Second-Generation Transfer Mediates Efficient Propagation of ICEBs1 in Biofilms

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ABSTRACT Horizontal gene transfer (HGT) by integrative and conjugative elements (ICEs) is an important mechanism in the spread of antibiotic resistance genes. However, little is known about the spatiotemporal dynamic of ICE propagation in bacterial biofilms, which are multicellular structures ubiquitous in natural and clinical environments. We report here that a high level of biofilm matrix production favors ICEBs1 acquisition. Also, using a fluorescently marked ICEBs1, we observed that conjugation appears restricted to clusters of bacteria in a close neighborhood in which a high level of ICEBs1 transfer occurs. These conjugative clusters are heterogeneously distributed in the biofilm, forming close to the air-biofilm interface. Importantly, we established that transconjugant cells are the main contributors to ICEBs1 propagation in biofilms. Our findings provide a novel spatiotemporal understanding of ICEs propagation in biofilms, which should have an important role in our understanding of horizontal gene transfer in relevant settings.

IMPORTANCE The transfer of mobile genetic elements between bacteria is the main cause of the spread of antibiotic resistance genes. While biofilms are the predominant bacterial lifestyle both in the environment and in clinical settings, their impact on the propagation of mobile genetic elements is still poorly understood. In this study, we examined the spatiotemporal propagation of the well-characterized ICEBs1. Using the Gram-positive Bacillus subtilis, we observed that the main actors of ICEBs1 propagation in biofilms are the newly formed transconjugants that allow rapid transfer of ICEBs1 to new recipients. Our study provides a better understanding of the spatiotemporal dynamic of conjugative transfer in biofilms.

KEYWORDS Bacillus subtilis, biofilm, ICEBs1, integrative and conjugative elements, horizontal gene transfer

H orizontal gene transfer (HGT) is a fundamental phenomenon that drives the adaptation and evolution of bacteria in their environment (1). Conjugation is a preeminent HGT mechanism (2) that mediates the transfer of genetic material from a donor to a recipient cell upon direct cellular contact (3, 4). Since they encode complete mating machinery, conjugative plasmids and integrative and conjugative elements (ICEs) are genetic elements capable of self-transfer. They often carry accessory genes involved in metabolism, antibiotic resistance, and/or pathogenicity, which confer the cells bearing them a selective advantage (5–7). Consequently, they represent important actors in the emergence of multidrug resistant bacteria (8).

ICEBs1 is a 20.5-kb ICE present in multiple strains of Bacillus subtilis, a low G+C Gram-positive bacterium also able to form robust biofilms (9, 10). Since ICEBs1 gene function and regulation are very well understood, it constitutes an excellent model to study the propagation of ICE in Gram-positive bacteria (11). Activation of ICEBs1 can result from the activation of the SOS response following DNA damage in a RecA-dependent fashion (9, 11, 12). ICEBs1 activation is also mediated by RapI, a protein from the tetratricopeptide-repeat family encoded on ICEBs1 whose activity is inhibited by the small quorum-sensing peptide PhrI encoded downstream of rapI (9). Thus, the extracellular concentration of PhrI increases with...
the number of ICEBs1-containing cells in a population, ensuring that the transfer will not be activated if nearby cells already contain the element (9). Since PhrI is taken up prior to sensing, its propagation in space is limited, which leads to short-range communication (13).

Upon activation, ICEBs1 is excised from its integration site downstream of trnS-leu2, circularized, and then replicated by a rolling circle mechanism initiated at its origin of transfer (oriT) by the relaxase NicK (14). NicK is also involved in single-strand cleavage of the oriT to initiate the transfer via the type IV secretion system (T4SS) encoded by ICEBs1, and the single-strand DNA is translocated to the recipient bacteria (15, 16). ICEBs1 is then recircularized before the complementary strand is synthesized (17–19). The newly completed ICEBs1 then integrates the chromosome via the attB site in the recipient genome. Importantly, transconjugants can also be immediately involved in another transfer to neighboring bacteria (3). In addition to the RapI-PhrI signaling, two other mechanisms limit ICEBs1 transfer to cells already bearing a copy of the element (20). The repressor ImmR mediates an immunity analogous to phage immunity (12), while YddJ, also encoded on ICEBs1, mediates an exclusion mechanism by inhibiting the transfer from the ICEBs1 conjugation machinery (20).

