An integrative and conjugative element encodes an abortive infection system to protect host cells from predation by a bacteriophage

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running head: *ICEBsI* interferes with the phage SP β

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Abstract

Bacteriophages and integrative and conjugative elements (ICEs, a.k.a. conjugative transposons) are mobile genetic elements that can move from one bacterial cell to another and often coexist in a host genome. Many bacterial species contain at least one temperate (lysogenic) phage and an integrative and conjugative element. Most strains of *Bacillus subtilis* are lysogenic for the phage SP β and also contain the integrative and conjugative element *ICEBs1*. We found that the presence of *ICEBs1* in cells inhibited production of SP β , during both activation of a lysogen and following *de novo* infection. The *ICEBs1* gene *yddK* (renamed *spbK* for SP β killing) was both necessary and sufficient for the anti-SP β activity. Co-expression of *spbK* and *yonE*, in the absence of other *ICEBs1* and SP β genes, resulted in inhibition of cell growth and loss of cell viability. These results indicate that together *spbK* and *yonE* affected a host process needed for normal growth and that *spbK* constitutes an abortive infection system. We found that this anti-SP β phenotype protected populations of *B. subtilis* from predation by SP β , likely providing selective pressure for the maintenance of *ICEBs1* in *B. subtilis* populations. *spbK* encodes a TIR (Toll-interleukin-1 receptor)-domain protein with similarity to both plant antiviral proteins and animal innate immune signaling proteins. We postulate that selective many uncharacterized cargo genes in ICEs may confer selective advantage to cells by protecting against other mobile elements.

Introduction

Mobile genetic elements can move between host genomes or within a host's genome. The genomes of many bacterial species contain multiple functional and defective mobile elements, including insertion sequences, transposons, lysogenic phages, genomic islands, and integrative and conjugative elements (ICEs; also called conjugative transposons). In some cases, these elements constitute a substantial portion of the host genome (Perna *et al.*, 2001; Paulsen *et al.*, 2003; Sebaihia *et al.*, 2006; Darmon and Leach, 2014). Multiple elements within a given host have the potential to interact with each other, and likely co-evolve.

ICEs are mobile genetic elements that reside integrated in a host chromosome and are replicated, segregated, and passed to daughter cells along with the host genome (Wozniak and Waldor, 2010; Johnson and Grossman, 2015; Delavat *et al.*, 2017). Under certain conditions, or stochastically, an ICE can excise from the chromosome and transfer itself to a recipient cell via the element-encoded conjugation machinery, typically a type IV secretion system.

ICEs frequently carry cargo genes that are not essential for their own lifecycle, but instead benefit the host. Most well-studied ICEs were discovered because of such phenotypes (Johnson and Grossman, 2015). For example, the ICE Tn916 was discovered because it confers tetracycline resistance to host cells and can move between cells via conjugation (Franke and Clewell, 1981a; Franke and Clewell, 1981b). Likewise, many other ICEs were identified because they carry genes that confer specific phenotypes including: antibiotic resistance (Shoemaker *et al.*, 1980; Stuy, 1980; Roberts and Smith, 1980; Nugent, 1981; Mays *et al.*, 1982; Magot, 1983), pathogenesis (Carter *et al.*, 2010), symbiosis (Ramsay *et al.*, 2006), and metabolic functions (Rauch and De Vos, 1992; Hochhut *et al.*, 1997; Ravatn *et al.*, 1998; Nishi *et al.*, 2000).

Many ICEs have been identified by means other than the phenotype conferred by their cargo genes. In these cases, the functions of the cargo genes are largely unknown. We suspect that many of these cargo genes encode functions that are beneficial to the host under certain conditions, but that the appropriate conditions have not been identified.

Many strains of *Bacillus subtilis* contain at least two functional mobile genetic elements, the integrative and conjugative element *ICEBs1* (Auchtung *et al.*, 2005; Auchtung *et al.*, 2016) and the lysogenic phage SP β (Warner *et al.*, 1977). *B. subtilis* strains also contain several defective mobile genetic elements (Kunst *et al.*, 1997; Barbe *et al.*, 2009; Smith *et al.*, 2014). We found that *ICEBs1* contains a cargo gene that protects cells from predation by the lysogenic phage SP β .

ICEBs1 (Fig. 1) is found integrated in the *B. subtilis* genome in *trnS-leu2*, the gene for a leucine-tRNA. While integrated, most *ICEBs1* genes are repressed (Auchtung *et al.*, 2005; Auchtung *et al.*, 2007). *ICEBs1* is activated during the *recA*-dependent SOS response to DNA damage or in the presence of *B. subtilis* cells that do not have the element (Auchtung *et al.*, 2005). Under these conditions, *ICEBs1* gene expression is derepressed, the element excises from the chromosome and can transfer to an available recipient via the element-encoded conjugation machinery. *ICEBs1* was identified because of homology to other ICEs (Burrus *et al.*, 2002) and because it is regulated by cell-cell signaling (Auchtung *et al.*, 2005). At the time of its discovery, it was not known if *ICEBs1* conferred a beneficial phenotype to its host.

