A mobile genetic element increases bacterial host fitness by manipulating development

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Abstract

Horizontal gene transfer is a major force in bacterial evolution. Mobile genetic elements are responsible for much of horizontal gene transfer and also carry beneficial cargo genes. Uncovering strategies used by mobile genetic elements to benefit host cells is crucial for understanding their stability and spread in populations. We describe a benefit that *ICEBsI*, an integrative and conjugative element of *Bacillus subtilis*, provides to its host cells. Activation of *ICEBsI* conferred a frequency-dependent selective advantage to host cells during two different developmental processes: biofilm formation and sporulation. These benefits were due to inhibition of biofilm-associated gene expression and delayed sporulation by *ICEBsI*-containing cells, enabling them to exploit their neighbors and grow more prior to development. A single *ICEBsI* gene, *devI* (formerly *ydcO*), was both necessary and sufficient for inhibition of development. Manipulation of host developmental programs allows *ICEBsI* to increase host fitness, thereby increasing propagation of the element.

Introduction

Conjugative elements and phages are abundant mobile genetic elements in bacteria, capable of transferring DNA between cells (Frost *et al.*, 2005). Integrative and conjugative elements (ICEs) appear to be the most widespread type of conjugative element (Guglielmini *et al.*, 2011). ICEs are found integrated in a host genome. When activated, they excise and produce conjugation machinery that transfers the element DNA from the host cell to recipients (Carraro and Burrus, 2015; Johnson and Grossman, 2015; Wozniak and Waldor, 2010).

ICEs often carry “cargo” genes that are not necessary for transfer but confer a phenotype to host cells. In fact, ICEs (conjugative transposons) were first identified because of the phenotypes conferred by cargo genes (Franke and Clewell, 1981). Cargo genes include those encoding antibiotic resistances, metabolic pathways, and determinants of pathogenesis and symbiosis (Johnson and Grossman, 2015). Transfer of mobile elements between cells contributes to rapid evolution and spread of associated cargo genes and phenotypes (Frost *et al.*, 2005; Treangen and Rocha, 2011).

Despite the benefits cargo genes can provide, the maintenance and transfer of mobile genetic elements requires host cellular resources and in some cases is lethal (Baltrus, 2013). Maintenance of a mobile genetic element in host cells requires balancing the costs and benefits to the host or a sufficiently high transfer frequency. Many mobile elements, especially ICEs, have been identified bioinformatically (Bi *et al.*, 2012; Guglielmini *et al.*, 2011). Many of these ICEs contain putative cargo genes. However, the phenotypes conferred by these genes cannot be inferred from sequence nor are they easily detected experimentally (Cury *et al.*, 2017).

ICEBs1, a relatively small (~20 kb) ICE found in most strains of *Bacillus subtilis*, was identified bioinformatically (Burrus *et al.*, 2002) and experimentally based on its regulation by

cell-cell signaling (Auchtung *et al.*, 2005). Most of the *ICEBsI* genes needed for conjugation are grouped together in an operon that is repressed until activating signals are sensed. Two pathways activate *ICEBsI*, both of which lead to cleavage of the repressor ImmR by the protease and anti-repressor ImmA (Auchtung *et al.*, 2007; Bose *et al.*, 2008; Bose and Grossman, 2011). *ICEBsI* contains the cell-cell signaling genes, *rapI* and *phrI*, which regulate *ICEBsI* activation by sensing population density and the relative abundance of *ICEBsI*-containing host cells (Auchtung *et al.*, 2005). RapI is produced at high cell density and during the transition to stationary phase and stimulates the proteolytic cleavage of the repressor ImmR by the protease ImmA (Bose and Grossman, 2011). Overproduction of RapI stimulates activation of *ICEBsI* in >90% of cells (Auchtung *et al.*, 2005). RapI activity (and therefore *ICEBsI* activation) is inhibited by PhrI, a peptide that is secreted by cells that contain *ICEBsI*. PhrI levels indicate the relative abundance of *ICEBsI*-containing cells in the population, preventing the activation and possible redundant transfer of *ICEBsI* if most nearby cells already contain the element. *ICEBsI* is also activated during the RecA-dependent DNA damage response (Auchtung *et al.*, 2005).

Biofilms appear to be hotspots of horizontal gene transfer for bacteria growing in natural settings (Madsen *et al.*, 2012; Molin and Tolker-Nielsen, 2003). Undomesticated strains of *B. subtilis* form complex biofilms on agar plates and at the air-liquid interface in standing cultures (Vlamakis *et al.*, 2013). There is also extensive spore formation in *B. subtilis* biofilms (Branda *et al.*, 2001; Vlamakis *et al.*, 2008). In addition, during growth in a biofilm, *ICEBsI* is naturally activated and transfers efficiently, generating on the order of 10 new *ICEBsI*-containing host cells (transconjugants) per donor cell (Lécuyer *et al.* 2018). *B. subtilis* biofilms are held together by a matrix composed of secreted exopolysaccharides, protein fibers, and DNA (Vlamakis *et al.*, 2013). This matrix reinforces cell-cell contacts, likely promoting rapid spread of *ICEBsI* by

conjugation. Additionally, the conditions that promote biofilm formation (high cell density) also promote activation and transfer of *ICEBsI* and sporulation (Auchtung *et al.*, 2005; Grossman and Losick, 1988). Though biofilm growth is clearly beneficial to conjugation, it is unknown how *ICEBsI* impacts its host cells under these conditions.

In this study, we describe a selective advantage provided by *ICEBsI* to its host cells during growth in biofilms. This fitness benefit was due to inhibition of host biofilm and spore development. We identified the *ICEBsI* gene *devI* (formerly *ydcO*) as necessary and sufficient to inhibit host development and provide a selective advantage to *ICEBsI*-containing cells. We also provide evidence indicating that *devI* inhibits the key developmental transcription factor Spo0A, reducing its ability to stimulate biofilm and sporulation gene expression. *devI* (*ydcO*) is conserved in other *ICEBsI*-like elements, indicating that manipulation of host development may be a conserved strategy among this family of mobile genetic elements. We postulate that manipulation of host pathways may be a common function of many of the as yet uncharacterized cargo genes in ICEs.

Results

ICEBsI spreads efficiently in biofilms by conjugation

Biofilm formation is characteristic of many bacteria growing in natural settings, including *B. subtilis*. We used biofilm growth to determine if *ICEBsI* affected the fitness of its host cells under conditions that naturally promote its spread. We performed competition experiments in biofilms using strains of undomesticated *B. subtilis* (NCIB3610 plasmid-free) with or without *ICEBsI*.