Bacterial biofilms are microbial communities surrounded by a self-produced extracellular matrix (21, 22). Biofilms are ubiquitous in the environment and are involved in most chronic bacterial infections (23). Bacteria within these multicellular structures have an increased tolerance to antimicrobials, mainly due to the surrounding matrix, and this densely packed community provides rich intercellular interactions (22–25). Because of these characteristics, biofilms are favorable environments for HGT (4, 26, 27). Indeed, population-level analysis revealed that the transfer of conjugative plasmids was increased in various biofilms such as activated sludge communities, which are well-studied environmental biofilms (28–30). Examination of conjugative plasmid propagation in biofilms by using fluorescence microscopy and microfluidics revealed a low infiltration of the plasmids in an already established biofilm (29). In some cases, this poor efficacy was attributed to the low metabolic activity of recipient cells (31). However, other studies suggested that a low nutrient availability did not affect the transfer ability (26). While the capacity of conjugative plasmids to invade a biofilm appears limited, analysis of the conjugative plasmids RP4 and pKJK5 displayed efficient transfer in growing biofilms (28, 32). Of note, transconjugant cells were shown to have an important role in driving the transfer of RP4 in dual-species biofilm while having a minor impact in a complex activated sludge community (28). While these observations led to a better understanding of conjugation in biofilms, the spatiotemporal dynamics and factors impacting the conjugative transfer of ICEs in these multicellular communities have not yet been characterized (27).

We previously demonstrated that B. subtilis biofilm formation promotes the transfer of ICEBs1 and that the production of the biofilm matrix exopolysaccharide (EPS) and fibers (TasA) is required for this high efficiency (33). Here, we took advantage of the high propagation of ICEBs1 in B. subtilis biofilms to better understand the spatiotemporal parameters of conjugation in biofilms using fluorescence microscopy. We report that although biofilms are considered a favorable environment for conjugative transfer, ICEBs1 propagates in confined, relatively small areas that display a strong transfer level. We also observed that most conjugation events occur near the air-biofilm interface and that transconjugant bacteria are the key actors in the propagation of ICEBs1 in biofilms.

RESULTS

Bacteria-producing matrix components do not preferentially acquire ICEBs1. In a previous study, we observed that the production of biofilm matrix by recipient cells, but not by donor cells, drives the strong conjugative transfer of ICEBs1 in biofilm. However, matrix expression is heterogeneous in a biofilm, and thus, we wanted to examine if ICEBs1 conjugative transfer would increase in a scenario where all recipient cells expressed matrix. We performed a conjugation assay with recipient cells deleted for sinR, the transcriptional repressor of the operons encoding exopolysaccharide
biosynthesis (epsA-O), and protein fibers (tapA-sipW-tasA) of the matrix (34). Donor and recipient cells were mixed at a 1:5 ratio, and conjugation efficiency was examined after 20 h of incubation on a biofilm-inducing medium (MSgg). As seen in Fig. 1A, deletion of sinR, and thus overproduction of the extracellular matrix by all recipient cells, led to a significant increase (2-fold) in conjugative transfer. Deletion of one or both matrix operons in a ΔsinR background caused a decrease in transfer, suggesting that both matrix components are needed for a high transfer efficacy, and a high level of one component cannot palliate for the absence of the other.
To confirm the high ICEBs1 transfer to matrix-secreting recipient cells, we imaged conjugation and matrix production in parallel. The gene encoding the red fluorescent protein mKate2 was inserted in ICEBs1, which allowed us to follow its propagation by microscopy. We noticed that ICEBs1-mKate2 had a slightly diminished transfer level compared to ICEBs1 bearing the \textit{kan} resistance gene used previously, but it still displayed robust conjugation (see Fig. S1 in the supplemental material). To visualize recipient bacteria, we integrated at a genomic locus the gene encoding cyan fluorescent protein (CFP). Finally, the transcriptional reporter P\textit{tapA}\textit{yfp}, which transcribes the \textit{yfp} gene when cells are producing at least one component of the biofilm matrix (24), was inserted in ICEBs1-mKate2 donors and \textit{cfp} recipient cells. After a 20-h mating period, we inverted biofilms on a coverslip to directly image conjugation and determine the proportion of recipients and transconjugants expressing matrix genes (Fig. 1B). Since the biofilm matrix has a strong positive effect on transfer, we expected the proportion of transconjugant cells to express P\textit{tapA}\textit{yfp} more often than recipient cells. However, we did not observe a significant difference in the proportion of transconjugants cells expressing P\textit{tapA}\textit{yfp} from the proportion observed for all recipient cells (Fig. 1C). This discrepancy might be due to the fact that biofilm formation can be inhibited in transconjugants following the acquisition of ICEBs1 via expression of DevI, as previously reported by Jones et al., thus limiting the expression of the P\textit{tapA}\textit{yfp} reporter in transconjugant cells (35).

