineered B. subtilis strains to distinguish donors from recipients and transconjugants (Fig. 2), using detection systems similar to those previously used to visualize conjugation (25, 26). Recipients did not contain ICEBs1 and had a relatively uniform green fluorescence (Fig. 2A) from expression of a green fluorescent protein (GFP) fused to the Escherichia coli Lac repressor (LacI-GFP). Donors had a relatively uniform red fluorescence from constitutively expressed mCherry (Fig. 2A). Donors also contained ICEBs1 with a lac operator array (lacO, to which the Lac repressor binds). When ICEBs1::lacO transfers to a recipient, LacI-GFP binds the lacO array and appears as a green focus in the transconjugant (Fig. 2B). We induced ICEBs1 gene expression, excision, and conjugation in donor cells by overproducing RapI. Donors and recipients were mixed and spotted onto agarose pads on a microscope slide. Images of cells were captured every 30 min for up to 3 h and then analyzed. Transconjugants were identified as cells with at least one green LacI-GFP focus (Fig. 2C and D). In 8 cases, it was difficult to determine the orientation of the donor. The orientations were determined from the relative positions of cells at the earliest time point, shortly after donors and recipients were mixed and placed on the microscope slide. This ~4:1 ratio corresponds to the approximate ratio of lateral to polar surface area of the rod-shaped bacilli, indicating that mating appears to occur randomly along the donor cell surface. These results contrast with previous predictions that conjugation would occur predominantly at a donor cell pole (23, 24), predictions that were based on observations that some conjugation proteins (including one ICEBs1 mating protein) appear concentrated at cell poles (21–24). Occasionally, we observed transfer from a donor that was internal in a chain of cells and flanked at the poles by other donors. That such a cell can serve as a donor is consistent with the conclusion that transfer need not occur at a donor pole.

Rapid and efficient transfer of ICEBs1 in cell chains. Like many bacteria, B. subtilis often grows in chains (≥4 connected cells). Each cell in a chain is distinct and surrounded by a membrane and cell wall, but the cells remain connected by the polar cell wall. We observed rapid spread of ICEBs1 to many cells in a chain when an initial transconjugant was part of the chain. Among 53 cases in which a single cell in a chain initially received ICEBs1 from a donor, 43 (81%) of the transconjugants became donors and

![Diagram](https://example.com/diagram.png)

**FIG 1** Map of ICEBs1 and the constructs used. (A) ICEBs1 is ~20 kb and inserted in the 3' end of trnS-leu2. Large arrows indicate open reading frames and orientation. The shaded boxes with small arrowheads underneath at the ends of ICEBs1 represent the 60-bp direct repeats. Vertical lines with arrows between immR and xis represent the two promoters (PimmR, Pxis) that are controlled by the transcriptional repressor/activator ImmR. (B) Insertion of the lacO array and kan and concomitant removal of part of rapl through yddM. (C) Boundaries of the conG deletion. (D) Insertion of sspB and kan and deletion of rapI-phrI.
transmitted ICE* to neighboring cells in the chain, often within 30 min (Fig. 3A to E). It appeared that ICE* spread to cells preexisting in the chain before the initial transconjugant divided. In addition, the number of cells in a chain that acquired ICE* was greater than 2^n (the number expected from “n” cell divisions), indicating spread by a mechanism other than growth and division of the initial transconjugant. Two types of experiments, described below, verified that spreading through the chains was due to conjugation and not due to replication and segregation of the plasmid form of ICE* during cell division, or some unforeseen property of LacI-GFP bound to ICE*::lacO.

Efficient transfer of ICE* in cell chains depends on conjugation. We found that efficient spreading of ICE* in cell chains was dependent on conjugation. Null mutations in conG (yddG) of ICE* prevent mating (C. T. Leonetti, M. A. Hamada, S. J. Lauer, A. D. Grossman, and M. B. Berkmen, unpublished results). We used a donor carrying ICE*::lacO △conG and a functional copy of conG* elsewhere in the chromosome (see Materials and Methods), permitting the initial transfer of ICE*. However, transconjugants that receive ICE*::lacO △conG cannot retransfer the element because they lack conG. In 26 initial transconjugants that were each part of a chain, there was no detectable spreading of the ICE*::lacO △conG mutant to other cells in the chain, other than by cell division and segregation to daughters of the initial transconjugant (Fig. 3F to J). These results indicate that the rapid spreading of wild-type ICE* through cells in chains is due to conjugation.