SP β is a temperate phage of *B. subtilis*. In strains lysogenic for SP β the phage is integrated in *spsM*, near the terminus of replication (Zahler *et al.*, 1977; Lazarevic *et al.*, 1999; Abe *et al.*, 2017). SP β contains genes needed for production of and resistance to the peptide antibiotic sublancin (Paik *et al.*, 1998; Lazarevic *et al.*, 1999), providing a growth advantage to the host in the presence of cells sensitive to sublancin. Most phage genes are repressed in the lysogen, but

during the *recA*-dependent SOS response to DNA damage, SP β gene expression is induced, the phage excises from the host chromosome, enters the lytic cycle producing progeny phage, and causes cell lysis and release of phage particles.

We found that the presence of *ICEBsI* in *B. subtilis* inhibited production of active SP β , both when the phage was activated from the lysogenic state and during *de novo* infection. The *ICEBsI* gene *spbK*, although dispensable for conjugation, was necessary and sufficient for the inhibition of SP β . The *spbK* gene product contains a Toll/Interleukin-1 Receptor (TIR) domain that was needed for function. The anti-SP β phenotype (abortive infection) caused by *spbK* was dependent on the SP β gene *yonE*. We found that *yonE* was essential for SP β lytic growth, but not for establishing a lysogen. Co-expression of *spbK* and *yonE* inhibited host cell growth and caused a drop in cell viability, even in the absence of any other *ICEBsI* or SP β genes. The presence of *ICEBsI* in cells prevented the spread of SP β , thereby protecting nearby *B. subtilis* cells from infection and allowing the population to continue growing. This phenotype likely provides strong selective pressure to maintain *ICEBsI* in *B. subtilis*. We postulate that other ICEs might encode abortive infection, or other anti-phage systems, providing selective pressure for host cells to maintain these ICEs.

Results

ICEBsI prevents SP β from forming plaques

ICEBsI was not known to confer phenotypes to *B. subtilis*, aside from those directly related to conjugation. However, the left end of *ICEBsI* (Fig. 1) encodes a phage-like repressor ImmR (Auchtung *et al.*, 2007), anti-repressor ImmA (Bose *et al.*, 2008), and recombinase Int (Lee *et*

al., 2007), leading us to wonder if *ICEBsI* could provide immunity to phages that infect *B. subtilis*.

We tested if the presence of *ICEBsI* in *B. subtilis* altered the ability of different phages to make plaques. We spread different phages on lawns of two *B. subtilis* indicator strains, one that was missing *ICEBsI* and has been used as an indicator strain for SPβ (CU1050; *ICEBsI*⁰) (Zahler *et al.*, 1977; Johnson and Grossman, 2016) and an isogenic derivative that contained *ICEBsI* (CMJ81; *ICEBsI*⁺). SPβ formed plaques on a lawn of the *ICEBsI*⁰ strain (Fig. S1A), but not on a lawn of the isogenic *ICEBsI*⁺ strain (Fig. S1B), even when 100-fold more plaque forming units (PFUs) were mixed with cells (Fig. S1C). Based on these results, we conclude that something about *ICEBsI* was inhibiting a step in plaque formation by SPβ.

***ICEBsI* reduces phage production during infection**

To quantify the effects of *ICEBsI* on the production of SPβ, we measured the kinetics of phage production during a single round of infection (Fig. 2A). We mixed ~10⁵ PFUs of SPβ with ~10⁷ cells (MOI = 0.01) for 5 min at 37 °C, pelleted the cells by centrifugation, washed the cells to remove unattached phage, and resuspended the cells in LB medium at 37 °C to allow for phage growth. The initial number of infective cells in the medium was determined by measuring the number of infective centers (PFUs) following the initial adsorption, and new phage production was monitored by tracking the subsequent increase in infective centers. For a strain without *ICEBsI*, the initial number of infective centers in the culture was about 90% of the initial number of phage used to inoculate the culture (Fig. 2A). The number of infective centers in the culture began to increase about 25 minutes after initial infection, and plateaued about 45 minutes after initial infection. This indicated that SPβ had an eclipse period of about 25 minutes (Fig.

2A). The burst size (number of phage produced per infective cell) was 20 ± 7 , somewhat less than the previously reported burst size of about 30 phage (Warner *et al.*, 1977).

Cells with *ICEBsI* that were exposed to SP β were less likely to become infective centers, and produced fewer phage per initial infective center. At an MOI of 0.01, the number of cells that produced any phage was reduced at least 10-fold relative to cells without *ICEBsI* (Fig. 2A). Furthermore, the number of phage produced per initial infective center was $\sim 2.2 \pm 0.4$ (Fig. 2A). Based on these results, we conclude that the presence of *ICEBsI* in cells reduced the total number of phage released from the infected culture by at least 100-fold, or to about 0.1 progeny phage per infecting phage. This reduction will not support propagation of phage in the lytic cycle.