**ICEBs1 transfer in biofilm occurs in clusters.** Intriguingly, microscopy observation revealed that conjugation events in biofilms appeared to be concentrated in particular regions. To further examine this phenomenon, we used the ICEBs1-mKate2 reporter to image conjugation in cells incubated on biofilm and non-biofilm-inducing medium after 12 h, 16 h, and 20 h. In these assays, genes encoding CFP or green fluorescent protein (GFP) were integrated at a genomic locus of donor and recipient bacteria, respectively. After 12 h of incubation, we observed that ICEBs1 transfer was initiated in a few donor bacteria scattered through the biofilm and which showed strong mKate2 fluorescence; of note, most donor cells showed low fluorescent signal from mKate2 (Fig. 2A). This discrepancy between the fluorescence of various donor cells is likely due to the activation of ICEBs1 and its subsequent replication, which would increase the number of mKate2 copies (36, 37). Four hours later, those initial donors had transferred ICEBs1 to neighboring cells, forming clusters of mKate2-positive (mKate2\textsuperscript{+}) cells (Fig. 2A). By 20 h, the clusters were enlarged, and almost all the bacteria included in the area were transconjugant cells, many of which also strongly expressed mKate2 (Fig. 2A). The propagation of ICEBs1 was restricted to these active conjugative clusters (defined as containing at least 5 transconjugants) formed by donor cells surrounded by transconjugants.

We previously showed that matings performed on a nonbiofilm medium have 100-fold fewer conjugation events than mating on a biofilm-inducing medium (33). Similar to the biofilm medium, at 12 h on a nonbiofilm medium, we observed few donor cells with a high level of mKate2 fluorescence and very few transconjugants. At 16 h and 20 h, ICEBs1 was also disseminated to neighboring cells, but the conjugative clusters appeared significantly smaller than those formed on the biofilm medium (Fig. 2A). Further analysis confirmed that at 20 h in biofilm conditions, the conjugative clusters were composed of an average of 95 cells, while in nonbiofilm conditions, they contained approximately 20 cells (Fig. 2B). Our results show that ICEBs1 transfer in biofilm and nonbiofilm conditions was confined into clusters, but these were significantly larger in biofilm conditions.

**ICEBs1 transfer in biofilm is heterogeneous.** Imaging of biofilm inverted on a microscope coverslip does not recapitulate the complexity of the biofilm structure, which is a very heterogeneous environment. Thus, the presence of conjugative clusters in a fully formed colony biofilm was examined using transversal imaging. Matings were performed by dropping a mix of donor and recipient cells, bearing the same fluorescent reporters as described in Fig. 2, at a 1:5 ratio on a solid biofilm-inducing medium. Biofilms were fixed and included in optimal cutting temperature (OCT) compound after
12 h, 16 h, 20 h, 24 h, and 28 h of incubation, and thin sections of the biofilms were prepared using a cryomicrotome to obtain slices containing a view of the entire depth of the biofilm. Slices were then mounted between a slide and the coverslip with an aqueous montage solution for imaging by confocal microscopy.

