



Biofilm Formation Drives Transfer of the Conjugative Element ICEBs1 in *Bacillus subtilis*

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ABSTRACT Horizontal gene transfer by integrative and conjugative elements (ICEs) is a very important mechanism for spreading antibiotic resistance in various bacterial species. In environmental and clinical settings, most bacteria form biofilms as a way to protect themselves against extracellular stress. However, much remains to be known about ICE transfer in biofilms. Using ICEBs1 from *Bacillus subtilis*, we show that the natural conjugation efficiency of this ICE is greatly affected by the ability of the donor and recipient to form a biofilm. ICEBs1 transfer considerably increases in biofilm, even at low donor/recipient ratios. Also, while there is a clear temporal correlation between biofilm formation and ICEBs1 transfer, biofilms do not alter the level of ICEBs1 excision in donor cells. Conjugative transfer appears to be favored by the biophysical context of biofilms. Indeed, extracellular matrix production, particularly from the recipient cells, is essential for biofilms to promote ICEBs1 transfer. Our study provides basic new knowledge on the high rate of conjugative transfer of ICEs in biofilms, a widely preponderant bacterial lifestyle in the environment, which could have a major impact on our understanding of horizontal gene transfer in natural and clinical environments.

IMPORTANCE Transfer of mobile genetic elements from one bacterium to another is the principal cause of the spread of antibiotic resistance. However, the dissemination of these elements in environmental contexts is poorly understood. In clinical and environmental settings, bacteria are often found living in multicellular communities encased in a matrix, a structure known as a biofilm. In this study, we examined how forming a biofilm influences the transmission of an integrative and conjugative element (ICE). Using the model Gram-positive bacterium *B. subtilis*, we observed that biofilm formation highly favors ICE transfer. This increase in conjugative transfer is due to the production of extracellular matrix, which creates an ideal biophysical context. Our study provides important insights into the role of the biofilm structure in driving conjugative transfer, which is of major importance since biofilm is a widely preponderant bacterial lifestyle for clinically relevant bacterial strains.

KEYWORDS *Bacillus subtilis*, ICEBs1, biofilms, extracellular matrix, horizontal gene transfer

Acquisition of genetic material via horizontal gene transfer (HGT) is a fundamental phenomenon for bacterial adaptation and evolution (1). Conjugation, which is regarded as the broadest and most efficient mechanism of HGT, allows bacteria to transfer genetic material such as conjugative plasmids and integrative and conjugative elements (ICEs) through direct cellular contact (2). These mobile genetic elements are autonomous since they encode their own mating apparatus. They often contain genes responsible for a wide range of functions, including virulence, antibiotic resistance, and symbiosis (3–5). Conjugative plasmids and ICEs can often transfer between different bacterial species and genera and mobilize genomic islands or plasmids that are

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otherwise not self-transmissible, granting these elements an extensive role in bacterial evolution (6–9).

ICEBs1 is a 20.5-kb ICE that is present in many strains of *Bacillus subtilis* (10, 11), a low-G+C Gram-positive bacterium that is well studied for its plant growth-promoting effect (12–14). While ICEBs1 can mobilize genetic elements lacking mobilization functions (8), whether it provides any advantage for its host cell remains unclear (15). ICEBs1 transmission is initiated in the donor cell by its excision from the 3' end of the chromosomal *trnS-leu2* gene (10). The resulting double-stranded circular intermediate undergoes rolling circle replication initiated at the origin of transfer (*oriT*) by the relaxase NicK, which cleaves the DNA strand to be transferred (16, 17). The nicked strand of ICEBs1 is then translocated into the recipient cells by an ICEBs1-encoded type IV secretion system (17, 18). In the recipient cell, the transferred strand is recircularized, and its complementary strand is synthesized. The circular copy of ICEBs1 then integrates at the 3' end of *trnS-leu2*, the *attB* site of the chromosome of the recipient (19).

Interestingly, two distinct cellular pathways regulate ICEBs1 excision. One is the global DNA damage response, which is mediated via the DNA repair protein RecA, which acts as an activator of conjugation. The other pathway is the ICEBs1-encoded quorum-sensing system RapI-PhrI, consisting of RapI, an inducer of ICEBs1 excision that can be inhibited by the coexpressed oligopeptide PhrI (10), which is secreted in the extracellular environment and imported back into the cell through a permease. In this pathway, ICEBs1 excision is repressed in a community where ICEBs1-harboring cells are widely present since the extracellular PhrI level is sufficient to inhibit RapI (10).

Biofilms are microbial communities surrounded by an extracellular matrix that protects bacterial cells from external stressors such as antibiotics and heavy metals (20, 21). In the environment and during chronic infections, most bacteria live within biofilms (22). *B. subtilis* biofilm matrix is mostly composed of exopolysaccharides and amyloid-like fibers, synthesis of which is encoded by the *epsA* to *-O* (*epsA-O*) operon and the *tapA-sipW-tasA* operon, respectively (23). Matrix production, and thus, biofilm formation, is triggered by a variety of environmental and physiological signals, including the lipopeptide surfactin, plant polysaccharides, and a combination of glycerol and manganese (24–26). In a planktonic population, the expression of the matrix production operons *epsA-O* and *tapA-sipW-tasA* is inhibited by the transcriptional repressor SinR (27).