***ICEBsI* has little or no effect on entry of phage into cells**

The *ICEBsI*-dependent reduction in plaque formation and phage production could be due to reduced entry of phage into cells. Alternatively, a step in the phage lifecycle after entry could be inhibited. If the presence of *ICEBsI* was causing a block in phage entry, then there should be a corresponding reduction in the frequency of lysogen formation. We used SP β that contained *spc*, conferring resistance to spectinomycin, to measure the frequency of lysogenization. Cells with or without *ICEBsI* were mixed with SP β ::*spc98* (MOI = 0.001), unbound phage were washed off, and cells were spread on plates containing spectinomycin to select for lysogens. The lysogenization frequency of cells without *ICEBsI* (CMJ472) was $\sim 1\%$ ($1.1 \times 10^{-2} \pm 0.46 \times 10^{-2}$), or approximately one lysogen per 100 initial phage. Similarly, the lysogenization frequency of *ICEBsI*+ cells (CMJ827) was $\sim 0.4\%$ ($4.3 \times 10^{-3} \pm 1.3 \times 10^{-3}$), or about 40% of that of the *ICEBsI*⁰ cells. These results indicate that *ICEBsI* has a relatively minor (if any) effect on lysogenization

frequency and that the anti-SP β phenotype conferred by *ICEBsI* was not due to a block in adhesion or entry of the phage.

***ICEBsI* reduces the number of phage released by SP β lysogens**

We found that the presence of *ICEBsI* in an SP β lysogen inhibited phage production. We grew lysogens in liquid medium and measured the number of PFUs present in the supernatant. We found that cultures of a lysogen without *ICEBsI* had approximately 100-fold more PFUs/ml than cultures of an ICE+ lysogen (Fig. 2C). Together our results demonstrate that *ICEBsI* acts primarily by blocking production of phage by infected cells, rather than by preventing infection of cells in the first place.

We also found that the presence of *ICEBsI* prevented production of SP β following induction of a temperature sensitive lysogen. We grew strains with a temperature sensitive SP β lysogen (SP β c2) in rich medium, induced the lysogen by heat shock, and measured phage release. Phage production was reduced by ~1,000-fold in cells with *ICEBsI* compared to cells without (Fig. 2D). Although production of functional phage particles was reduced, the cells were still killed following phage induction. Cell viability, as measured by colony forming units (CFUs), was reduced to ~0.1% after phage induction compared to right before phage induction for strains with (CMJ826) and without *ICEBsI* (CMJ114). Based on these results we conclude that *ICEBsI* was probably not preventing induction of SP β but rather was inhibiting production of active phage particles post-induction.

The *ICEBsI* gene *spbK* is necessary and sufficient to inhibit SP β

We were interested in determining which *ICEBsI* gene was responsible for the inhibition of SP β . Most *ICEBsI* genes are repressed when *ICEBsI* is integrated in the host genome. Because the inhibition of SP β did not appear to depend on activation of *ICEBsI*, we focused on the

handful of *ICEBsI* genes that are constitutively expressed, including genes toward the left and right ends of the element (Fig. 1). In preliminary testing of SP β lysogens containing deletions of different regions of *ICEBsI*, we found that strains in which *spbK* (formerly *yddK*) had been deleted did not inhibit phage production, whereas all strains in which *spbK* was intact inhibited production. Based on these results, we inferred that *spbK* was likely needed for *ICEBsI*-mediated inhibition of spontaneous release of SP β from a lysogen.

We used three different assays to test the effects of *spbK* on SP β . In all three assays, we compared three *B. subtilis* strains: an *ICEBsI*⁺ strain with *spbK* ($\Delta(rapI-phrI)::kan$, Fig. 1C), an *ICEBsI*⁺ strain lacking *spbK* ($\Delta(spbK-phrI)::kan$, Fig. 1D), and an *ICEBsI*⁰ strain expressing *spbK* from its own promoter at an ectopic locus (*ICEBsI*⁰ *amyE*::{*spbK kan*}, Fig. 1E). We measured: 1) the appearance of infective centers following a single round of infection with SP β (Fig. 2B); 2) the number of phage spontaneously released from an SP β lysogen (Fig. 2C); and 3) the number of phage produced after induction of a temperature sensitive SP β lysogen (Fig. 2D). In all cases, we found that *spbK* was necessary for *ICEBsI* to inhibit the formation of infective centers and the production of phage, and that *ICEBsI*⁺ Δ *spbK* strains were indistinguishable from strains entirely lacking *ICEBsI*. Furthermore, ectopic expression of *spbK* was sufficient to inhibit phage production in the absence of *ICEBsI* in all three assays.

Expression of the SP β gene *yonE* inhibits acquisition of *ICEBsI* and this inhibition is dependent on the *ICEBsI* gene *spbK*

Based on the results described above, we thought that there might be at least one gene in SP β that was needed for the *spbK*-mediated inhibition of phage production. Results described below indicate that *yonE* is this SP β gene.