After 12 h of incubation, donor bacteria with strong mKate2 fluorescence were disseminated through the biofilm, but there were few transconjugants, similar to what was observed in inverted biofilms (Fig. 3A). After 16 h of incubation, small independent clusters of mKate2\(^+\) (magenta) cells composed of donors and transconjugants were visible (Fig. 3A and B). Longer biofilm incubation times led to larger clusters composed of transconjugant cells, suggesting a strong multiplication of transconjugants and/or a sustained transfer of ICEBs1-mKate2 (Fig. 3A and B). Of note, most of these clusters appeared to have a vertical expansion and to be heterogeneously distributed in the biofilm. Positional analysis was performed on the clusters by measuring the distance between the top of the cluster and the air interface (Fig. 3C, light blue) and between the bottom of the cluster and the medium interface (purple). The results clearly demonstrate that most clusters were found close to or directly at the air-biofilm interface of the biofilm, while the bottoms of the cluster were more randomly distributed, respectively, to the medium interface (Fig. 3C).

**Second-generation transfer mediates efficient ICEBs1 propagation inside the biofilm.** Using three-dimensional (3D) imaging of the conjugative cluster on inverted biofilm, we noticed that many transconjugant cells were not adjacent to donor cells (Video S1). This observation suggests that the transfer of ICEBs1 mediated by transconjugants could play an important role in ICEBs1 propagation in biofilms. To examine the importance of this second-generation transfer, we designed a conjugation assay in which only the donor cells, but not the transconjugants, could transfer ICEBs1. We first...
performed an in-frame markerless deletion of \textit{conG}, a gene present on ICE\textit{Bs1} which encodes an essential protein of its conjugative machinery (16). The \textit{conG} complementation was constructed in \textit{trans} under the control of the inducible promoter \textit{P}_{hyperspank}. As previously reported, deletion of \textit{conG} completely abrogated ICE\textit{Bs1} transfer, but the presence of \textit{conG} in \textit{trans} in both the donor and recipient cells restored conjugation to wild-type levels (Fig. 4A). Importantly, if the complementation was only present in the donor cells, ICE\textit{Bs1}\text{Δ}\textit{conG} was able to transfer from donor cells to recipient cells, but the newly formed transconjugants were unable to further propagate ICE\textit{Bs1}\text{Δ}\textit{conG}.

**FIG 3** Spatiotemporal analysis of ICE\textit{Bs1} propagation within a biofilm. Vertical thin section of biofilm composed of donor cells expressing ICE\textit{Bs1}-\textit{mkate2} with recipient cells expressing a \textit{gfp} gene at a 1:5 donor-to-recipient ratio. At the indicated time, biofilms were fixed prior to cryosectioning. (A) Wide view of the thin section representing approximately one-third of the biofilm diameter, starting from the edge (left) toward center (right). (B) Closeup of the conjugative clusters. Donors appear purple, recipients are shown in green, and transconjugants are in light pink to gray color; for each image, the air-biofilm interface is at the top, and the biofilm-agar interface is at the bottom. The scale bars indicate a size of 250 \(\mu\)m (A) and 20 \(\mu\)m (B). (C) Relative distance of the conjugative cluster from the air-biofilm (light green) or agar-biofilm (purple) interface. Relative distance is reported as the distance separating the conjugative cluster to the interface relative to the depth of the biofilm at the cluster’s location. The darker dotted lines represent the median, and lighter dotted lines represent the 25th and 75th percentiles. Results are a compilation of at least 19 conjugative clusters from at least 3 independent biofilms. Statistical analysis was done using a \(t\) test and shows that the bacterial cluster is closer to the air-biofilm interface than the agar-biofilm interface. (**, \(P < 0.01\); ***, \(P < 0.001\); ****, \(P < 0.0001\)).
in biofilm and nonbiofilm conditions when conG was complemented only in donor cells compared to when it was complemented in both donor and recipient cells (Fig. 4A). This result confirmed that transconjugant bacteria propagate ICEBs1 after its acquisition. Importantly, transfer by transconjugant cells represented about 99% of ICEBs1 transfer in biofilm conditions, while it represented only 43% of the transfer in nonbiofilm conditions (Fig. 4B). These results indicate that ICEBs1 propagation in biofilm is mediated by a rapid spread via transconjugant cells.