We observed highly efficient spread of *ICEBsI* at low donor to recipient ratios (Table 1) during growth in biofilms, similar to results reported previously (Lécuyer *et al.*, 2018). To measure mating, we mixed *ICEBsI*-containing cells (potential donors) with cells that did not contain *ICEBsI* (*ICEBsI*⁰, potential recipients) and co-cultured the mix on standard biofilm-stimulating growth medium (MSgg agar) (Branda *et al.*, 2001). Since *ICEBsI* induction is regulated by cell-cell signaling, we varied the initial frequency of *ICEBsI*⁺ cells between approximately 0.01 and 0.9. We inserted unique selectable markers (antibiotic resistances) in the chromosomes of the donors and recipients as well as within *ICEBsI*. After four days of growth at 30°C (approximately 17 net doublings of the initial population), biofilms were disrupted and the number of transconjugants was determined by selective plating.

We found that after four days of biofilm growth, the frequency of transconjugants ranged from ~0.4 – 0.6 of total cells in the biofilm for starting donor frequencies of ~0.01 – 0.5 (Table 1). The highest frequency of transconjugants was observed when the starting frequency of *ICEBsI*-containing cells was ~0.1. Enhanced conjugation at low donor to recipient ratios is likely due to regulation of *ICEBsI* by cell-cell signaling (induction is inhibited by the presence of other potential donors) and the higher likelihood of contacting potential recipients at low frequencies of donors.

Table 1: Frequency of transconjugants generated in biofilm matings

Initial frequency <i>ICEBsI</i> donors ¹	Final frequency transconjugants ²
0.008 ± 0.002	0.42 ± 0.13
0.10 ± 0.03	0.64 ± 0.15
0.47 ± 0.05	0.44 ± 0.07
0.89 ± 0.03	0.063 ± 0.008

¹ ICEBsI-containing cells (JMJ592) were mixed with ICEBsI-cured cells (JMJ550). The initial frequencies reported are the average $\pm$ standard deviation from three independent experiments.

² The final frequencies of transconjugants reported are the average $\pm$ standard deviation from a total of 9 biofilms from 3 independent experiments.

The high levels of ICEBsI conjugation during growth in biofilms presented a challenge for quantifying the fitness of ICEBsI-containing host cells relative to ICEBsI⁰ cells. Mating converts a large fraction of ICEBsI⁰ cells to transconjugants (ICEBsI-containing), reducing the ICEBsI⁰ proportion of the population in a manner unrelated to host fitness. To measure the effect of ICEBsI on host fitness, we blocked conjugative DNA transfer using the *conEK476E* mutation (Berkmen *et al.*, 2010). We then compared the proportion of ICEBsI-containing hosts to ICEBsI⁰ cells without the confounding influence of conjugation.

ICEBsI provides a frequency-dependent selective advantage in biofilms

We found that cells containing ICEBsI that is incapable of conjugation {ICEBsI(*conEK476E*)} had a fitness advantage over cells lacking ICEBsI during biofilm growth when they were initially present as a minority of the population. As before, we varied the initial frequency of ICEBsI-containing host cells in the inoculum between approximately 0.01 and 0.9. To measure fitness we determined the frequency of ICEBsI-containing cells (f_{ICE}) and ICEBsI⁰ (f_{NULL}) cells in the initial inoculum and in mature biofilms (4 days of growth at 30°C) by selective plating. The relative fitness of the ICEBsI-containing cells was calculated as the fold change in the ratio of f_{ICE} / f_{NULL} over the course of the competition.

We found that the fitness of ICEBsI-containing cells was dependent on their initial frequency in the population (Fig. 1A). The frequency-dependence was most likely due to regulation of ICEBsI gene expression by the cell-cell signaling genes *rapI-phrI* or some other function of *rapI*. Cells with ICEBsI had a selective advantage at low frequencies (0.01 or 0.1) when the

element is most strongly activated. At high frequencies in the population (0.5 or 0.9), when there is little or no activation, fitness of *ICEBsI*-containing cells was approximately neutral (Fig. 1A). The final growth yields of the populations were similar regardless of the frequency of *ICEBsI*-containing cells (Fig. 1C).

We performed control competitions of two differentially marked *ICEBsI*⁰ strains to verify that the enhanced fitness we observed was due to the presence of *ICEBsI* rather than an inherent fitness difference associated with antibiotic resistances (see Methods and Materials). There was a small cost associated with the marker used to select cells containing *ICEBsI* (relative fitness 0.7 ± 0.09), leading to a slight underestimate of the selective advantage to these cells.

There is a large amount of sporulation in *B. subtilis* biofilms (Branda *et al.*, 2001; Vlamakis *et al.*, 2008). Consistent with this, we found that approximately 80% of viable colony-forming units (CFUs) in a mature biofilm after 3 days were from spores. The selective advantage to cells containing *ICEBsI* growing in biofilms could be due to sporulation and/or biofilm development.

164

Figure 1. The fitness of ICEBs1-containing cells during development depends on their initial frequency in the population. The fitness of ICEBs1-containing cells (JMJ593) relative to ICEBs1-cured cells (JMJ550) was measured by competition during biofilm growth (A) or growth on sporulation medium without biofilm formation (B). The total growth yields in biofilms (C) and on sporulation medium (D) were determined by counting CFUs derived from both cells and spores. Data shown are pooled from 3 independent experiments. A total of 9 populations were analyzed per initial frequency. Boxes extend from the lower to upper quartiles of the data, and the middle line indicates the median fitness. Whiskers indicate the range of the fitness measurements.

ICEBsI confers a selective advantage in biofilms without sporulation

We blocked sporulation using a mutation that causes a reduction in the amount of the transcription factor Spo0A that is required for spore formation. The *spo0AΔPs* mutation is a deletion of the sigma-H-dependent promoter upstream of *spo0A* (Siranosian and Grossman, 1994). This mutation reduces production of Spo0A, and cells do not achieve the threshold concentration required to initiate sporulation (Chung *et al.*, 1994). *spo0AΔPs* mutant cells formed biofilms that were morphologically similar to those formed by wild type cells.

In biofilms without sporulation, *spo0AΔPs* mutant cells containing ICEBsI (JMJ788) had a selective advantage compared to *spo0AΔPs* mutant cells without ICEBsI (JMJ786). Notably, the median fitness for *spo0AΔPs* mutant cells containing ICEBsI at a low frequency in the population was approximately six. Thus, sporulation was not required for a fitness benefit to ICEBsI-containing cells in biofilms.

Fitness of cells in biofilms can be affected by production of the biofilm matrix. For example, cells that “cheat” by contributing less to biofilm matrix production reap the benefits of growing with other cells that bear the cost of matrix gene expression (Dragoš *et al.*, 2018). We showed that cells containing ICEBsI “cheat” by decreasing expression of biofilm matrix genes compared to cells without ICEBsI (see below).