In previous work, we identified transposon insertions in recipients that reduced the ability of cells to acquire *ICEBsI* from donor cells (Johnson and Grossman, 2014). Recipients were SP β lysogens, and we found that certain mutations in the SP β gene *yonF* reduced the ability of would-be recipients to acquire *ICEBsI* from donors in conjugation (Johnson and Grossman, 2014). Because the pattern of *yonF* mutations was not uniform, we hypothesized that the reduction in *ICEBsI* acquisition was not due to loss of *yonF*, but rather due to increased expression *yonE*, the gene immediately downstream of *yonF*, driven by the promoter the drives transcription of the antibiotic resistance gene in the transposon.

We found that expression of *yonE* reduced the ability of cells to acquire a copy of *ICEBsI*. We made a series of mutations in SP β (Fig. 3A) and tested these for effects on the ability of cells to act as *ICEBsI* recipients during conjugation. We found that an insertion of *spc* into a deletion of *yonF* ($\Delta yonF::spc$) reduced acquisition of *ICEBsI* only when *spc* was co-directional with *yonE*. Furthermore, deletion of *yonE* in this context eliminated the defect in acquisition of *ICEBsI* (Fig. 3B). In the absence of all other SP β genes, expression of *yonE* from the IPTG-inducible promoter Pspank(hy) was sufficient to inhibit acquisition of *ICEBsI* (Fig. 3B). We conclude that *yonE* in SP β is both necessary and sufficient to cause the decrease in acquisition of *ICEBsI*.

The decreased acquisition of *ICEBsI* by recipients expressing *yonE* was dependent on the *ICEBsI* gene *spbK*. We tested strains expressing *yonE* for the ability to acquire *ICEBsI* that was missing *spbK* (*ICEBsI* $\Delta spbK$), and found that they all acquired the $\Delta spbK$ element at the same frequency as wild type recipients not expressing *yonE* (Fig. 3B, right end of panel). From these results we conclude that expression of *yonE* caused a defect in *ICEBsI* acquisition and that this defect was dependent on the presence of *spbK* in the incoming *ICEBsI*. We note that loss of

spbK caused no reduction in conjugation efficiency (Fig. 3B), demonstrating that it is dispensable for conjugation.

Co-expression of *yonE* and *spbK* causes a defect in cell growth and a drop in cell viability

We found that expression of *spbK* (from its own promoter) and *yonE* (from Pspank(hy)) together caused a severe growth defect. We grew cells containing both *spbK* and *yonE* in defined minimal medium and added IPTG (time = 0) to increase expression of *yonE* (Fig. 4A). This caused a rapid growth arrest as measured by optical density (Fig. 4A). In contrast, expression of either gene alone, *spbK* from its own promoter (*lacA::spbK*), or *yonE* from an inducible promoter (*amyE::Pspank(hy)-yonE*), had no obvious effect on growth (Fig. 4A). This growth arrest was accompanied by a ~1000-fold drop in viability as measured by plating for CFUs on LB plates made with Noble agar (Fig. 4B; see below). Together, these results indicate that co-expression of *yonE* and *spbK* is detrimental to cell growth.

Despite growing normally in defined liquid medium prior to adding IPTG, cells containing both *lacA::spbK* and *amyE::Pspank(hy)-yonE* had a substantial plating defect (~200-fold) when plated on LB plates made with standard bacto-agar (Difco), and had a distinct small colony morphology even in the absence of IPTG. The plating and colony size defects were eliminated when the cells were plated on LB plates made with Noble agar (Difco) (Fig. S2), a purified form of agar that is used to culture some fastidious organisms. We hypothesize that a component of bacto-agar sensitizes cells to the detrimental impact of co-expressing *spbK* and *yonE*, such that leaky expression from Pspank(hy)-*yonE* is sufficient to trigger the growth defect.

***yonE* is needed for phage production**

To determine the effect of *yonE* on phage production, we made an unmarked deletion of *yonE* ($\Delta yonE443$) in a temperature-sensitive SP β lysogen. We found that cultures of this inducible $\Delta yonE$ lysogen cleared comparably to a *yonE*⁺ strain following a shift to high temperature (Fig. 5A), demonstrating that *yonE* is not needed for induction of SP β from a lysogenic state, nor is it needed to cause host cell lysis.

Despite the fact that the $\Delta yonE$ host cells lysed, there were no detectable viable phage (< 10 PFUs/ml) produced by the mutant lysogen (Fig. 5B, first two columns). This defect in phage production was partially complemented by expression of *yonE* from an ectopic chromosomal locus. These $\Delta yonE$ phage (recovered from the complemented strain) were capable of forming plaques on an indicator strain that also expressed *yonE* (Fig. 5B, last three columns). Although a small number of phage produced by a $\Delta yonE$ lysogen were able to form plaques on a *yonE*-indicator strain, analysis of lysogenized cells obtained from these plaques revealed that the phage had a wild type copy of *yonE*, likely obtained through homologous recombination with the *yonE* allele on the chromosome of the original host strain. Based on these results, we conclude that *yonE* is essential for production of SP β .