It was previously reported that ICEBs1 can be efficiently transmitted via transconjugants by spreading rapidly through bacterial chain cells (3). Since B. subtilis can adopt this morphology in biofilm (38), we investigated if our observation of the importance of second-generation transfer could be explained by propagation in cell chains. Thus, we used single and combinatorial deletion mutants of genes (lytABC, lytD, lytE, and lytF) which encode autolysins responsible for the peptidoglycan cleavage following cell division, allowing the cells to separate (39, 40). While these deletion mutants forced the formation of cell chains (Fig. S2A), they did not display an increase in ICEBs1 transfer in nonbiofilm conditions (Fig. 5A). Similarly, overexpression of the autolysin

![Graph](image-url)
locus lytABC in biofilm formation conditions did not impair the ability of ICEBs1 to transfer in biofilms (Fig. 5B) while reducing the presence of cell chains in biofilm conditions (Fig. S2B). From these results, we conclude that ICEBs1 transfer by transconjugant is key for its efficient propagation in biofilms but that this effect is not mediated by cell chains.

**DISCUSSION**

The molecular mechanisms underlying conjugation are well understood for many mobile genetic elements, but little is known about the spatiotemporal parameters of their propagation in more natural contexts (27). Here, we used the well-characterized ICEBs1 and its host, B. subtilis, to demonstrate that although propagation of ICEBs1 in biofilm is extremely efficient, its transfer is confined to clusters of cells where ICEBs1 replicates and rapidly spreads through transconjugant bacteria.

We recapitulated our previous observations that the production of matrix by recipient cells plays an important role in the propagation of ICEBs1 in biofilm. Using a deletion of sinR to force production of biofilm matrix, we uncoupled general phenotypic differentiation from epsA-O and tapA-sipW-tasA expression, further confirming the importance of the matrix components (Fig. 1A). However, microscopy observations revealed that transconjugants did not systematically express matrix genes, which might reflect the activity of the ICEBs1-encoded devl. Devl was recently shown to inhibit expression of genes related to sporulation and biofilm formation, including epsB and tasA. Following ICEBs1 acquisition and devl expression in transconjugant cells, Devl then inhibits biofilm formation and sporulation, thus providing a fitness advantage, allowing cells bearing ICEBs1 to grow more (35). Our current hypothesis is that matrix would be required only at the beginning of transfer to stabilize the contact between donor and recipient cells.

We observed that ICEBs1 forms conjugative clusters, which are mostly located close to the air-biofilm interface, showing a clear heterogeneity in vertical distribution (Fig. 3C). Many of these conjugative clusters also display an uneven appearance with a preference for vertical expansion, which could suggest a polar growth of the transconjugant cells, a polar transfer, or both (Fig. 3B). Similarly, propagation of the TOL plasmid pWWO in an already formed biofilm also appeared to be confined to the few upper