Visualization of horizontal gene transfer using conditional protein degradation. To further confirm that the spreading of ICE* in chains was due to conjugation, we observed conjugation using a tracking system based on conditional protein degradation. Recipients expressed a fusion of GFP to a modified SsrA degradation tag (GFP-SsrA*). This fusion protein is rapidly degraded if cells produce SspB (29), a protein that delivers SsrA-tagged proteins to the cellular proteolytic machinery. Recipients did not produce SspB and were green (Fig. 3K).

sspB was inserted into ICE* (ICE*::sspB, without lacO) in a donor strain expressing mCherry (Fig. 3K to N). Transconjugants turn from green to dark due to the instability of GFP-SsrA* in the presence of SspB (29) expressed from newly transferred ICE*::sspB (Fig. 3K to N). When the initial transconjugant was in a chain of cells, the other cells in the chain (that were not contacting a red donor) also became dark (Fig. 3L to N), indicating the transfer of ICE*::sspB through the chain. These results also indicate that spreading was not due to growth and division, as once a transconjugant turns dark, all the progeny from division should initially be dark and should not start as green cells that subsequently turn dark. Based on these findings, we conclude that the initial transconjugants become donors and ICE* rapidly spreads through cells in chains via efficient conjugation.

DISCUSSION
We used two different methods to visualize conjugal DNA transfer between donor and recipient cells. In one case, we visual-
ized the DNA that was transferred from cell to cell. In the second, we used conditional protein degradation to identify cells that acquired the horizontally transferred element. We found that successful conjugation of the integrative and conjugative element of *B. subtilis*, ICEBs1, occurred with no obvious orientation of the donor and recipient. That is, transfer of DNA from a donor into a recipient appeared to occur at a cell pole or along the lateral cell surface. Furthermore, when acquired by a cell in a chain of cells, ICEBs1 spread rapidly to other cells in the chain through sequential transfer events as transconjugants quickly became donors.

Integration and stable maintenance of ICEBs1 in the host chromosome requires repression of ICEBs1 gene expression from the...
rightward promoter Pxis (Fig. 1). Derepression of Pxis leads to expression of genes needed for ICEBs1 excision and conjugation (11, 15). The excised circular form of ICEBs1 is required for its dissemination to recipients. Our results indicate that soon after receiving ICEBs1, a very high percentage of transconjugants become donors by expressing conjugation genes. The ability of a transconjugant to become a donor is likely influenced by the kinetics of repression of Pxis, which in turn is influenced by the kinetics of accumulation of the ICEBs1 repressor ImmR. ImmR both activates and represses its own expression, creating a homeostatic autoregulatory loop (15). Initially, there is no ImmR in a newly formed transconjugant, permitting transcription from Pxis and expression of ICEBs1 conjugation genes. However, in the absence of an inducing signal, expression and accumulation of ImmR in the transconjugant will eventually repress Pxis, allowing integration of ICEBs1 into the chromosome. This type of regulatory circuit is common in mobile genetic elements, notably in bacteriophages (30, 31), and is important in fate determination for such elements. In ICEBs1, this circuit likely allows switching between an active dissemination mode (excision and gene expression) and a quiescent inactive mode (integration and repression). Our studies indicate that a delay in ICEBs1 integration and transcriptional repression in transconjugants contributes to the spread of ICEBs1 in cell populations.

Much is known about conjugation and conjugative elements of both Gram-negative and -positive bacteria (4, 32, 33). In most cases, transfer efficiencies of a few percent are considered high. Our results indicate that conjugation efficiencies in cell chains can be ≥50%. A different mechanism for efficient dispersal of a mobile element has been described for Streptomyces ICEs that can exist as stable plasmids. Plasmid spreading through Streptomyces mycelia depends on spreading proteins (Spd) and is independent of conjugation proteins (summarized in references 32 and 34). In contrast, transfer of ICEBs1 to cells in a chain requires the conjugation machinery and is not due to replication and segregation of the plasmid form of ICEBs1.

Many bacterial species, including pathogenic, commensal, symbiotic, and nitrogen-fixing organisms, grow in chains and harbor conjugative elements. In addition, microbial biofilms are often composed of long chains or aggregates of connected cells (17). It seems likely that efficient intrachain spreading is a general feature of conjugative DNA transfer and probably serves to rapidly amplify the number of cells that acquire conjugative mobile genetic elements. When cells are present in a chain, they are in intimate contact with other cells in a pole-to-pole configuration. The high efficiency of intrachain conjugation is likely due to close and stable cell-cell contact. The high concentration of conjugation proteins at donor cell poles (21–24) might also contribute to the efficient pole-to-pole transfer in cell chains.