ICEBsI confers a selective advantage during sporulation, in the absence of biofilms

We found that cells containing ICEBsI also had a frequency-dependent selective advantage during sporulation, in the absence of biofilms. We prepared mixtures of cells with and without ICEBsI as described above. These mixtures were spotted onto a medium (DSM agar) that promotes high levels of sporulation. During growth on this medium, there are no complex colony features found in biofilms. As in the biofilm competitions, cells containing ICEBsI had a

frequency-dependent selective advantage during sporulation (Fig. 1B). At an initial frequency of approximately 0.01 the median relative fitness of the *ICEBsI*-containing cells was approximately 14 (14.5 ± 4.3). As in biofilms, the total growth yields of the populations were similar regardless of *ICEBsI* host frequency (Fig. 1D). These results demonstrate that *ICEBsI* confers a selective advantage to cells growing on DSM agar, outside the context of biofilms. This could be due to sporulation or growth under these specific conditions.

We found that sporulation was required for the strong selective advantage during growth on sporulation medium (DSM agar). The fitness benefit associated with the *ICEBsI*-containing cells at a low frequency in the population was greatly reduced in the *spo0AΔPs* mutant (no sporulation). The sporulation mutant with *ICEBsI* had a median fitness of approximately 1.5 compared to approximately 9 for wild-type. Based on these results, we conclude that the presence of *ICEBsI* confers a frequency-dependent selective advantage during sporulation.

Together, our results demonstrate that cells containing *ICEBsI* have a frequency-dependent selective advantage in biofilms and during sporulation. This selective advantage is independent of the ability of *ICEBsI* to actually transfer from one cell to another. Biofilm formation (Hamon and Lazazzera, 2001) and sporulation (Hoch, 1993; Sonenshein, 2000) are both regulated by the transcription factor Spo0A. Our results indicate that the presence of *ICEBsI* could somehow be inhibiting the activity or activation of Spo0A.

***ICEBsI*-containing cells have a frequency-dependent delay in sporulation**

We hypothesized that some *ICEBsI*-encoded gene(s) inhibit host cell development. This inhibition could delay development and enable cells to continue growth for a small number of generations. This model derives from analogous phenotypes of mutants that do not enter the sporulation pathway (Dawes and Mandelstam, 1970). Mutants that delay the start of sporulation

have a growth advantage as they are able to divide one or a few more times while other cells in the population stop growing and start to sporulate.

We found that in mixed populations, sporulation was delayed in cells containing *ICEBsI* compared to cells without *ICEBsI*, in a frequency-dependent manner. As above, we used an *ICEBsI* mutant that is incapable of conjugation {*ICEBsI(conEK476E)*}. We started several replicate populations, each of which we sampled once at different times to create a time-course. {This was done because sampling to quantify CFUs (spores and cells) disrupts and prevents monitoring a single population over time.} Spore frequency was determined by measuring heat-resistant CFUs as a fraction of total CFUs for *ICEBsI*-containing and *ICEBsI*-cured strains that contained different antibiotic resistance markers to distinguish the strains.

Sporulation is delayed in *ICEBsI* host cells during biofilm formation. We found that sporulation of *ICEBsI*-containing cells was delayed in a frequency-dependent manner during growth in biofilms (Fig. 2A, 2B). When *ICEBsI*-containing cells were started at a frequency of approximately 0.01, they reached their maximum sporulation frequency (>80% spores) roughly 17 hours later than the cells without *ICEBsI* (Fig. 2A). After three days of biofilm growth, the sporulation frequencies of *ICEBsI*-containing and *ICEBsI*-cured cells were indistinguishable. Over this period of time the total frequency of *ICEBsI*-containing cells in the population typically rose from ~0.01 to ~0.03, giving a relative fitness (~3) consistent with results above (Fig. 1A). When the *ICEBsI*-containing cells were the majority in the population (initial frequency ~0.9) the timing and sporulation frequencies of the *ICEBsI*-containing and *ICEBsI*-cured cells were indistinguishable (Fig. 2B).

233

Figure 2. *ICEBs1*-containing cells delay sporulation in a frequency-dependent manner.

ICEBs1-containing cells (JMJ593, black circles) were mixed with *ICEBs1*-cured cells (JMJ550, open squares) at an initial frequency of 0.01 and spotted onto biofilm growth medium (A) or sporulation medium (C). Mixtures with *ICEBs1*-containing cells at an initial frequency of 0.9 were also spotted onto biofilm medium (B) and sporulation medium (D). Biofilms and colonies were harvested at the indicated times to determine the fraction of CFUs derived from heat-resistant spores for both the *ICEBs1*-containing and the *ICEBs1*-cured cells. Boxes below each graph indicate the frequency of *ICEBs1*-containing CFUs at each timepoint. Data shown are the average from two populations per timepoint with error bars indicating the standard deviation. A representative experiment is shown.

Sporulation is delayed in *ICEBsI* host cells during sporulation in the absence of biofilms.

We also found that sporulation of *ICEBsI*-containing cells was delayed in a frequency-dependent manner during sporulation in the absence of biofilm formation (Fig. 2C, 2D). When the *ICEBsI* containing cells were inoculated at a low frequency (approximately 0.01), the delay in sporulation was qualitatively similar to that observed in biofilms (Fig. 2C). However, the increase in the total frequency of *ICEBsI*-containing cells in the population was approximately 10-fold, giving a relative fitness of approximately 10, consistent with results described above (Fig. 1B). This increase was much greater than the approximately 3-fold increase during biofilm formation.

We suspect that the stronger selective advantage of *ICEBsI*-containing cells on sporulation medium is due to the earlier onset of sporulation. By 16 hours of growth on sporulation medium, spores made up about 40% of the total CFUs. By the same time in biofilms spores were undetectable (limit of detection ~0.03% spores).

***rapI-phrI* are necessary but not sufficient for enhanced fitness**

The fitness benefits provided by *ICEBsI* were dependent on the relative abundance of *ICEBsI*-containing cells, indicating that the cell-cell signaling genes *rapI-phrI* in *ICEBsI* were likely involved, either directly or indirectly. Other Rap proteins in *B. subtilis* are known to regulate development by inhibiting phosphorylation (activation) of the transcription factor Spo0A (Sonenshein, 2000). RapI, like other Rap proteins in *B. subtilis*, can inhibit the pathway needed to phosphorylate (activate) the transcription factor Spo0A, and overexpression of *rapI* in vivo inhibits sporulation (Parashar *et al.*, 2013, Singh *et al.*, 2013). However, it was unknown whether RapI regulates development in vivo under physiological conditions. Results described below demonstrate that the *rapI-phrI* system is required for the fitness advantage of *ICEBsI*-

containing cells, but that this requirement is by virtue of causing induction of *ICEBsI* gene expression. Another gene in *ICEBsI* is both necessary and sufficient for the selective advantage of *ICEBsI*-containing cells during development.

rapI-phrI are required for the fitness benefit of cells containing *ICEBsI*. We deleted *rapI-phrI* (Δ *rapI-phrI*) in *ICEBsI* and compared the fitness conferred by this mutant to that conferred by *ICEBsI* with *rapI-phrI*. Because loss of *rapI* prevents induction of gene expression, excision, and replication of *ICEBsI*, we used *ICEBsI* mutants ('locked-in') that are incapable of excision or replication (see Methods and Materials), regardless of the presence or absence of *rapI*. Preventing excision and replication of *ICEBsI* allowed us to compare the fitness of wild-type *ICEBsI* to *ICEBsI* Δ *rapI-phrI* (and other mutants), which would otherwise have a lower gene copy number due to a lower frequency of induction.