**FIG 5** Cell chains are not involved in the propagation of ICEBs1 in a biofilm. (A) WT donor cells were mated with recipient cells lacking different autolysins and incubated on a nonbiofilm (MSNc) medium. (B) WT donor cells were mated with recipient cells overexpressing lytABC under the control of an IPTG-inducible promoter and incubated on a biofilm-inducing (MSgg) medium with 50 μM IPTG. For panels A and B, cells were mixed at a 1:5 donor-to-recipient ratio and incubated for 20 h at 30°C. Statistical analysis was performed using an ANOVA for panel A and t tests for panel B; (ns, $P > 0.05$) and showed no difference. The results shown are representative of at least 3 independent experiments, and error bars represent the standard deviation (SD).
layers of cells at the liquid medium interface of the biofilm (29). A lack of nutrients and oxygen inside the biofilm was proposed as an explanation for the confined transfer of pWWO at the liquid medium interface since the conjugative transfer requires a high energy cost (22). In our mating assay on solid biofilm-inducing medium, nutrient availability does not appear to influence ICEBs1 conjugal transfer since the cells present in the upper part of a colony biofilm have access to the least nutrients. Indeed, sporulation occurs rapidly in this specific biofilm layer (24). However, the air-biofilm interface was also shown to be the site of early TasA expression and faster accumulation of the biofilm matrix (24), which could explain the preferential localization of clusters close to this interface prior to occurrence deeper in the biofilm.

We observed that, initially, in biofilm, only a small number of bacteria produce a bright mKate2 fluorescent signal resulting from ICEBs1 excision and its replication in plasmid form (Fig. 2). This low number is in agreement with our previous observation that ICEBs1 is excised in less than 0.1% of the donors in both biofilm and nonbiofilm conditions (33). These initial events might be driven by the local density of donor and recipient cells since a high density of donor cells will lead to a repressing extracellular concentration of PhrI (9, 13). A similar observation was reported for the conjugal plasmid RP4 whose propagation in a growing biofilm also appears to arise from distinct areas prior to the expansion through most of the biofilm (28). Since transconjugant clusters can expand to almost a hundred cells, repression by PhrI is unlikely to have an effect on second-generation transfer. We hypothesize that this phenomenon could be explained by the kinetic repression by PhrI, which needs to be synthesized, exported, and then imported back to inhibit RapI, a process that might be slower than the conjugal transfer of an already active ICEBs1 present in transconjugants.

ICEBs1 propagation mediated by transconjugant cells was previously shown, but its importance for overall conjugative transfer was not evaluated (3). Our approach allowed us to precisely determine that the transfer initiated by transconjugant cells represents more than 99% of the total conjugative events observed in a biofilm (Fig. 4B). Because of the significant fitness advantage provided by ICEBs1 in biofilm, one could hypothesize that transconjugant cells would be abundant in the biofilm despite absence of second-generation transfer (35). However, our results confirm that second-generation transfer, and not the inherent growth advantages, explains the large number of transconjugant cells in biofilm. Additionally, even if ICEBs1 can rapidly spread through bacterial cell chains (3), this ability does not appear to contribute significantly to conjugation in biofilms (Fig. 5). Of note, a complementation of ICEBs1-encoded ConG in donor cells was previously shown to restore completely the conjugation efficiency in filter mating (16), suggesting that the importance of second-generation transfer under nonbiofilm conditions might be condition dependent.

The high transfer efficiency by transconjugants might be explained by the initial absence of the negative regulator ImmR, upon transfer to a new cell (41). Following translocation, ICEBs1, in its plasmid form, will be able to maintain its activated state, replicating and transferring efficiently, as proposed in another study (3). A high replication before the conjugative transfer was also observed for ICEclc, an ICE present in Pseudomonas putida that induces a transfer-competent (tc) differentiation in donor bacteria after activation of ICEclc genes (42). Activation of these genes led to a transient replication step prior to the conjugative transfer (43). Thus, bacteria in which ICEclc replicates are most likely to propagate the element. While ICEBs1 replication in the donor bacteria was shown to be unnecessary for the conjugative transfer of this element (44), a high copy number of ICEBs1 in transconjugants could favor the high secondary transfer while also supporting integration in the genome (19, 44). Our current model is that the strong ICEBs1 transfer in biofilm results from the combination of high ICEBs1 activity in newly formed transconjugant cells with the stabilizing effect of the extracellular matrix on cell-to-cell contacts between transconjugants and recipient cells.