Studies have suggested that biofilms are hot spots for the transfer of conjugative plasmids due to the high proximity of cells within this multicellular structure, but the importance of the extracellular matrix in this process is unexplored (28, 29). Also, many of the pathogens that have acquired antibiotic resistance through conjugative elements can form biofilms (30–32). However, despite their fundamental importance in antibiotic resistance gene dissemination, ICE propagation in biofilms has not yet been examined. Here, we take advantage of the extensive knowledge of ICEBs1 and *B. subtilis* biofilms to evaluate the dynamics of ICEBs1 dissemination within biofilms. Using medium that does or does not induce biofilm formation as support for conjugation, we report here that natural ICEBs1 transmission is 100- to 10,000-fold more efficient when cells are forming a biofilm, even when recipient cells outnumber donor cells. However, while biofilm formation increases conjugation, it does not influence ICEBs1 excision, suggesting that its effect occurs at the contact level. Accordingly, we observed that the biofilm extracellular matrix is crucial for enhanced ICEBs1 transfer in biofilms.

RESULTS

Biofilm enhances the conjugative transfer of ICEBs1. Throughout the years, many aspects of ICEBs1 regulation and transmission have been characterized by using a domesticated strain of *B. subtilis*, incapable of forming a biofilm (10, 33). Here, we wanted to assess the real impact of biofilm on ICEBs1 transfer, and thus, without artificial induction. Mating assays were performed using NCIB3610, an undomesticated *B. subtilis* strain that forms strong and well-characterized biofilms and contains a kanamycin selection marker in ICEBs1, as donor cells. Recipient cells were constructed

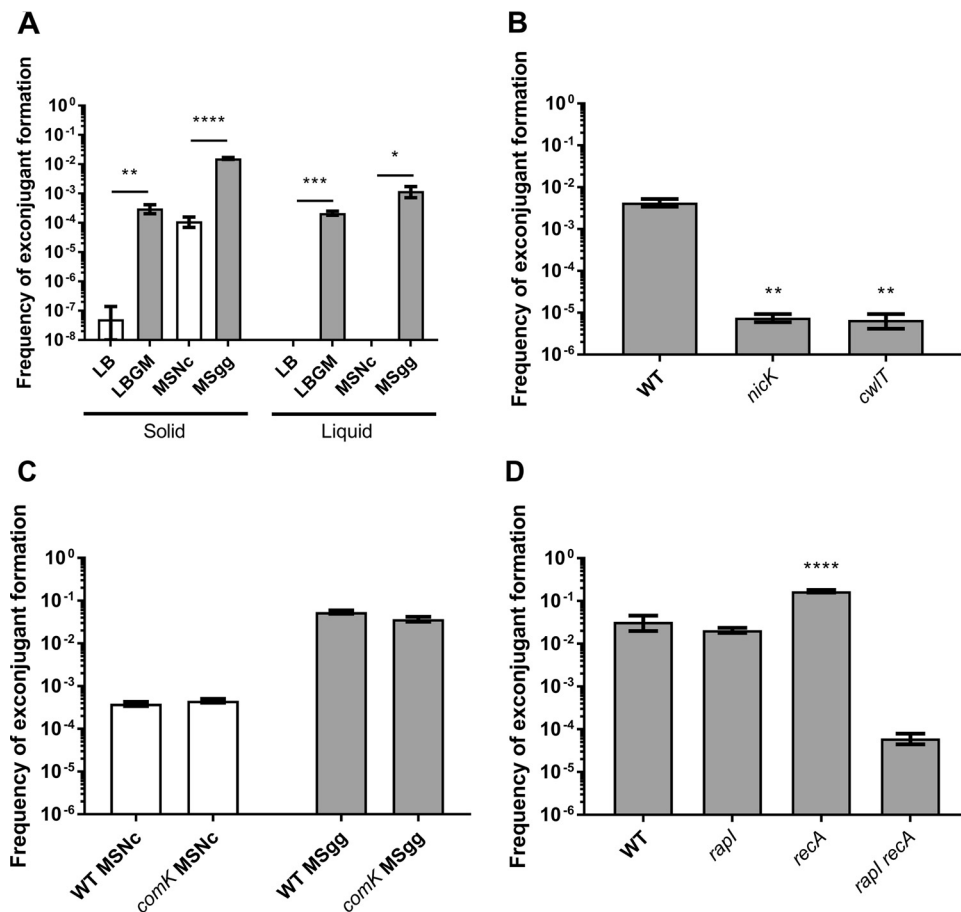


FIG 1 Biofilm formation enhances *ICEBs1* transfer. (A) Donor cells with a kanamycin resistance cassette inserted in *ICEBs1* were mated with recipient cells bearing an intergenic chloramphenicol resistance cassette in a 1:1 ratio on non-biofilm-inducing solid and liquid media (LB and MSNc [white bars]) and biofilm-inducing solid and liquid (pellicle-inducing) media (LBGM and MSgg [gray bars]). Statistical analysis indicates a significant increase in *ICEBs1* transfer when biofilm is formed (Student's *t* test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). (B) Conjugation-deficient donor cells (*nick* and *cwIT*) were mated with WT cells on MSgg to assess mating efficiency. Statistical analysis shows a significant decrease in mating efficiency with the *nick* and *cwIT* donor cells (one-way ANOVA; **, $P < 0.01$). (C) Transformation-deficient cells (*comK*) were mated on MSNc and MSgg, and mating efficiency was compared to that of WT cells. Statistical analysis shows no significant difference in mating efficiencies between *comK* and WT cells (Student's *t* test). (D) Single and double mutant *ICEBs1* activation pathway donor cells were mated with WT recipient cells on MSgg. Statistical analysis shows a significant increase of *ICEBs1* mating efficiency between *recA* and WT donor cells, but not with the *rapI* mutant (one-way ANOVA; ****, $P < 0.0001$). While the double mutant showed a decrease in mating efficiency, that difference was not significant. For all panels, mating efficiency was measured after 20 h for solid media and 28 h for liquid media at 30°C. The results shown are representative of at least three independent experiments, and error bars represent the standard error of the mean (SEM).