We verified that locked-in *ICEBsI* still conferred a fitness benefit to host cells. During sporulation in biofilms (MSgg agar), cells containing locked-in *ICEBsI* had a relative fitness of approximately 14 when they were started at a low frequency in the population (~ 0.01) (Fig. 3A). This benefit was much greater than that conferred by wild-type *ICEBsI* that can excise and replicate. We suspect that replication of *ICEBsI* incurs a fitness cost to the host cell that reduces the apparent benefit. The sources of this burden could include use of host resources, the host's response to single-stranded DNA produced by rolling-circle-replication of *ICEBsI*, and increases in *ICEBsI* gene expression due to increased copy number.

We found that *rapI-phrI* were required for the fitness benefit conferred by *ICEBsI*. During sporulation in biofilms (MSgg agar), the relative fitness of the Δ (*rapI-phrI*) host strain was approximately neutral (Fig. 3A), in contrast to the high fitness (median ~ 14) provided by *ICEBsI* containing *rapI-phrI* when the *ICEBsI*-containing cells were started at a low frequency (~ 0.01).

280

Figure 3. The *ICEBs1* cell-cell signaling genes, *rapI-phrI*, are necessary but not sufficient to confer a selective advantage. (A) The fitness of cells containing locked-in *ICEBs1* (JM1646) or an isogenic *rapI-phrI* mutant (JM1686) was measured relative to *ICEBs1*-cured cells (JM1550) during biofilm competitions. The *ICEBs1*-containing cells were started at a frequency of 0.01. (B) The fitness of cells containing *rapI-phrI* alone (JM1576) or cells containing both *rapI-phrI* and locked-in *ICEBs1 $\Delta rapI-phrI$* (JM1785) was measured relative to *ICEBs1*-cured cells (JM1714) during biofilm and sporulation medium competitions. JM1576 and JM1785 were started at a frequency of 0.01. Data shown are pooled from 3 independent experiments with a total of 9 populations analyzed per strain mixture. Boxes extend from the lower to upper quartiles, and the middle line indicates the median fitness. Whiskers indicate the range of the fitness measurements.

The requirement for *rapI-phrI* could be due to a direct role for one of these, likely RapI, or an indirect role in activating expression of ICE genes.

rapI-phrI are not sufficient in the absence of other *ICEBsI* genes to provide a fitness benefit.

We found that *rapI-phrI* alone were not sufficient to provide a fitness benefit during sporulation or during sporulation in biofilms. We cloned *rapI-phrI* and their native promoters and inserted them in an ectopic locus (*bcaP*) in a strain that did not contain *ICEBsI*. When this strain was started at a low frequency (~0.01), fitness of this strain was neutral relative to a control strain without *rapI-phrI* (Fig. 3B). To verify that *rapI-phrI* were functional, we added back *ICEBsI* that was missing *rapI-phrI*. Adding the rest of *ICEBsI* restored the fitness advantage during sporulation and in biofilms, indicating that the ectopic copy of *rapI-phrI* was functional (Fig. 3B). The requirement for *rapI-phrI* and some other *ICEBsI* gene(s) indicated that the selective advantage was likely dependent on induction of *ICEBsI* by RapI.

Activation of *ICEBsI* is required for the fitness benefit. We found that expression of one or more *ICEBsI* genes controlled by the promoter *Pxis* was required for the selective advantage in biofilms with sporulation. *Pxis* drives most of the genes in *ICEBsI* and is indirectly activated by RapI in a frequency-dependent manner (Auchtung *et al.*, 2005; Bose and Grossman, 2011). We deleted *Pxis* in a strain in which *ICEBsI* was unable to excise or replicate (locked-in-*ICEBsI*). In this strain, only genes not dependent on *Pxis* could be expressed, including *rapI-phrI*. Fitness of this strain was approximately neutral during sporulation in biofilms (Fig. 4B). This indicated that expression of one or more genes controlled by *Pxis*, either alone or in combination with *rapI*, was required for the fitness benefit conferred by *ICEBsI*.

Since most of the genes controlled by *Pxis* have known roles in the conjugative life cycle, we focused our search on genes without a known function, starting with the genes near *Pxis*. Of

these, we found that a deletion of *devI* (*ydcO*) reduced fitness. Results described below demonstrate that a single *ICEBsI* gene, *devI* (*ydcO*), is both necessary and sufficient to inhibit development and provide a selective advantage. The primary role of *rapI* in the fitness benefit appears to be the induction of *devI* expression.

devI* is necessary for the fitness benefit conferred by *ICEBsI

We found that an *ICEBsI* gene of unknown function, *devI* (*ydcO*), was necessary for the fitness advantage of *ICEBsI* host cells. We constructed a deletion of *devI* in the locked-in-*ICEBsI* strain. When started at a low frequency in the population (~0.01) the relative fitness of the *devI* mutant was approximately 3.5 (Fig. 4D), much less than that of the isogenic *devI*⁺ cells (median fitness ~14) in biofilms with sporulation (Fig. 4A). Interestingly, the deletion of *devI* did not reduce fitness fully to neutral, indicating a possible role for other *ICEBsI* genes.

devI (*ydcO*) is predicted to encode an 86 amino acid protein. A search for conserved motifs and structural similarity between DevI (YdcO) and other proteins did not significantly inform our understanding of DevI function. However, *devI* (*ydcO*) homologs are found in other *Bacillus* species (see below).

***devI* is sufficient to inhibit sporulation and provide a fitness benefit**

We found that when expressed constitutively, *devI* alone, in the absence of all other *ICEBsI* genes, was sufficient to inhibit sporulation and provide a selective advantage. We cloned *devI* under the control of *P_{xis}* at an ectopic locus (*lacA*) in a strain without *ICEBsI*. In the absence of *ICEBsI* (and its repressor ImmR), *P_{xis}* is constitutively active (Auchtung *et al.*, 2007). Fitness was measured relative to a control strain that had *P_{xis}* with no gene downstream.