To determine if *yonE* is needed to form a lysogen, we made a stock of *spc*-marked $\Delta yonE$ phage by growing the $\Delta yonE$ mutant on a *B. subtilis* strain ectopically expressing *yonE*. The frequency of lysogenization of *spc*-marked *yonE*⁺ and $\Delta yonE$ phage were both approximately 1%, indicating that *yonE* is not needed for lysogen formation.

The function of *yonE* is not known. However, there are homologs in other phages, including the phage C-ST from *Clostridium botulinum*. The region of homology between C-ST and SP β extends from *yonG* to *yomZ*, indicating that these genes may encode conserved phage functions

(Sakaguchi *et al.*, 2005). The phage E3 from *Geobacillus* encodes a putative portal protein (accession number AJA41333) that is 25% identical to YonE (van Zyl *et al.*, 2015). Portal proteins are one of three molecular components involved in packaging the phage genome into the capsid during maturation. The other two components are the large and small terminase subunits (Rao and Feiss, 2015). Additional homology searches using NCBI BLAST revealed that YonF is a member of the terminase 1 superfamily and encodes a terminase 6 multidomain, typical of large terminase subunit proteins. These results indicate that YonE and YonF may be a part of the SP β head packaging machinery. This notion is consistent with the need for *yonE* in production of functional phage, but not in host cell lysis or formation of lysogens.

SpbK contains a TIR domain involved in protein-protein interaction

spbK is predicted to encode a 266 amino acid protein. Using the NCBI Delta-BLAST search tool (Boratyn *et al.*, 2012) we found that the C-terminal region of SpbK (amino acids 113-266) contains a Toll Interleukin-1 Receptor (TIR) domain in the TIR_2 superfamily (accession: cl23749) (Fig. S3A). Proteins containing TIR domains have been found in animals, plants, and bacteria. In animals, such proteins are involved in signaling cascades in development and in immune activation (Narayanan and Park, 2015). In plants they mediate disease resistance, often in response to infectious agents (McHale *et al.*, 2006). Some pathogenic bacteria encode TIR domain proteins that interact with eukaryotic host TIR domain proteins to modulate the host immune response (Rana *et al.*, 2013). Many non-pathogenic bacteria also contain TIR domain proteins and it is thought that the TIR domains mediate protein-protein interaction (Spear *et al.*, 2009). Recent work has also implicated some bacterial TIR domain proteins as components of anti-phage defense systems, though the mechanism of defense is not understood (Doron *et al.*, 2018).

Where they have been studied, TIR domains mediate protein-protein interactions by interacting with other TIR domains. *spbK* is the only gene in *B. subtilis*, including all horizontally acquired sequences (e.g, *ICEBsI* and SP β), predicted to encode a TIR domain. Using a yeast two-hybrid assay, we found that full-length SpbK multimerizes *in vivo* (Fig. S2B). Additionally, we found that the TIR domain alone interacted with both full-length SpbK and with the TIR domain, but that deleting the TIR domain abolished all interaction between SpbK proteins (Fig. S2B). We also tested for, but were unable to detect, interaction between SpbK and YonE.

***ICEBsI* protects *B. subtilis* populations from attack by SP β**

Cells containing *ICEBsI* and SP β die when SP β enters the lytic cycle, and very few infectious phage are produced. We found that in a population of cells, this abortive infection system in *ICEBsI* protected cells from killing by SP β . We grew SP β -cured strains of *B. subtilis* that either contained or did not contain *ICEBsI*, infected the cultures with SP β at a low multiplicity of infection (MOI = 0.01), and tracked the growth (optical density) of the culture, the concentration of viable cells (including lysogens), and free phage over time (Fig. 6). When cultures of an *ICEBsI*⁰ strain were infected with a clear plaque mutant of SP β (incapable of forming lysogens) the cells continued to grow at the same rate as an uninfected culture for approximately 1.5 hours, then the majority of the cells abruptly died, as evidenced by a decrease in optical density (Fig. 6A) and an approximately 5,000-fold decrease in CFUs (Fig. 6B). During this time (1.5 hrs) the concentration of phage in the culture (Fig. 6C) surpassed the concentration of cells (Fig. 6B).

In contrast, when an *ICEBsI*⁺ strain was infected with the clear plaque mutant of SP β (MOI = 0.01), cell growth was indistinguishable from an uninfected culture as measured by both the

optical density (Fig. 6D) and the number of CFUs (Fig. 6E). The population of phage in the culture generally decreased to below the initial inoculum (Fig. 6F). These results indicate that the presence of *ICEBsI* is beneficial to the population of cells even though individual infected cells may not survive.

Experiments described above were done with SPβ phage that were unable to form lysogens. We also found that the presence of *ICEBsI* in cells caused a decrease in the formation of lysogens in a population exposed to SPβ. We repeated the experiments described above with SPβ::*spc* that is otherwise wild type and able to form lysogens. Lysogens were detected as spectinomycin-resistant colonies. When cells without *ICEBsI* were infected with SPβ::*spc* (MOI = 0.01), there was a 10-fold drop in both the optical density of the culture (Fig. 6A) and the number of CFUs (Fig. 6B). During the experiment, cells became lysogenized with SPβ. Cells lysogenized by SPβ are protected from killing by new SPβ infection (Warner *et al.*, 1977). These lysogens continued to grow, and after about five hours the population of cells had increased and virtually all cells were SPβ lysogens (Fig. 6B).