by curing NCIB3610 of *ICEBs1* (*ICEBs1*⁰) as described previously (10). To discriminate between the effects of medium composition versus biofilm induction, pairs of complex rich media and defined minimal media were used as conjugative support. For each pair, one medium induces biofilm formation (LBGM and MSgg [see Materials and Methods]), while the other does not (LB and MSNc [see Materials and Methods]) (24, 25, 34) (see Fig. S1A in the supplemental material). Donor and recipient cells were mated in a 1:1 ratio, spotted, and incubated for 20 h on the various solid media. Conjugation efficiency was determined by plating serial dilutions of mating mixtures on selective media. Strikingly, we observed that the efficiency of exconjugant formation on biofilm-inducing media, compared to the conjugation on noninducing media, increased between 100-fold (minimal media; compare MSgg with MSNc) and 10,000-fold (rich media; compare LBGm with LB) (Fig. 1A). Importantly, these very high levels of transfer were obtained without the need to artificially activate *ICEBs1* excision or add a

DNA-damaging reagent such as mitomycin C. Similarly, formation of floating biofilms (pellicles) in liquid biofilm-inducing media induced high levels of *ICEBs1* transfer, while media not inducing biofilms, in which cells are in planktonic form, showed no *ICEBs1* transfer (Fig. 1A).

To confirm that increased conjugative *ICEBs1* transfer explains the high level of exconjugant formation in biofilms, we performed mating assays on MSgg using *nick* and *cwlT* deletion mutants: *nick* and *cwlT* encode the relaxase and a cell wall hydrolase associated with *ICEBs1* type IV secretion system, respectively (17, 35). As expected, transfer efficiencies dropped significantly for donors with either deletion (Fig. 1B), indicating that conjugation is the main HGT mechanism used for *ICEBs1* acquisition in biofilm. Of note, on MSgg, *nick* and *cwlT* mutants exhibited a residual frequency of exconjugant formation of 10^{-5} : i.e., less than 1% of wild type (WT). Since extracellular DNA is a common feature of biofilm matrix, these exconjugants could result from acquisition of genetic material via transformation (27, 36). We tested this hypothesis by doing a conjugation assay with donor and recipient cells mutated for *comK*, the competence transcription factor of *B. subtilis* required for transformation (37), and *nick* donor cells. As shown in Fig. S2 in the supplemental material, conjugation- and transformation-deficient cells did not produce any *kan⁺ cat⁺* (exconjugants) cells on MSgg, confirming that natural transformations is responsible for approximately 1 out of 100 *kan⁺ cat⁺* cells formed in biofilm. However, since the contribution of natural competency is negligible, mating assays performed with *comK* or WT cells showed similar efficiencies of exconjugant formation in biofilms (Fig. 1C).

In *B. subtilis*, RapI and RecA are both capable of lifting inhibition on *ICEBs1* excision and transfer (10). To evaluate which pathway regulates *ICEBs1* transfer during biofilm formation, we performed mating assays on MSgg using *rapI* or *recA* null mutants as donors. We observed that neither led to a significant decrease of mating efficiency and that *recA* donor cells exhibited 5- to 10-fold higher mating efficiency. These results suggest either that a third unknown *ICEBs1* activation pathway is active in biofilms or that both RapI and RecA pathways are redundant in biofilms. To discriminate between these two hypotheses, we tested the mating efficiency of a *rapI recA* donor strain and observed a 1,000-fold decrease in mating efficiency compared to the WT (Fig. 1D). These results suggest that both *ICEBs1* activation pathways are active during biofilm formation and that they are redundant in biofilm. Interestingly, the frequencies of exconjugant formation with *nick*, *cwlT*, or the *rapI recA* donors were comparable (Fig. 1B and D), further strengthening the notion that *rapI* and *recA* are the only *ICEBs1* activators in biofilm.

Biofilm allows for highly efficient conjugation in a low donor/recipient ratio.

The 1:1 ratio of donor to recipient cells often used to assess conjugation *in vitro* is probably not frequently encountered in the environment. Therefore, we performed mating assays on MSgg using donor/recipient ratios ranging from 1:1 to 1:10⁶. We observed that exconjugant formation frequency was at its highest when recipient cells outnumbered donor cells by 10 to 100 times, whereas similar efficiencies were obtained between the 1:1 and the 1:10³ ratios and lower ratios showed decreased efficiency (Fig. 2). These observations can be partially explained by the *ICEBs1*-encoded quorum-sensing system RapI-PhrI, since extracellular PhrI would not be sufficient to inhibit the RapI activator upon low donor cell density in the population. Alternatively, diffusion of PhrI could be hampered in the presence of the biofilm matrix. Of note, it is possible that newly formed exconjugants transfer *ICEBs1* immediately after receiving it, which would compensate for the low initial level of donor cells. These results indicate that a small population of donor cells can efficiently transfer *ICEBs1* in a biofilm community.