While ICEBs1 propagation is driven by transconjugants, it is not the case for most conjugal elements. Previous reports showed complete recovery of the conjugal
levels of multiple elements upon deletion of a protein from their T4SS machinery and complementation only within the donor cells (45–47). These elements are members of multiple incompatibility groups present in both Gram-positive and Gram-negative bacteria, which indicates that a high level of second-generation transfer might be key for a certain subset of conjugative elements. However, these complementation assays were done in nonbiofilm conditions; since our study demonstrates that the epidemic transfer is important, particularly in biofilm, more investigation might reveal if it is a biofilm-specific feature of conjugation or not.

Our study provides new insights into the spatiotemporal dynamic of conjugative element propagation in biofilms. Since biofilms are ubiquitous in clinical environments where multiresistant bacteria can emerge, a better understanding of ICE propagation under these conditions is required to develop efficient strategies to prevent or decrease horizontal gene transfer. Further studies on the epidemic transfer of ICEs to better understand their real impact in natural environments are warranted.

MATERIALS AND METHODS

Strains and media. The strains used in this study were derived from the ancestor strain NCIB3610 (see Table S1 in the supplemental material). The bacterial growth media used is Luria-Bertani medium (LB; 1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, and 0.5% [wt/vol] NaCl), and the different media used for mating assays were MSNc (5 mM potassium phosphate buffer, pH 7.0, 0.1 M morpholinepropane-sulfonic acid [MOPS], pH 7, 2 mM MgCl₂, 50 μM MnCl₂, 1 μM ZnCl₂, 2 μM thiamine, 700 μM CaCl₂, 0.2% [wt/vol] NH₄Cl, and 0.5% [wt/vol] cellobiose) (48) and MSgg (5 mM potassium phosphate buffer, pH 7.0, 0.1 M MOPS, pH 7, 0.025 mM FeCl₃, 2 mM MgCl₂, 50 μM MnCl₂, 1 μM ZnCl₂, 2 μM thiamine, 700 μM CaCl₂, 0.5% [vol/vol] glycerol, and 0.5% [wt/vol] glutamate) (38) solidified with 1.5% [wt/vol] agar. When needed, the following antibiotics were added to the media: MLS (1 μg mL⁻¹ erythromycin and 25 μg mL⁻¹ lincomycin), spectinomycin (5 μg mL⁻¹), and kanamycin (10 μg mL⁻¹).

Strain and plasmid construction. Most B. subtilis strains were made by transferring genetic constructions into NCIB3610 using SPP1-mediated generalized transduction (49). JMA384 (ICEBs1::kan) was a gift from Alan D. Grossman’s lab (Massachusetts Institute of Technology, MA); BJM396 (lytC), BJM402 (lytD), BJM76 (lytE), and BJM104 (lytF) were gifts from David Rudner’s lab (Harvard Medical School, MA). pminiMAD2 (50) was a gift from Richard Losick’s lab (Harvard, MA). The Escherichia coli strain used for routine cloning was NEB Sx5 (New England Biolabs). The plasmids used in this article are listed in supplemental material (Table S2). tapA-sinR deletion was created by long-fragment homology PCR. The flanking regions were amplified using P746-P747 and P748-P749, respectively, and the erm was listed in supplemental material from pDG646. Plasmid constructions were done using pDR111 as the backbone plasmid unless indicated otherwise. Cells were then centrifuged for 3 min at 5,000 rpm, and the pellet was resuspended in 10 mM Na₂HPO₄ and 1.8 mM KH₂PO₄, pH 7.4) and mixed at a 1:5 donor-to-recipient ratio unless indicated otherwise. Cells were then centrifuged for 3 min at 5,000 rpm, and the pellet was resuspended in 1/10th of the volume. Ten microliters of the mix was dropped onto the appropriate medium and incubated for 20 h (unless time specified [Fig. 2 and 3]) at 30°C. When necessary, expression of the different constructs was induced by adding IPTG (isopropyl β-D-1-thiogalactopyranoside) to the medium. Fluorescent constructions were induced by adding 50 μM IPTG to both the liquid preculture and biofilm.
medium (Fig. 1, 3, and 4; Fig. S2; Video S1). Complementation of conG was done by adding 50 μM IPTG to the biofilm-inducing medium (Fig. S5).