In contrast, when an *ICEBsI*+ strain was infected with SPβ::*spc* (MOI = 0.01), cell growth continued and there was no obvious drop in optical density (Fig. 6D) or the number of CFUs (Fig. 6E). Five hours after the initial infection, the number of phage in the culture was below the initial inoculum (Fig. 6E) and the number of SPβ lysogens remained at approximately 10^4 - 10^5 per ml (Fig. 6F), a relatively small fraction of the total number of cells. Together, these results indicate that the presence of *ICEBsI* in cells limits phage production, thereby protecting a population of cells from predation by SPβ and limiting formation of SPβ lysogens.

Discussion

Results presented here demonstrate that *ICEBsI* encodes an anti-phage system that inhibits production of the phage SP β . This inhibition occurs upon *de novo* infection by SP β and also upon induction of an SP β lysogen. There is little or no direct effect on the formation of lysogens. The *ICEBsI* gene *spbK* is both necessary and sufficient to inhibit SP β : deleting *spbK* from *ICEBsI* abolishes the phenotype, and expressing *spbK* in a strain missing *ICEBsI* fully inhibits SP β . Expression of *yonE* apparently triggers this anti-phage response, and co-expression of *spbK* and *yonE* in a strain that otherwise lacks *ICEBsI* and SP β rapidly kills the cells. We conclude that *ICEBsI* encodes an abortive infection system that protects its host from predation by SP β .

Genes involved in protection against SP β

yonE is essential for the phage lytic cycle, but is not required for lysogen formation. Bioinformatic analysis of *yonE* and *yonF* revealed a possible role for these genes as components of the phage head-packing machinery, needed during the final stages of a phage's lytic cycle.

SpbK contains a TIR domain. Where TIR domains have been studied they generally mediate protein-protein interactions through recognition of other TIR domains. However, examples of heterotypic interactions of TIR domains with non-TIR domain proteins have been described (Nimma *et al.*, 2017). In some bacterial pathogens, TIR-domain proteins modulate the host immune response (Newman *et al.*, 2006; Cirl *et al.*, 2008; Spear *et al.*, 2009; Alaidarous *et al.*, 2014). Recent work has implicated some bacterial TIR-domain proteins as being essential components of a class of anti-phage defense systems ("Thoeris") (Doron *et al.*, 2018). Two of these Thoeris systems (from *Bacillus cereus* and *Bacillus amyloliquefaciens*) have been shown to non-specifically confer resistance to some myophages when reconstituted in *B. subtilis*, although the mechanism of this resistance is not understood.

Although SpbK and the Thoeris systems appear to have a common purpose, SpbK does not appear to be a component of a *B. subtilis* Thoeris antiphage system. The hallmark of Thoeris systems is a single gene encoding a NAD⁺ binding protein (ThsA) in proximity to (typically multiple) genes encoding TIR-domain proteins (ThsB) (Doron *et al.*, 2018). *spbK* is the only gene encoding a TIR-domain protein in *B. subtilis*. Furthermore, of all the genes in *ICEBs1*, *spbK* is both necessary and sufficient for protection from SP β , and there is no indication that SpbK contains a nucleotide binding domain. Irrespective of these differences, our analysis of SpbK raises the possibility that Thoeris anti-phage systems might function as abortive infection systems.

Abortive infection systems

The anti-phage phenotype encoded by *ICEBs1* resembles abortive infection systems that have been described for *Lactococcus*, *Escherichia coli*, and other bacteria. Such systems detect infection of a bacterial cell by phage and respond by inhibiting a host process needed for phage maturation and release (Labrie *et al.*, 2010). The mechanisms of inhibition vary widely, but often target a critical host process. The cellular process that is inhibited by SpbK is evidently essential for the host, as co-expression of *spbK* and *yonE* results in cell death. We have not yet determined what essential process (es) are targeted to cause SpbK-YonE-induced cell death.

Cargo genes in ICEs

The cargo genes of mobile genetic elements, including ICEs, are those genes that are not necessary for the function of the mobile element, but are part of and transferred with the element. Cargo genes on a mobile element can often allow bacteria to rapidly acquire a new phenotype through acquisition of the element. Historically, most well studied ICEs were identified because

of the phenotype (s) conferred by the cargo genes (Johnson and Grossman, 2015). Identification and characterization of the responsible genes revealed that they were in an ICE.

Many ICEs are being identified by bioinformatic analyses of sequenced bacterial genomes (Burrus *et al.*, 2002; Guglielmini *et al.*, 2011). In most of these analyses, it is not clear what, if any, phenotype is conferred by the ICE to its host. We suspect that many other ICEs with cargo genes of unknown function likely assist their hosts in mediating interactions with other mobile genetic elements.