Conjugation activation and biofilm formation are simultaneous. Since biofilm formation positively influences *ICEBs1* transfer, we examined the timing of conjugation in relation to biofilm formation. Mating efficiency was assessed on MSgg at different time points using donor and recipient cells carrying the P_{tapA} -*yfp* reporter. In this reporter gene construction, the yellow fluorescent protein (YFP) is under the control of a matrix gene promoter, thus, allowing its expression concomitantly to matrix produc-

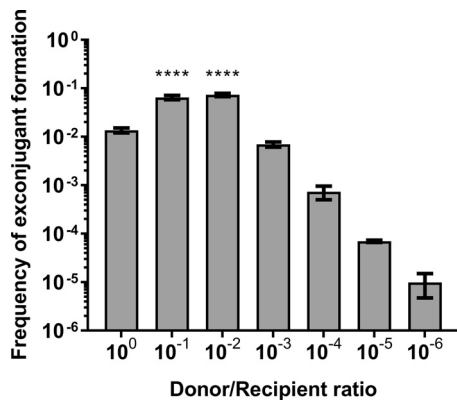


FIG 2 Lower donor/recipient ratio allows increased ICEBs1 transfer in biofilm. WT donor cells were diluted and mated with a fixed number of WT recipient cells on MSgg. Transfer efficiency was measured after 20 h at 30°C. Donor/recipient ratios of 1:10 and 1:100 show significantly more ICEBs1 transfer efficiency than the 1:1 ratio (one-way ANOVA; ****, $P < 0.0001$). The results shown are representative of at least three independent experiments, and error bars represent the SEM.

tion, which can therefore be evaluated by quantification of fluorescent cells in a population by flow cytometry (24, 38). On solid MSgg medium, we observed a steady increase in both mating efficiency and biofilm matrix induction from 8 to 20 h after inoculation (Fig. 3), indicating a temporal correlation between biofilm formation and conjugation.

Biofilm formation does not alter excision of ICEBs1. The increase of conjugation throughout biofilm formation could be due to a gradual increase in ICEBs1 excision, which is under the control of several signaling pathways. To examine this hypothesis, we determined the ICEBs1 excision level under biofilm-forming conditions by monitoring the formation of the *attB* site in donor cells using quantitative PCR (qPCR). Since quorum sensing can influence ICEBs1 excision, we used a 1:1 donor/recipient ratio. Accordingly, we constructed recipient cells in which an erythromycin resistance cassette was inserted at the hybridization site of one of the qPCR primers. In this context, the recipient unoccupied *attB* site cannot be amplified, although the site remains functional (see Fig. S3 in the supplemental material). Surprisingly, there was no significant increase of ICEBs1 excision in donor cells on biofilm-inducing media compared to noninducing media (Fig. 4A). We also followed ICEBs1 excision in donor cells over time using the same time points as the conjugation assay previously described and observed low levels of excision between 4 h and 24 h (see Fig. S4 in the supplemental material). Of note, this method does not allow us to evaluate excision rates in exconjugants, which may lead to an underestimation of the subset of cells bearing

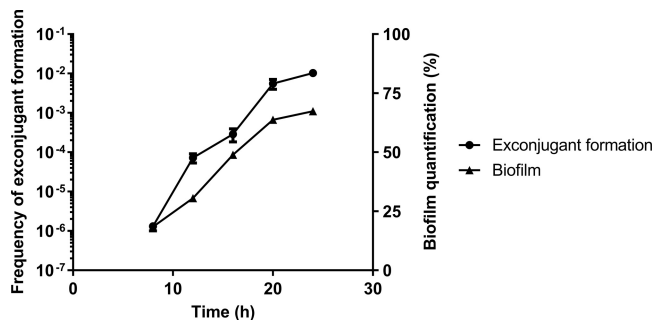


FIG 3 Biofilm formation and ICEBs1 conjugation activation are simultaneous. Recipient and donor cells harboring a P_{topA} -*yfp* fluorescent marker in the *amyE* locus were mated on MSgg. Biofilms were then harvested after 8, 12, 16, 20, and 24 h. For each time point, cells were used to quantify biofilm expression by fluorescence-activated cell sorter (FACS) and to assess mating efficiency. The results shown are representative of at least three independent experiments, and error bars represent the SEM.

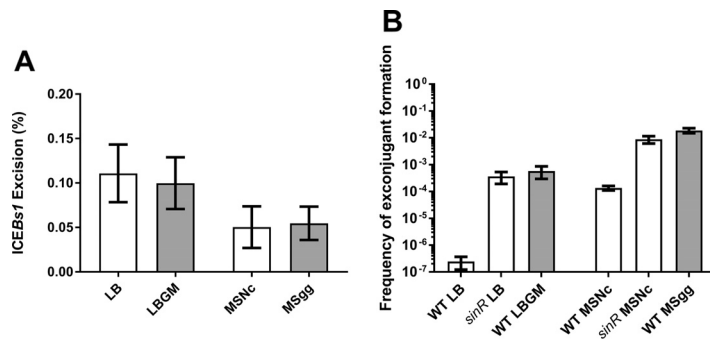


FIG 4 Matrix production is important for conjugation. (A) WT donor cells and ICEBs1⁰ attB-down recipient cells were mated on LB, LBGM, MSNc, and MSgg for 20 h at 30°C, and the donor attB site was amplified by qPCR. There was no significant difference in the ICEBs1 excision rate when there was biofilm formation (Student's *t* test). (B) *sinR* donor and recipient cells were mated on non-inducing media, and mating efficiency was compared to that of WT cells mated on non-biofilm-inducing (LB and MSNc [white bars]) and biofilm-inducing solid media (LBGM and MSgg [gray bars]). Mating efficiency was measured after 20 h at 30°C. *sinR* mutants led to higher ICEBs1 transfer efficiency compared to WT cells on noninducing media. For all panels, the results shown are representative of at least three independent experiments, and error bars represent the SEM.

excised ICEBs1. However, these results show that biofilm formation does not alter ICEBs1 excision in donor cells.