To determine conjugation levels, colonies were collected from agar media with a bent pipette tip and put in 1 mL 1× PBS and then sonicated at 30% amplitude for 20 s twice with a 1:1 pulse. Cell suspensions were serially diluted and plated on LB with the appropriate antibiotic and allowed to grow until the next day. Donor, recipient, and transconjugant CFU were then counted. The frequency of transconjugants formation was expressed as a function of the number of recipients CFU (number of transconjugants divided by the number of recipient CFU) because transconjugants are an important actor in the propagation of ICEβ1 (Fig. 4).

Thin section. Preparation of the mating assay was done as previously described, but 2 μL instead of 10 μL of the mixture was dropped on MSgg medium with 1.5% (wt/vol) agar poured into 6-well plates. At the appropriate time, biofilms were fixed using paraformaldehyde 4% (wt/vol) for 10 min and washed twice with PBS. Samples of biofilm and agar were harvested from the plates and transferred into a homemade aluminum foil mold, overlaid with Tissue-Plus optimal cutting temperature (OCT) compound clear (catalog no. 4585; Thermo Fisher), and fast-frozen on dry ice. The frozen samples were stored at −80°C before being sent to Plateforme d’histologie de l’Université de Sherbrooke and sliced into 8-μm-thick slices using a cryomicrotome set to −20°C. Thin sections were placed on a positively charged slide (Globe Scientific Inc.; catalog no. no. 1358W), and an aqueous mounting medium was added (0.5% [wt/vol] N-propyl gallate, 50% [vol/vol] glycerol, and PBS) and covered with a number 1.5 coverslip. Slides were kept at 4°C prior to imaging. Thin sections were analyzed using an Olympus FV3000 confocal microscope at ×60 magnification using PlanApo objective (60×, 1.4 numerical aperture [NA]) with the following laser settings: 405 nm, 488 nm, and 561 nm. Laser intensity and sensor sensitivity were the same under all conditions. Fields of view are composed of multiple pictures taken sequentially to cover the area of the biofilm. To allow stitching of the picture together and form a large field of view, the overlapping region between 2 pictures needs to be imaged twice causing small bleaching of the area in the second picture. As the software used for the stitching calculated the mean of the overlapping region, some darker lines appeared at the border of two individual pictures (Fig. 3A). The distances separating the conjugative clusters with either the air-biofilm or biofilm-media interface were measured using the ImageJ software. The relative distance was calculated as the distance measure divided by the total thickness of the biofilm at the area of the picture taken.

Inverted microscopy on biofilm. Preparation of the mating assay was done as previously described, but 2 μL instead of 10 μL of the mixture was dropped on MSgg or MSNC medium with 1.5% (wt/vol) agar. At the appropriate time point, agar containing the biofilm was cut with a razor blade. Excess agar was removed carefully before inversion of the whole biofilm into a 35-mm petri dish containing a coverslip number 1.5 (part no. P35G-1.5-14C; MatTek Corporation). Pictures of the inner part of the biofilm into a 35-mm petri dish were taken sequentially to cover the area of the biofilm. To allow stitching of the picture together and form a large field of view, the overlapping region between 2 pictures needs to be imaged twice causing small bleaching of the area in the second picture. As the software used for the stitching calculated the mean of the overlapping region, some darker lines appeared at the border of two individual pictures (Fig. 3A). The distances separating the conjugative clusters with either the air-biofilm or biofilm-media interface were measured using the ImageJ software. The relative distance was calculated as the distance measure divided by the total thickness of the biofilm at the area of the picture taken.

Statistical analysis. Statistical analyses were performed using GraphPad Prism 9. Comparisons were done as specified in the figure legends.

SUPPLEMENTAL MATERIAL
Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 4.3 MB.
SUPPLEMENTAL FILE 2, AVI file, 0.2 MB.

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