Methods

Media and growth conditions

E. coli cells were grown in LB medium and on LB plates containing 1.5% agar at 37°C. *S. cerevisiae* cells were grown in YPAD and on YPAD plates or synthetic dropout (SD) plates containing 2% agar and appropriate supplements to test for the indicated auxotrophies (James *et al.*, 1996; Gietz and Schiestl, 2007). *B. subtilis* cells were grown in LB or S7₅₀ defined minimal medium with 0.1% glutamate (Jaacks *et al.*, 1989) with either glucose or arabinose (1% w/v) as a carbon source and on LB plates containing 1.5% bacto-agar or on Noble Agar (1.5%) for strains expressing both *spbK* and *yonE*. Antibiotics and other additives were used at the following concentrations for *E. coli*: carbenicillin (100 µg/ml), *B. subtilis*: kanamycin (5 µg/ml), spectinomycin (100 µg/ml), chloramphenicol (5 µg/ml). The Pspank(hy) promoter was activated with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the Pxyl promoter was activated with 1% (w/v) xylose, typically in cells grown in arabinose.

Strains and alleles

SP β ::*spc*. *spc* (spectinomycin resistance) was inserted between *yolB* and *yolC* in SP β . *spc* was amplified by PCR, and Gibson assembly (Gibson *et al.*, 2009) was used to join this fragment to genomic sequences containing the apparent bidirectional terminator located between the convergently transcribed genes *yolB* and *yolC* (de Hoon *et al.*, 2005) such that a copy of the terminator is located on each side of *spc*. This was then used to transform naturally competent *B. subtilis* cells selecting for resistance to spectinomycin. An antibiotic resistant strain (CMJ98) was identified and the location of the *spc* gene verified by sequencing. This strategy resulted in duplication of the terminator with *spc* located between the terminators. The resulting phage is referred to as SP β ::*spc*98, or SP β ::*spc*.

Δ (*spbK-rapI-phrI*)1500::*kan*. The region of ICEBsI encoding *spbK-rapI-phrI* was replaced with *kan*. The deletion replaces all of *spbK*, *rapI*, and *phrI*, and was designed such that the orientation of *kan* and the *phrI* deletion boundary would be identical to the Δ (*rapI-phrI*)342::*kan* allele of IRN342 (Auchtung *et al.*, 2005).

Δ yonEF396::*spc*. A deletion-insertion that replaces *yonE* and *yonF* with a co-directional *spc* insertion.

Δ yonE443. The unmarked Δ yonE443 allele was made by replacing *yonE* with the *cat* gene, flanked by *lox* sites (CMJ434). The Cre recombinase, expressed from the temperature-sensitive plasmid, pDR244, was then used to remove the *lox*-flanked *cat* gene by recombination. Strains were then cured of pDR244 by culturing them on LB + 1.5% agar at 45°C, as previously described (Meisner *et al.*, 2013; Johnson and Grossman, 2014), resulting in strain CMJ443.

Overexpression of *yonE*. The *yonE* coding sequence was cloned into a plasmid containing the IPTG-inducible Pspank(hy) promoter (Britton *et al.*, 2002), *lacI*, and either *spc* situated between

genomic sequence from *amyE*, or *mls* situated between genomic sequence from *lacA*. The resulting construct was then transformed into competent *B. subtilis* cells. The following strains carrying a double-crossover of the given construct were identified by antibiotic resistance and PCR: CMJ403, *lacA*::{Pspank(hy)-*yonE lacI mls*}; CMJ616, *amyE*::{Pspank(hy)-*yonE lacI spc*}. Pspank(hy) is only partly repressed by LacI and was fully derepressed upon addition of 1 mM IPTG. Constructs lacking the *yonE* insert were also transformed into *B. subtilis* to generate the control alleles *lacA*::{Pspank(hy)-empty *lacI mls*} and *amyE*::{Pspank(hy)-empty *lacI spc*}.

Expression of *spbK* To study expression of *spbK* in the absence of other ICEBsI genes, a fragment containing the *spbK* coding sequence and 330 bp upstream was amplified by PCR and cloned into a plasmid for double-crossover integration into *lacA* or *amyE*. For cloning into *lacA*, the *spbK* fragment was cloned by Gibson assembly into a plasmid containing *kan* and parts of *lacA* suitable for double crossover. For cloning into *amyE*, the *spbK* fragment was cloned into a plasmid containing *cat* flanked by genomic sequences flanking the *amyE* locus by Gibson assembly. The resulting constructs were transformed into naturally competent *B. subtilis* cells and strains carrying a double crossover were identified as above, resulting in strains CMJ74 {*amyE*::(*spbK cat*)} and CMJ684 {*lacA*::(*spbK kan*)}.