A second hypothesis to explain the positive effect of biofilm on conjugation is that the biophysical context provided by the extracellular matrix highly favors conjugative transfer. To test this idea, we used donor and recipient cells deleted for *sinR*, the transcriptional repressor of the *tapA-sipW-tasA* and *epsA-O* operons responsible for matrix production. A *sinR* mutant constitutively produces the biofilm matrix, even on non-biofilm-inducing media, with little effect on the upstream signaling pathways (27). This single mutation is sufficient to induce the formation robust biofilm, regardless of the media (Fig. S1B). As shown in Fig. 4B, transfer efficiency using *sinR* cells on both non-biofilm-inducing media was similar to that in WT cells on biofilm-inducing media. These results demonstrate that matrix production is sufficient to promote efficient transfer of ICEBs1 under non-biofilm conditions. To evaluate the importance of cell contact mediated by biofilm matrix for conjugative transfer versus the possible effects of *sinR* mutants or biofilm-inducing media on other cell processes, we examined ICEBs1 transfer in WT and *sinR* cells in planktonic (shaking) LB, LBGM, MSNc, and MSgg. Importantly, the WT in shaking biofilm-inducing media did not transfer ICEBs1 at all (LBGM) or transferred it at a lower rate than under biofilm conditions (floating pellicles) at similar donor and recipient cell densities (see Fig. S5 in the supplemental material). Also, *sinR* cells in all media and WT cells in MSgg rapidly clump despite agitation and show ICEBs1 transfer, suggesting that these cell aggregates mediated by matrix secretion are microenvironments favoring conjugative transfer (Fig. S5). Together, these results suggest that maximum transfer rates are obtained when biofilm matrix is produced and hold cells together.

TasA was shown to bind cell together in the biofilm, and matrix exopolysaccharides were suggested to favor adhesion on neighboring cell chains in complex community development (39, 40). To strengthen the hypothesis that biofilm formation can provide a favorable context for conjugation by bringing cells closer or by stabilizing cell-cell contacts, we mated donor and recipient cells incapable of producing matrix (*epsA-O* and *tasA* mutants) on MSNc. We decided to emulate the binding effect provided by the extracellular matrix by adding 1% agarose, which is expected to move the matrix-deficient cells closer and stabilize their contact. We observed that addition of agarose to *epsA-O* mutants increased ICEBs1 transfer efficiency (see Fig. S6 in the supplemental material), suggesting that the polymer helps to stabilize the contact between cells the same way the extracellular matrix can, albeit to a lesser degree.

Matrix production by recipient cells is important for optimum conjugation in biofilm. The results obtained with *sinR* mutants suggest that the biofilm matrix acts as

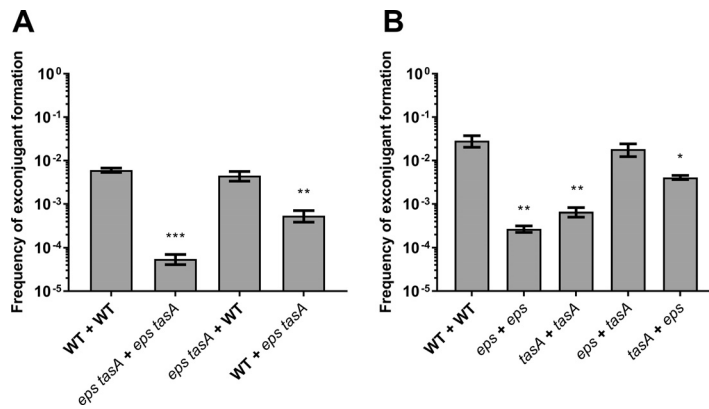


FIG 5 Both matrix components are important for ICEBs1 conjugation. (A) Donor and recipient cells deleted for the *epsA-O* (*eps*) and *tasA* operons were mated together or with WT cells on MSgg. The first genotype shown represents the donor genotype, while the second represent the recipient. Statistical analysis showed that absence of matrix and nonproduction from the recipient cells reduced significantly ICEBs1 transfer efficiency (one-way ANOVA; **, $P < 0.01$; ***, $P < 0.001$). (B) Donor and recipient cells mutated for either *eps* or *tasA* operon were mated on MSgg. Statistical analysis showed that absence of either exopolysaccharides or amyloid-like fibers in both donor and recipient decreases ICEBs1 transfer significantly. However, *eps* donors and *tasA* recipients can complement each other and restore the WT level of conjugation, while *tasA* donors and *eps* recipients are significantly different from WT pairs (one-way ANOVA; *, $P < 0.05$; **, $P < 0.01$). For both panels, mating efficiency was measured after 20 h at 30°C. The results shown are representative of at least three independent experiments, and error bars represent the SEM.

a structure promoting cell-cell contacts and optimal ICEBs1 conjugative transfer. This hypothesis was further verified by carrying out mating assays with cells incapable of secreting matrix (i.e., *eps tasA* mutants). In *B. subtilis*, the *epsA-O* (*eps*) and *tasA* operons produce two major matrix components: exopolysaccharides and the TasA amyloid-like fibers, respectively (23). As shown in Fig. 5A, production of the extracellular matrix polymers was required for efficient ICEBs1 transfer on biofilm-inducing medium. Indeed, mating assays using mutant donor and recipient cells yielded transfer efficiencies similar to those observed on a minimal non-biofilm-inducing medium. Interestingly, while donor cells deficient for matrix production could still efficiently conjugate with WT recipient cells, mating WT donor with non-matrix-producing recipient cells significantly reduced transfer (Fig. 5A). This result suggests that the biofilm matrix production of the recipient cells is particularly important for efficient ICEBs1 transfer.