326

Figure 4. Effects of deletions in ICEBsI on host fitness. (A) Abbreviated genetic map of locked-in-ICEBsI showing genes as open block arrows, promoters as thin right-angle arrows, and the left attachment site (*attL*) as a black bar. (B to E) Brackets under the map of ICEBsI indicate regions contained in isogenic derivatives of locked-in-ICEBsI. Open spaces represent regions deleted. The fitness of strains containing locked-in-ICEBsI (JMJ646) and its derivatives (ΔP_{xis} , JMJ662; $\Delta ydzL$, JMJ704; $\Delta devI$, JMJ703; $\Delta sncO$, JMJ688) is indicated at the right. Fitness was measured relative to ICEBsI-cured cells (JMJ550) during competitions in biofilms. ICEBsI-containing cells were started at a frequency of 0.01. Data shown are the median $\pm$ standard deviation from at least 3 independent experiments (at least 9 total competitions per strain mixture), with the exception of JMJ688 which was measured only in 1 experiment (3 total competitions).

Sporulation of the *Pxis-devI* strain was strongly inhibited under conditions that normally support robust sporulation, including in biofilms (Fig. 5). During sporulation either with (Fig. 5A) or without biofilm formation (Fig. 5B), the frequency of the *Pxis-devI* strain in the population rose from ~0.01 to ~0.05, giving a relative fitness of ~5. This is greater than the typical fitness conferred by *ICEBsI* in biofilms, but less than that observed during sporulation without biofilms. We suspect these differences are due to constitutive expression of *devI* in the absence of *ICEBsI*'s regulatory systems and the earlier onset of starvation on DSM agar compared to MSgg agar; cells that are unable to sporulate eventually die.

DevI likely targets the developmental transcription factor Spo0A

Results described above demonstrated that *devI* is a robust inhibitor of sporulation. Sporulation is controlled by the transcription factor Spo0A {reviewed in (Hoch, 1993; Sonenshein, 2000)}, which both directly and indirectly regulates the expression of many genes needed for development, including biofilm formation (Hamon and Lazazzera, 2001). The results described below indicate that DevI most likely targets Spo0A.

devI inhibits early sporulation gene expression. We found that *devI* inhibits expression of genes normally activated early during sporulation. Sporulation is initiated when Spo0A~P directly stimulates transcription of several genes, including the three sporulation operons, *spoIIA*, *spoIIIE*, and *spoIIG* (Sonenshein, 2000). Using *lacZ* fusions to the promoters of each of these operons, we found that *Pxis-devI* inhibited activity of each promoter compared to wild-type during sporulation in liquid sporulation medium (Fig. 6A). This indicates that DevI inhibits the initiation of sporulation.

devI inhibits biofilm gene expression. We also found that *devI* inhibits expression of genes needed for extracellular matrix production during biofilm formation. We measured expression of

350

Figure 5. *devI* alone is sufficient to inhibit sporulation and provide a selective advantage. ICEBs1-cured cells that constitutively express *devI* (JM725, black circles) were mixed at an initial frequency of 0.01 with ICEBs1-cured cells containing an empty expression construct (JM727, open squares). The mixture was spotted onto biofilm growth medium (A) or sporulation medium (B). Biofilms and colonies were harvested at the indicated times to determine the fraction of CFUs derived from heat-resistant spores for both the *devI*-containing and control cells. Boxes below each graph indicate the frequency of *devI*-containing CFUs at each timepoint. Data shown are the average from two populations per timepoint with error bars indicating the standard deviation.

biofilm matrix genes *epsB* and *tasA* by RT-qPCR with primers internal to each gene. In early stationary phase in liquid biofilm medium, transcript levels of *epsB* and *tasA* were reduced by about 5-fold and 3-fold, respectively, in the *Pxis-devI* strain compared to wild-type (Fig. 6B). We suspect that inhibition of biofilm matrix genes, in addition to delaying sporulation, is an important mechanism of selection for ICEBsI host cells during growth in a biofilm. This is consistent with the selective advantage of ICEBsI host cells in biofilms without sporulation described earlier. Inhibition of biofilm and early sporulation genes is consistent with DevI functioning as an inhibitor of Spo0A or its activation by phosphorylation.

***devI* is conserved among ICEs homologous to ICEBsI**

We found that *devI* (*ydcO*) is conserved among *Bacillus* species and in many cases is located within what appear to be ICEs similar to ICEBsI. We used NCBI BLAST to search for homologous protein sequences using both pBLAST (protein database) and tBLASTn (translated nucleotide database). Homologs with 100% sequence coverage and greater than 70% identity to YdcO from *B. subtilis* NCIB3610 were found in dozens of other *B. subtilis* strains and in closely related species including *B. licheniformis*, *B. atrophaeus*, and *B. amyloliquefaciens*. Excluding *Bacillus* species from the searches to possibly identify more distantly related proteins with known functions produced no hits.

We analyzed the sequence surrounding the *devI* (*ydcO*) homologs identified to determine if there is similarity to ICEBsI. All of the *devI* (*ydcO*) homologs appear to be within mobile element regions similar to ICEBsI, though some are clearly missing genes present in ICEBsI. Although we cannot infer whether any of these regions are functional mobile elements, we suspect that the ability to inhibit host development may be a conserved strategy among ICEBsI-like elements and possibly other ICEs with cargo genes of unknown function.

374

Figure 6. *devI* inhibits expression of genes associated with sporulation initiation and biofilm formation. (A) ICEBs1-cured cells that constitutively express *devI* (black circles) or contain an empty expression construct (open squares) were grown in liquid sporulation medium. Cells were harvested at the indicated times and β -galactosidase specific activity was measured. Strains: *PspolIIA-lacZ* *Pxis-devI* (JM1732), *PspolIIA-lacZ* WT (JM1735), *PspolIIE-lacZ* *Pxis-devI* (JM1731), *PspolIIE-lacZ* WT (JM1734), *PspolIIG-lacZ* *Pxis-devI* (JM1733), *PspolIIG-lacZ* WT (JM1736). A representative experiment is shown. (B) ICEBs1-cured cells that constitutively express *devI* (JM1725, black circles) and ICEBs1-cured cells containing an empty expression construct (JM1727, open squares) were grown in liquid biofilm medium, and cells were harvested at the indicated times. cDNA was synthesized using reverse transcriptase and RT-qPCR was used to measure expression of biofilm-associated genes *epsB* and *tasA*. The transcript copy numbers of these genes were measured relative to a housekeeping gene *gyrA*. The data reported are the average of three technical replicates from one experiment. The relative expression levels are normalized to wild-type at T₋₁. A representative experiment is shown.

Discussion

Our work demonstrates that *ICEBsI* confers a selective advantage on its host cells by delaying biofilm and spore development, enabling the host to grow more than cells without *ICEBsI*. When *ICEBsI*-containing cells are the minority in a mixed population, *ICEBsI* genes are induced. One of these genes, *devI*, is necessary and sufficient to inhibit biofilm- and sporulation-associated gene expression, likely by inhibiting the key developmental regulator Spo0A. Together with previous findings we conclude that *ICEBsI* encodes at least three distinct strategies to benefit its host cells. 1) Inhibition of development (described here) provides a growth advantage in biofilms and during sporulation. 2) Exclusion, mediated by *yddJ*, blocks the conjugation machinery and protects the host cell from lethal excessive transfer (Avello *et al.*, 2019). 3) An abortive infection mechanism, mediated by *spbK* (*yddK*) protects populations of *ICEBsI* host cells from predation by the lysogenic phage SP β (Johnson *et al.*, 2020). We propose that all three strategies provide a competitive advantage for *ICEBsI* and its host cells in different conditions.