**MATERIALS AND METHODS**

**Bacterial strains and alleles.** The _B. subtilis_ strains used are listed (Table 1). All are derivatives of JH642 and contain trpC and penA mutations (not indicated). Strains were constructed by standard procedures using natural transformation (35). Strains cured of (missing) ICEBs1 (11) are indicated as ICEBs1. Rapl was overproduced from the xylose-inducible promoter Psy1 from amyE::[(Psy1-rapl) spc] as described previously (23).

(i) ICEBs1::lacO/λlac-gfp. A deletion-insertion in ICEBs1 was made by inserting an array of ~120 Lac operators (lacO) (36) along with kat (kanamycin resistance), by double crossover, into the region of ICEBs1 from bp 879 (of 1176) at the 5’ end of rapl and leaving 156 bp (of 942 bp) at the 3’ end of ydm (Fig. 1). This allele, ICEBs1 [Δ(rapl-ydm)::lacO kan], is simply referred to as ICEBs1::lacO. The ICEBs1::lacO allele used here was present in donor strains in the absence of lac-gfp. The presence of LacI-GFP (or LacI) interfered with kanamycin resistance, probably by silencing expression of the adjacent kan gene.

The lacO array contained on a plasmid was previously integrated into ICEBs1 by single crossover (23). We found that the single-crossover array was not transferred to recipients during conjugation, necessitating the integration of a lacO array by double crossover. LacI-GFP was produced from thrC::[(P::gfp-lacI°, as described previously (37). This construct fuses lacI-GFP to a constitutive promoter and is integrated at thrC (making the cells threonine auxotrophs). Strains containing this fusion without a lacO array have relatively uniform green fluorescence. The presence of a lacO array in a cell with LacIGFP results in a green focus. Strains containing lacI-GFP were used as recipients in conjugation experiments with ICEBs1::lacO donors.

(ii) Construction and complementation of ΔconG. ΔconG (yddG) is an in-frame markerless deletion of codons 5 to 805 (of 815). It was constructed in a manner analogous to that constructed for _E. coli_ (88-808) (23). conG function was provided in trans by thrC::ICEBs1-311 [Δ(xyl-rapI::lacO kan)], an ICEBs1 inserted in thrC that is incapable of excision (27). The mating efficiency of this complemented mutant (strain AB101), determined by filter mating (23), is normal.

(iii) _Ppen-mCherry at cgeD_. We used two different constructs that expressed a version of _mCherry_ (38) that was codon optimized for _E. coli_ (provided by S. Sandler) from the constitutive promoter _Ppen_. _Ppen_ was obtained from upstream of _lacI_ from a plasmid derived from pSI-1 (35). Plasmid pMMB1010 contains _Ppen-mCherry_ with a linked _kan_ flanked by sequences from _cgeD_ in the pGEMcat (35) backbone. This was integrated by single crossover into _cgeD_ selecting for chloramphenicol resistance. This construct was used in donor strains AB86, AB101, and AB110 (Table 1).

We also used a _Ppen-mCherry_ fusion at _cgeD_ that is integrated by double crossover. This allele, _ΔcgeD1388::[(Ppen-mCherry) cat]_ is an insertion-deletion containing _Ppen-mCherry_ followed by _cat_ (chloramphenicol resistance) inserted between base pairs 160 and 490 of the 1,278-bp _gfpD_ open reading frame. The inserted genes are cooriented with _cgeCD_ in the _B. subtilis_ chromosome. _Ppen-mCherry_ was obtained from _B. subtilis_ strain MM1023 containing _gfpD::[(Ppen-mCherry) kan]_ as a double crossover from pMMB1010, as described above. _cat_ was obtained from pGEMcat (35). The _ΔcgeD1388::[(Ppen-mCherry) cat]_ was contained on plasmid pCAL1387 and was introduced into the _B. subtilis_ chromosome by transformation and double-crossover homologous recombination. This construct was used in donor strain CAL1391 (Table 1).
(iv) IECESI:1::[(Peps-spB) kan] and gfp-ssrA*. The allele (Δrpl-phr)1366c::[(Peps-spB) kan] [simply IECESI:1::(Peps-spB)] is an insertion-deletion, removing the region of IECESI1 from 100 bp upstream of the rpl open reading frame through the stop codon of phr (Fig. 1). E. coli spB fused to the constitutive promoter Peps is inserted in this region, followed by kan. The inserted genes are cooriented with downstream yhdM (Fig. 1). spSB with a ribosome-binding site was obtained from pKG1266 (29). kan was obtained from pGK67 (39). Δ(rpl-phr)1366c::[(Peps-spB) kan] was constructed as a linear PCR product and introduced into IECESI1 in the B. subtilis chromosome by transformation and homologous recombination.

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