To examine the importance of both components for conjugation, mating assays with donor and recipient lacking either *eps* or *tasA* were performed. As shown in Fig. 5B, both components of the matrix were instrumental for efficient ICEBs1 transfer, stressing the importance of the matrix integrity for maximal conjugation. Various reports have shown that *eps* and *tasA* mutants can complement each other extracellularly to establish biofilm both *in vitro* and on plant roots (23, 24). Interestingly, combination of these mutants can also restore conjugation efficiency, but only when TasA is produced by donor cells (*eps* mutant) and the exopolysaccharides are produced by recipient cells (*tasA* mutant). The reverse combination resulted in a 5-fold reduction of transfer efficiency compared to WT cells (Fig. 5B). This result confirms that the extracellular matrix is essential for ICEBs1 transfer in biofilms and that matrix production by recipient cells is essential for optimal transfer.

DISCUSSION

ICEBs1 regulation and its transfer mechanism have been thoroughly characterized in the last decade. However, as is also the case for most conjugative elements, its transfer between cells within a biofilm has not been previously studied. Here, we show that biofilm formation greatly increases conjugation of ICEBs1, allowing for high-efficiency transfer in the absence of added DNA-damaging reagents.

Using donor cells carrying *nick* and *cwlT* deletion mutations, both unable to transfer

ICEBs1, we validated that the high transfer observed in biofilm is due to conjugation events (Fig. 1B). However, we also observed a 10^{-5} background level of *kan⁺ cat⁺* “exconjugant” cell formation using donor cells with *nick*, *cwlT*, or *rapI recA* deleted, the latter being unable to excise ICEBs1 (Fig. 1B and D). Further experiments allowed us to determine that these *kan⁺ cat⁺* cells arose by natural transformation, via transfer of the *Kan^r* gene present in ICEBs1 to recipient cells or the transfer of the *cat* gene present in recipient cells to donor cells (Fig. S2) (data not shown). These observations suggest that natural transformation contributes to approximately 1 out of 100 HGTs observed on MSgg and thus is also fairly efficient to promote gene transfer in biofilms formed by nondomesticated strains.

The minimal biofilm-inducing medium (MSgg) provided the highest frequency of ICEBs1 exconjugant formation (i.e., 10^{-2}). Interestingly, *rapI* overexpression in a domesticated, non-biofilm-inducing *B. subtilis* strain also gives similar transfer efficiency, suggesting that it might be the upper limit for ICEBs1 transfer in a 1:1 ratio (10, 41). This transfer efficiency, obtained without artificial activation of ICEBs1 excision, demonstrates the high mobility of this element. ICEBs1 is therefore one of the ICEs transferring at the highest rate in the *Firmicutes*. Indeed, *Staphylococcus aureus* ICE6013, an ICE closely related to ICEBs1, has a mating efficiency of around 10^{-5} (42). Tn916, an ICE found in a variety of Gram-positive bacteria, was reported to transfer at frequencies ranging from 10^{-9} to 10^{-4} (43, 44), while *Streptococcus agalactiae* TnGBS1 and TnGBS2 transfer at around 10^{-5} (45). Finally, *Streptococcus thermophilus* ICES_{t3} transfers at a rate of 3.4×10^{-6} , and only one conjugation event was ever reported for ICES_{t1} (46). However, in the studies mentioned above, filter mating assays, which generally do not promote biofilm formation, were used to assess mating efficiencies. Our study demonstrates that to evaluate naturally relevant conjugation transfer of ICEs, the transfer rate within a biofilm must be examined.

Variable donor/recipient ratios ranging from 1:1 to 1:10³ in the mating population did not decrease the frequency of recipient cells acquiring ICEBs1. This observation is very important, since it reflects how a genetic element, present in a small subset of an initial population, can propagate rapidly and efficiently. In fact, a ratio of 1:10 to 1:10² led to a higher mating efficiency than a ratio of 1:1 (Fig. 2). This observation was previously reported for ICEBs1 (10), but was never explored in a biofilm-related setting. This increase in efficiency could be explained by the RapI-PhrI quorum-sensing system, since a smaller amount of donor cells in the biofilm leads to a low level of PhrI in the extracellular environment, thus favoring the action of the RapI activator (10). A similar system was described in *Enterococcus faecalis*, where a lower donor cell density led to higher transfer efficiency of conjugative plasmids pAD1 and pAM373, which both encode a secreted conjugation inhibitor (iAD1 and iAM373, respectively) (47). However, quorum-sensing regulation of excision has been observed only in a limited subset of genetic elements. For many others, DNA damage and/or environmental conditions such as reaching stationary phase or the presence of subinhibitory concentrations of antibiotics trigger ICE excision (48–50). ICEBs1 excision is also induced by DNA damage via the *recA* pathway, but *recA* donors showed better conjugation efficiency than WT donors (Fig. 1D). A similar increase of mating efficiency with a *recA* donor was observed previously (51). This result can be explained by the poor growth of *recA* mutants, known to have a slower doubling time (52). While cells were initially mated at a 1:1 donor/recipient ratio, we observed that after 20 h, the ratio had become approximately 1:50 (see Table S1 in the supplemental material), leading to a higher mating efficiency (Fig. 2).