Expression of *devI* reduces biofilm matrix expression and delays the initiation of sporulation. Production of the biofilm matrix is a public good, benefiting the whole community (Dragoš *et al.*, 2018). Avoidance of matrix production can therefore be considered an exploitative behavior. Exploitation can be detrimental to the population as a whole (Smith and Schuster, 2019), but we did not observe any negative effects on populations under conditions where *ICEBsI* host cells had an advantage. This is in agreement with the facultative nature of *ICEBsI* cheating (Even-Tov *et al.*, 2016; Pollak *et al.*, 2016). Quorum-sensing by *rapI-phrI* ensures that *ICEBsI* cheats only as a minority, where its impact on total public goods levels is negligible. Interestingly, the pBS32 plasmid utilizes direct regulation of biofilm formation by a Rap receptor to its benefit (Bendori *et*

al., 2015; Pollak *et al.*, 2015), while in *ICEBsI* this regulation was moved from the Rap receptor to one of its regulated genes.

The fitness consequences of sporulation inhibition are complicated (Mutlu *et al.*, 2018). Delaying sporulation too long would result in a loss of viability of the starved cells. Inhibition of sporulation by *ICEBsI* appears to be transient; *ICEBsI* host cells eventually sporulate and do not lose significant viability as a consequence of delaying sporulation. Regulation of *devI* expression by cell-cell signaling is likely critical for transient developmental inhibition.

***ICEBsI* stability during sporulation**

We hypothesize that in addition to providing a fitness advantage to its host cell, delaying sporulation may also improve stability of *ICEBsI* in the host during development. Sporulation involves the formation of an asymmetric division septum generating the larger mother cell and the smaller forespore (Errington, 2001; Higgins and Dworkin, 2012). Sporulation is induced when cells are at a high population density and running out of nutrients, conditions that also activate *ICEBsI* (Auchtung *et al.*, 2005; Grossman and Losick, 1988). The plasmid form of *ICEBsI* that is generated after excision from the chromosome is not known to have a mechanism for active partitioning and is more likely to remain in the larger mother cell if the cells do enter the sporulation pathway and divide asymmetrically. Therefore, the ability of *ICEBsI* to delay the initiation of sporulation under conditions where the element is activated could help prevent loss of the element and maintain *ICEBsI* in host cells.

Mobile genetic elements employ various strategies to promote their maintenance during sporulation. Rates of curing during sporulation for various plasmids in *Bacillus* species vary widely and do not necessarily correlate with their stability during normal cell division (Tokuda *et al.*, 1993; Turgeon *et al.*, 2008). Mechanisms encoded by plasmids to promote their stability

during growth and sporulation include the production of dynamic cytoskeletal filaments (Becker *et al.*, 2006) and post-segregational killing of plasmid-cured pre-spores with toxin-antitoxin systems (Short *et al.*, 2015). Interestingly, even lytic phage genomes can be incorporated into spores (first described in the 1960s) by co-opting the host's chromosomal partitioning system (Meijer *et al.*, 2005).

Diversity of cargo genes and associated phenotypes

Mobile genetic elements, especially ICEs, are widespread in bacteria (Frost *et al.*, 2005; Guglielmini *et al.*, 2011). Many known mobile genetic elements encode cargo genes that confer easily recognizable phenotypes, notably antibiotic resistance. Other cargo genes provide less obvious phenotypes but still fundamentally alter the physiology of the host cell. A large (500-kb) ICE was discovered in *Mesorhizobium loti* because its horizontal transfer conferred the ability to form nitrogen-fixing symbiotic root nodules on *Lotus* plant species (Sullivan and Ronson, 1988). In many pathogens, cargo genes in mobile elements are largely responsible for virulence. For example, *Vibrio cholerae* is capable of a pathogenic lifestyle in human hosts due to the toxin-encoding phage CTXΦ (Waldor and Mekalanos, 1996). In the sporulating pathogen *Bacillus anthracis*, mobile genetic elements regulate both virulence and host development. Two plasmids, pXO1 and pXO2, provide the genes for toxin synthesis and production of a protective capsule, respectively (Green *et al.*, 1985; Mikesell *et al.*, 1983). pXO1 also contains a regulatory gene, *atxA*, that regulates virulence factor production and inhibits host cell sporulation (Dale *et al.*, 2018). Co-regulation of virulence factors and sporulation is likely important during infection, as *B. anthracis* spores are thought to be more susceptible than vegetative cells to eradication by the immune system (Mock and Fouet, 2001).

Mobile elements are also known to alter the host's interaction with other horizontally acquired DNA, which has implications for the fitness and evolvability of the host. For example, the plasmid pBS32 in *B. subtilis* encodes an inhibitor of the host's DNA uptake machinery, blocking natural transformation (Konkol *et al.*, 2013). Interestingly, genes with roles in defense against foreign DNA, CRISPR-Cas systems, are also identified within mobile elements (Faure *et al.*, 2019; McDonald *et al.*, 2019; Millen *et al.*, 2012). Competition between mobile elements not only shapes the repertoire of cargo genes in a given cell, but it may also protect the host from harmful elements.

Many mobile genetic elements have been identified bioinformatically from genome sequences or discovered by means other than the phenotypes they provide (Bi *et al.*, 2012; Guglielmini *et al.*, 2011; Johnson and Grossman, 2015). Many elements lack obvious cargo genes, or at least lack cargo genes that have recognizable functions (Cury *et al.*, 2017). We suspect that many elements with uncharacterized cargo genes provide important traits to their hosts beyond the scope of the phenotypes currently attributed to mobile elements. Though mobile genetic elements can have remarkably broad host ranges, such as the Tn916-Tn1545 group of ICEs (Clewett *et al.*, 1995; Roberts and Mullany, 2009) and the IncP-1 group of plasmids (Popowska and Krawczyk-Balska, 2013), cargo genes and their associated functions could be highly specific to certain hosts.

Characterization of unknown cargo genes is likely to expand the diversity of traits currently attributed to mobile genetic elements. We speculate that many of these genes modulate normal host functions rather than provide entirely new phenotypes. Understanding cargo gene function is critical for understanding interactions between and co-evolution of mobile elements and their hosts.