Somewhat surprisingly, we observed that biofilm formation does not induce excision of ICEBs1 in donor cells. Despite the low excision rate (~0.2%), transfer levels in biofilms were similar to those obtained with donor cells overexpressing *rapI*, for which excision rates reach approximately 90% (10, 51). Of note, ~0.2% is more than 10 times as high as the excision rate observed in an uninduced domesticated strain (51). ICEBs1 is known to replicate in a rolling circle and can be present in multiple copies in the donor cell (16). Thus, we hypothesize that under biofilm-forming conditions, ICEBs1

rapidly reintegrates into its host chromosome following replication. The extrachromosomal copies would then be transferred to recipient cells, explaining the efficient transfer despite low excision levels. Another hypothesis underlying the high conjugative transfer of ICEBs1 in biofilm could be the presence of abundant cell chains. Indeed, ICEBs1 transfers exceptionally well through bacterial chains (53), and these structures are frequently found in biofilms, which could help propagate ICEBs1 much more efficiently (23, 34). Considering the very low excision rate and the high conjugative transfer, it is also extremely likely that a single donor cell can propagate ICEBs1 to multiple recipients in biofilms. It is also likely that once a recipient receives ICEBs1, it can immediately become a donor, further spreading it in the population. Importantly, the excision rate is not necessarily correlated with conjugation efficiency, as shown for Tn916 (43).

Many conjugative elements encode surface factors that stabilize the contact between donor and recipient cells, such as conjugative pili in Gram-negative bacteria and adhesins in Gram-positive bacteria (54, 55). While it is unknown whether ICEBs1 encodes surface factors, its low transfer efficiency in liquid compared to solid media suggests that no such factors are expressed under these conditions (56). Here, we have shown that both components of the extracellular matrix are required for the positive effect of biofilm on conjugation, suggesting that these polymers could help stabilize the donor-recipient pair and compensate for the lack of adhesion factors of ICEBs1. Other conjugative elements that are not known to encode adhesion factors, such as pCW3 and Tn916, are found in the biofilm-forming bacteria *Clostridium perfringens* and *E. faecalis*, respectively (57–59). Lack of adhesion factors in those elements could be compensated for by the ability of their host cells to form biofilms.

Experiments with single and double biofilm mutants allowed us to determine the individual importance of both matrix components in conjugation. Interestingly, matrix production from the recipient cells, but not from the donor cells, is likely essential for efficient transfer (Fig. 5A). This observation could be explained by the fact that recipients that do not produce matrix will not form cell chains, and thus, lead to less-efficient ICEBs1 transfer. It also suggests that cells within a biofilm might be able to receive ICEs from either biofilm- or non-biofilm-forming cells, making the biofilm a very receptive environment for genetic element transfer. These results allow us to better understand conjugative element dynamics in natural and clinical environments, where biofilms are ubiquitous. Biofilm matrix could thus have a considerable impact on the dissemination of mobile genetic elements, such as for the clinically important bacteria *S. aureus* and *C. difficile*, which can acquire multiple-antibiotic resistance through ICEs (60, 61).

MATERIALS AND METHODS

Strains and media. The strains used in this study are derivatives of the ancestor strain NCIB3610 (see Table S2 in the supplemental material). The different media used for mating assays are LB (Luria Bertani; 1% tryptone, 0.5% yeast extract, 0.5% NaCl), LBGM (LB plus 1% glycerol and 0.1 mM MnCl₂ [25]), MSNc (5 mM potassium phosphate buffer, pH 7, 0.1 M morpholinepropanesulfonic acid [MOPS], pH 7, 2 mM MgCl₂, 0.05 mM MnCl₂, 1 μM ZnCl₂, 2 μM thiamine, 700 μM CaCl₂, 0.2% NH₄Cl, 0.5% cellobiose) (24), and MSgg (5 mM potassium phosphate buffer, pH 7, 0.1 M MOPS, pH 7, 0.025 mM FeCl₃, 2 mM MgCl₂, 0.05 mM MnCl₂, 1 μM ZnCl₂, 2 μM thiamine, 700 μM CaCl₂, 0.5% glycerol, 0.5% glutamate) solidified with 1.5% agar (34). Media did not affect significantly bacterial growth, with biofilm-inducing media leading to slightly more yield compared to noninducing media (see Table S3 in the supplemental material). When needed, the following antibiotics were added to media: MLS (1 μg ml⁻¹ erythromycin, 25 μg ml⁻¹ lincomycin), spectinomycin (100 μg ml⁻¹), tetracycline (10 μg ml⁻¹), chloramphenicol (5 μg ml⁻¹), and kanamycin (10 μg ml⁻¹).

Strain construction. Most strains were made by transferring genetic constructs present in domesticated strains in NCIB3610, using SPP1-mediated generalized transduction (62). JMA348 (ICEBs1::kan), CAL51 [(*rapI phrI*)342::kan], JMA208 (*immR*::cat), and CAL419 (ICEBs1^o *comK*::cat) were kind gifts from Alan D. Grossman (Massachusetts Institute of Technology, MA), and 3610 ICEBs1^o strains were cured from ICEBs1 and verified as described in reference 10. Briefly, MG9 (3610 *immR*::cat) was inoculated in LB, grown for 4 h, diluted at an optical density at 600 nm (OD₆₀₀) of 0.01 in fresh LB, and grown overnight at 37°C. The culture was then diluted back to an OD₆₀₀ of 0.01 in fresh LB and grown until the culture reached an OD₆₀₀ of 1. The cells were then plated on LB agar and grown overnight at 37°C, and colonies were streaked on LB with or without chloramphenicol. Colonies that lost the resistance were then PCR

verified for ICEBs1 excision with the following primers (5'→3'): P197 (GAC GAA TAT GGC AAG CCT ATG TTA C) and P198 (GGG TAT ACA ATC ATG GGT GAT CGA G).

Long-flanking homology PCR was used to insert a spectinomycin cassette between *ycbU* and *ImrB* and to create the recipient used for qPCR (JSB18). The following primers (5'→3') were used for the *ycbU-ImrB::spec* insertion: P246 (CCA TTG ATG TGA AGG AAT GGG GCG TA), P247 (CGT TAC GTT ATT AGC GAG CCA GTC ATG TTT ACT TGT GGA TCG TTT TCG CCG), P248 (CAA TAA ACC CTT GCC CTC GCT ACG CCT GAA CAC TAG TCA GGG GCT TTT CA), and P249 (GGC TTA GTC CTC ACT GCA TTT GCA TC). The following primers (5'→3') were used for the *attB-down::erm* deletion: P328 (CCG TTG GTC AAG CGG TTA AG), P329 (GAG GGT TGC CAG AGT TAA AGG ATC TAT TAT TGA GAT GCG GCC GAG), P330 (CGA TTA TGT CTT TTG CGC AGT CGG CGT GTG GAA AAT ACG GCT ATG GG), and P331 (AGT AAG CTT ATT CCA CCC ACT G). PCR products were then introduced in *B. subtilis* 168 by natural competency (63), verified by PCR, and transferred in derivatives of *B. subtilis* NCIB3610 by SPP1-mediated generalized transduction (62).

Mating assays. Donor and recipient cells were grown in 3 ml LB broth at 37°C overnight, diluted at an OD₆₀₀ of 1.5 in LB, and mixed at a 1:1 ratio (or the specified ratio [Fig. 2]). The mixture was then centrifuged for 3 min at 5,000 rpm. The cell pellet was resuspended in 50 μl LB, and 10 μl was dropped onto the appropriate medium and incubated for 20 h (or the time specified [Fig. 3]) at 30°C, which is the temperature at which *B. subtilis* biofilm grows efficiently. For mating assays in liquid, 10 μl of bacterial mixture was used to inoculate 3 ml of medium for shaking conditions, while 3.33 μl was used to inoculate 1 ml of medium found in a 24-well plate for pellicle mating; both were also done at 30°C. Pellicles were incubated for 28 rather than 20 h, since the growth dynamic is slower under nonshaking conditions. For mating assays using agarose, cells were resuspended in 50 μl of warm (55°C) 1% molecular-grade agarose before being dropped on agar medium. Cells were then collected with 1 ml LB broth and sonicated at 30% amplitude for 20 s two times for cells grown for 20 h on biofilm-inducing media. Microscopy observation allowed us to determine that sonication was sufficient to obtain single cells. Cells were then serially diluted and plated on LB with the appropriate antibiotics. Donor, recipient, and exconjugant CFU were then counted. We expressed the frequency of exconjugant formation as a function of the number of recipient CFU (number of exconjugant CFU divided by the number of recipient CFU), because the starting donor amount varied in some experiments (Fig. 2).

Flow cytometry. Mating assays were performed on MSgg as described above, using the FL60 and FL63 strains. For each time point, three biofilms were harvested with 500 μl phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4) and disrupted with up and down pipetting. Subsequent steps were performed as described previously (38). Flow cytometry analysis was performed on a BD FACSJazz (BD Biosciences).

ICEBs1 excision quantification. ICEBs1 excision was evaluated using qPCR. Donor and recipient cells were mated on MSgg as described above, and cells were harvested and flash-frozen at the appropriate time point. Genomic DNA was extracted using the BioBasic genomic DNA extraction kit, and qPCR was performed on the *attB* site (created when ICEBs1 is excised from the chromosome) using the following primers (5'→3'): P358 (GCC TAC TAA ACC AGC ACA AC) and P333 (AAA GGT GGT TAA ACC CTT GG). Since the recipient strain (JSB18) contains an erythromycin resistance cassette in the hybridization site of the P333 primer, amplification is only possible for donor cells in which ICEBs1 is excised. qPCR on a chromosomal chloramphenicol resistance cassette present only in the donor genome was performed for normalization (threshold cycle [ΔC_T]) of donor cells, using the following primers (5'→3'): P363 (AGA ACT GGT TAC AAT AGC GAC GGA GAG) and P366 (CCC CGA ACC ATT ATA TTT CTC TAC ATC AGA AAG G). The percentage of excision is calculated as the ΔΔC_T, using the culture of the ICEBs1⁰ *ylnF/yboA::Tn917::amyE::cat* control strain grown under the same conditions, which is considered as being 100% excised.

Stereomicroscopy. Donor and recipient WT and *sinR* cells were mated on LB, LBGM, MSNc, and MSgg as described above. Photographs of colonies were taken after 20 h at 30°C with a Leika M165 FC (Leika).

Statistical analyses. Statistical analyses were performed using GraphPad Prism 7. Comparisons were done using Student's *t* test or one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test, both with 95% confidence intervals.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/mSphere.00473-18>.

FIG S1, PDF file, 0.3 MB.

FIG S2, PDF file, 0.1 MB.

FIG S3, PDF file, 0.1 MB.

FIG S4, PDF file, 0.1 MB.

FIG S5, PDF file, 0.1 MB.

FIG S6, PDF file, 0.1 MB.

TABLE S1, PDF file, 0.1 MB.

TABLE S2, PDF file, 0.2 MB.

TABLE S3, PDF file, 0.1 MB.

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