Methods and Materials

Media and growth conditions

Prior to competition experiments, cells were grown as light lawns for approximately 20 hours at room temperature on 1.5% agar plates containing 1% w/v glucose, 0.1% w/v monopotassium glutamate, and 1x Spizizen's salts (2 g/l (NH₄)SO₄, 14 g/l K₂HPO₄, 6 g/l KH₂PO₄, 1 g/l Na₃citrate-2H₂O, and 0.2 g/l MgSO₄-7H₂O) (Harwood and Cutting, 1990). Cells were resuspended from light lawns and grown at 37°C with shaking in S7₅₀ defined minimal medium (Jaacks *et al.*, 1989) with 1% w/v glucose and 0.1% w/v monopotassium glutamate. Biofilms were grown at 30°C on MSgg agar plates (as defined in Branda *et al.*, 2001 with the exception of tryptophan and phenylalanine, which we did not include). The sporulation medium used was DSM (in liquid form or as plates solidified with 1.5% agar) (Harwood and Cutting, 1990). MSgg agar and DSM agar plates were dried for 20-24 hours at 37°C prior to use. Antibiotics were used at the following concentrations for selection on LB agar plates: chloramphenicol (5 µg/ml), kanamycin (5 µg/ml), spectinomycin (100 µg/ml), tetracycline (12.5 µg/ml), and a combination of erythromycin (0.5 µg/ml) and lincomycin (12.5 µg/ml) to select for macrolide-lincosamide-streptogramin (MLS) resistance.

Strains and alleles

The *B. subtilis* strains used are listed in Table 2. The strain background used in all experiments was a derivative of the undomesticated strain NCIB3610 lacking its endogenous plasmid pBS32 (NCIB3610 plasmid-free). ICEBsI⁰ indicates the strain is cured of ICEBsI. Standard techniques were used for cloning and strain construction (Harwood and Cutting 1990). Some alleles were previously described and are summarized below. Variants of ICEBsI that were blocked for transfer contained the *conEK476E* mutation derived from MMB1118 (Berkmen

et al., 2010). The *spo0AΔPs* allele was derived from AG1242 (Siranosian and Grossman, 1994). The *amyE::PspoIIA-lacZ cat* allele was derived from KI938 (Chung *et al.*, 1994). Essentially identical alleles with *PspoIIE* and *PspoIIG* were also used.

Construction of selective markers for mating and competition experiments. *ICEBsI* was marked with the *cat* gene (conferring chloramphenicol resistance) between the divergently transcribed genes *yddJ* and *spbK* (*yddK*). Markers used to select *ICEBsI*-containing and *ICEBsI*⁰ cells were all integrated at *lacA* and contained *spec* (spectinomycin resistance), *mls* (macrolide-lincosamide-streptogramin resistance), or *kan* (kanamycin resistance). The *mls* and *kan* constructs also contained constitutively expressed fluorescent proteins BFP and RFP, respectively. The plating efficiency of all markers was verified, and control competitions (described below) were performed to measure marker-associated fitness effects.

Construction of *ICEBsI* mutants. Fitness measurements of *ICEBsI* mutants were performed in a version of *ICEBsI* unable to excise and replicate (locked-in *ICEBsI*). All mutants were isogenic to JMJ646 (*ICEBsI* oriT* $\Delta attR::tet$), which is unable to excise due to the *attR::tet* deletion (Lee and Grossman, 2007). The origin of transfer was mutated (oriT*) to prevent *ICEBsI* replication while integrated, which is detrimental (Lee and Grossman, 2007; Menard and Grossman, 2013). The markerless oriT* mutation was constructed by cloning *nicK*(oriT*) from pJT245 and ~1 kb of upstream sequence into pCAL1422 (a plasmid that contains *E. coli lacZ*) by isothermal assembly (Gibson *et al.*, 2009), essentially as previously described (Thomas *et al.*, 2013). The resulting plasmid, pJMJ196, was integrated into the chromosome by single-crossover recombination. Transformants were screened for loss of *lacZ*, indicating loss of the integrated plasmid, and PCR was used to identify a clone containing the oriT* allele. Markerless deletions of *ICEBsI* sequence were also generated using pCAL1422-derived plasmids. The *rapI-phrI*

deletion was generated using pJMJ430 and removes the *rapI* and *phrI* ORFs. The *Pxis* deletion was generated using pJMJ199 and removes sequence from 149 bp to 27 bp upstream of the *xis* ORF. This removes the presumed -35 and -10 of the promoter but does not remove the known regulatory sites at the neighboring *immR* promoter (Auchtung *et al.*, 2007). The *ydzL* deletion was generated using pELS5 and fuses the first four and last two codons of *ydzL*. The *devI* deletion was generated using pELS1 and fuses the first four and last two codons of *devI*. The *sncO* deletion was generated using pELC815 and removes the *sncO* gene and 44 bp of upstream sequence.

Construction of ectopic *rapI-phrI* construct. The *rapI-phrI* ORFs plus the promoter region (352 bp upstream of *rapI*) and 112 bp of downstream sequence were cloned into pMMH253 (vector for integration at *bcaP*). The resulting plasmid (pJMJ354) was linearized and introduced to *B. subtilis* by transformation and selection for kanamycin resistance. The corresponding empty control construct was generated by transforming linearized pMMH253.

Construction of *lacA::Pxis-devI*. We expressed *devI* from the *Pxis* promoter by cloning existing elements from a *Pxis* gene expression construct marked with *mls* at *lacA* and inserting the *devI* ORF by isothermal assembly. The resulting product was introduced to the chromosome by transformation and selection for MLS resistance. The *Pxis*-empty control strain contains an identical construct lacking an ORF fused to *Pxis*.

Biofilm mating experiments

Cultures were started from resuspended light lawns (described above) diluted to an initial OD600 of 0.05 in S7₅₀ minimal medium. Cultures were grown to mid-exponential phase (OD600 ~0.5) at 37°C with shaking. Cells were pelleted, resuspended in 1x Spizizen's salts, and diluted to an OD600 of 0.01. Donor and recipient strains were mixed at the indicated frequencies and 50

μl of the mixture was spotted onto the center of MSgg agar plates. Spots were allowed to dry at 30°C before flipping the plates. Plates were incubated at 30°C for 4 days. At the time of inoculation, the strain mixes were diluted and plated on LB agar plates containing the appropriate antibiotics to determine the initial CFU/ml of the donor and recipient strains. After 4 days, the mature biofilms were scraped from the agar surface with sterile wooden sticks and resuspended in 5 ml 1x Spizizen's salts, followed by mild sonication to disperse the cells. Cells were diluted and selectively plated to determine the final CFU/ml of transconjugants.

Competition experiments

Cells were prepared for competition experiments as described above for biofilm mating experiments. Strain mixtures at the indicated frequencies were spotted onto MSgg agar plates for biofilm competitions and DSM agar plates for sporulation competitions. Plates were incubated at 30°C for 4 days unless otherwise indicated. Biofilms/colonies were collected, disrupted, and plated as described above. For time-course competitions, two replicate biofilms/colonies were collected at each of the indicated times. Sporulation frequency was determined by selective plating before and after a heat treatment at 85°C for 20 minutes to enumerate total CFUs and CFUs derived from heat-resistant spores. Relative fitness of *ICEBsI*-containing cells over *ICEBsI*⁰ cells was determined as $(p_f/(1-p_f))/(p_i/(1-p_i))$, where p_f , p_i are the frequencies of *ICEBsI*-containing cells in the final and initial populations, respectively. Control competitions between *ICEBsI*-cured cells were performed to determine the fitness associated with the *lacA::Pveg-mTagBFP mls* marker (JMJ574) used in *ICEBsI*-containing cells relative to the *lacA::Ppen-mApple2 kan* marker used in *ICEBsI*-null cells (JMJ550). When the *mls*-marked cells were started at a frequency of 0.01, their relative fitness was 0.7 ± 0.09 (average and standard deviation from 3 independent experiments and a total of 9 biofilms).

Gene expression assays

Expression of sporulation genes was measured in cultures grown from single colonies in liquid DSM at 37° with shaking. Cells were harvested at the indicated timepoints. For β -galactosidase assays, growth was stopped by the addition of toluene (~1.5% final concentration). β -galactosidase specific activity ($\{\Delta A_{420}$ per minute per ml of culture per $OD_{600}\} \times 1000$) was measured as described (Miller, 1972) after pelleting cell debris.

Biofilm gene expression was measured in cultures grown from single colonies in liquid MSgg at 37° with shaking. For RT-qPCR assays, cells were harvested directly into ice-cold methanol (1:1 methanol to culture volume) and pelleted. RNA was isolated using Qiagen RNeasy PLUS kit with 10 mg/ml lysozyme. iScript Supermix (Bio-Rad) was used for reverse transcriptase reactions to generate cDNA. Control reactions without reverse transcriptase were performed to assess the amount of DNA present in the RNA samples. RNA was degraded by adding 75% volume of 0.1 M NaOH and incubating at 70°C for 10 minutes, followed by neutralization with an equal volume of 0.1 M HCl. qPCR was done using SSoAdvanced SYBR master mix and CFX96 Touch Real-Time PCR system (Bio-Rad). Primers used to measure *epsB* were oJJ363 (5'-CGGAACAATATCGCACCATTTC-3') and oJJ364 (5'-CGCTGCACTGAACGATTTAC-3'). Primers used to quantify *tasA* were oJJ367 (5'-GGATCACTTGCGATCAAAGAAG-3') and oJJ368 (5'-CTTCAAACCTGGCTGAGGAAATC-3'). Primers used to measure the control locus *gyrA* were oMEA128 (5'-TGGAGCATTACCTTGACCATC-3') and oMEA129 (5'-AGCTCTCGCTTCTGCTTTAC-3'). The relative transcript copy numbers (as indicated by the C_p values measured by qPCR) of *epsB* and *tasA* were normalized to *gyrA* after subtracting the signal from control reactions without reverse transcriptase.

Table 2: *B. subtilis* strains used¹

Strain	Relevant genotype
JMJ550	ICEBsI ⁰ <i>lacA</i> ::{Ppen- <i>mApple2 kan</i> }
JMJ574	ICEBsI ⁰ <i>lacA</i> ::{Pveg- <i>mTagBFP mls</i> }
JMJ576	ICEBsI ⁰ <i>bcaP</i> ::{PrapI- <i>rapIphrI kan</i> } <i>lacA</i> ::{Pveg- <i>mTagBFP mls</i> }
JMJ592	ICEBsI <i>yddJ-cat-yddK lacA</i> ::{Pveg- <i>mTagBFP mls</i> }
JMJ593	ICEBsI <i>conEK476E yddJ-cat-yddK lacA</i> ::{Pveg- <i>mTagBFP mls</i> }
JMJ646	ICEBsI <i>oriT* attR::tet lacA</i> ::{Pveg- <i>mTagBFP mls</i> }
JMJ662	ICEBsI Δ <i>Pxis oriT* attR::tet lacA</i> ::{Pveg- <i>mTagBFP mls</i> }
JMJ686	ICEBsI <i>oriT* ΔrapIphrI attR::tet lacA</i> ::{Pveg- <i>mTagBFP mls</i> }
JMJ688	ICEBsI <i>oriT* ΔsncO attR::tet lacA</i> ::{Pveg- <i>mTagBFP mls</i> }
JMJ703	ICEBsI <i>oriT* ΔdevI attR::tet lacA</i> ::{Pveg- <i>mTagBFP mls</i> }
JMJ704	ICEBsI <i>oriT* ΔydzL attR::tet lacA</i> ::{Pveg- <i>mTagBFP mls</i> }
JMJ714	ICEBsI ⁰ <i>lacA</i> :: <i>spec bcaP::kan</i>
JMJ725	ICEBsI ⁰ <i>lacA</i> ::{Pxis- <i>devI mls</i> }
JMJ727	ICEBsI ⁰ <i>lacA</i> ::{Pxis- <i>empty mls</i> }
JMJ731	ICEBsI ⁰ <i>lacA</i> ::{Pxis- <i>devI mls</i> } <i>amyE</i> ::{PspoIIE- <i>lacZ cat</i> }
JMJ732	ICEBsI ⁰ <i>lacA</i> ::{Pxis- <i>devI mls</i> } <i>amyE</i> ::{PspoIIA- <i>lacZ cat</i> }
JMJ733	ICEBsI ⁰ <i>lacA</i> ::{Pxis- <i>devI mls</i> } <i>amyE</i> ::{PspoIIG- <i>lacZ cat</i> }
JMJ734	ICEBsI ⁰ <i>lacA</i> ::{Pxis- <i>empty mls</i> } <i>amyE</i> ::{PspoIIE- <i>lacZ cat</i> }
JMJ735	ICEBsI ⁰ <i>lacA</i> ::{Pxis- <i>empty mls</i> } <i>amyE</i> ::{PspoIIA- <i>lacZ cat</i> }
JMJ736	ICEBsI ⁰ <i>lacA</i> ::{Pxis- <i>empty mls</i> } <i>amyE</i> ::{PspoIIG- <i>lacZ cat</i> }
JMJ785	ICEBsI <i>oriT* ΔrapIphrI attR::tet bcaP</i> ::{PrapI- <i>rapIphrI kan</i> } <i>lacA</i> ::{Pveg- <i>mTagBFP mls</i> }
JMJ786	ICEBsI ⁰ <i>spo0AΔPs lacA</i> ::{Ppen- <i>mApple2 kan</i> }
JMJ788	ICEBsI <i>conEK476E yddJ-cat-yddK spo0AΔPs lacA</i> ::{Pveg- <i>mTagBFP mls</i> }

¹All strains derived from NCIB3610 plasmid-free

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