Plaque assays

To quantify the number of PFUs, samples with phage were diluted in LB and 100 µl of appropriate dilutions were mixed with 300 µl of an indicator strain at an OD600 of 0.5. Phage and cells were incubated at room temperature for 5 minutes, then mixed with 3 ml of soft agar (soft agar contains 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, 6.5 g/l agar). The soft agar was spread on warm LB plates and incubated overnight at 37°C, allowing a lawn of cells to form. Plaques in the lawn were then counted. To photograph plaques, bacterial lawns were

stained with 2,3,5 - triphenyltetrazolium chloride (Pattee, 1966) (TTC, Sigma). Briefly, 8 ml of 0.1% TTC in LB was pipetted onto plates and incubated at 37°C for 30 minutes. The TTC solution was then aspirated off and the petri dishes were photographed.

Single-round infection experiments

Cells of the strain to be infected were cultured in rich medium to mid- to late exponential phase. The OD600 was then adjusted to 0.5 and 100 µl of cells was mixed with 10 µl medium containing 10⁵ PFU of SPβ (MOI 1:100). Cells and phage were co-incubated for 5 min at 37°C, then washed 3 times by adding 1 ml LB, pelleting the cells in a microcentrifuge and removing the supernatant. The washed pellet was resuspended in 10 ml LB and incubated at 37°C with aeration to allow the phage to develop. Samples of the culture were taken at various time points and used immediately for plaque assays to quantify the concentration of infective centers (free phage + infected cells) in the culture.

Quantification of lysogeny

The frequency of lysogenization was determined using SPβ::*spc98*. 10⁴ PFUs of SPβ::*spc98* in 10 µl LB were added to 100 µl of an indicator strain at an OD600 of 0.5 (an MOI of approximately 1:1000). Phage and cells were incubated at room temperature for 5 minutes, then cells were washed 3x with 1 ml LB to remove unbound phage. Cells were then spread on LB plates with spectinomycin to select for cells that had become lysogenized with SPβ::*spc*.

Yeast two-hybrid assays

The yeast two hybrid strains and vectors used in this study have been previously described (James *et al.*, 1996). Briefly, the coding sequence for *spbK* from amino acids 1-104 (N-terminus), 97-266 (TIR domain) and full length *spbK* were cloned into pGAD-C1 and fused to the *GAL4* activation domain or pGBDU-C3 and fused to the *GAL4* DNA binding domain. These

vectors were then transformed into competent PJ69-4A cells using the LiAc method of Gietz and Schiestl (Gietz and Schiestl, 2007) and plated on synthetic dropout (SD) medium with appropriate supplements to select for acquisition of the plasmids. The ability to grow in the absence of leucine (pGAD-based plasmids) or uridine (pGBDU-based plasmids) was used to select clones that acquired each plasmid. To test for interaction between peptides, yeast strains carrying the plasmids of interest were spotted on SD medium and scored for growth in the absence of adenine, with growth indicating an interaction. As a control, strains carrying each individual plasmid were also scored for growth in the absence of adenine (all were negative).

Mating assays

Mating assays were performed as previously described (Lee *et al.*, 2007; Johnson and Grossman, 2014). Briefly, donors and recipients were grown separately in minimal medium with 1% arabinose as a carbon source. RapI expression was induced in donors for 2 hours with 1% xylose. Approximately equal numbers of donors and recipients were then mixed, collected on a filter and placed on 1.5% agar plates buffered with Spizizens minimal salts (SMS agar contains 15 mM ammonium sulfate, 80 mM dibasic potassium phosphate, 44 mM monobasic potassium phosphate, 3.4 mM trisodium citrate, 0.8 mM magnesium sulfate, and 1.5% agar at pH 7.0) (Harwood and Cutting, 1990) for 90 minutes. Cells were rinsed off the filter, diluted, and spread on LB plates with selective antibiotics and incubated at 37°C overnight before quantification of colony forming units.

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Table 1. *B. subtilis* strains used.

<i>B. subtilis</i> Strain	Genotype (reference)
AG174	<i>trpC2 pheA1</i> a.k.a., JH642 (Smith <i>et al.</i> , 2014)
CAL321	<i>trpC2 pheA1</i> Δ (<i>rapI-yddM</i>)318:: <i>kan</i> (Lee and Grossman, 2007)
CAL322	<i>trpC2 pheA1</i> Δ (<i>yddG-yddM</i>)319:: <i>kan</i> (Lee and Grossman, 2007)
CAL323	<i>trpC2 pheA1</i> Δ (<i>yddB-yddM</i>)320:: <i>kan</i> (Lee and Grossman, 2007)
CAL347	<i>trpC2 pheA1</i> Δ (<i>ydcR-yddM</i>)347:: <i>kan</i> (Lee and Grossman, 2007)
CAL348	<i>trpC2 pheA1</i> Δ (<i>ydcQ-yddM</i>)348:: <i>kan</i> (Lee and Grossman, 2007)
CAL1500	<i>trpC2 pheA1</i> Δ (<i>spbK-rapI-phrI</i>)1500:: <i>kan</i>
CMJ48	PY79 (ICEBsI ⁰) SP β + <i>amyE</i> ::{Pspank(hy)-empty <i>lacI spc</i> }
CMJ74	JH642 <i>trpC2 pheA1</i> ICEBsI ⁰ <i>amyE</i> ::{ <i>spbK cat</i> }
CMJ81	CU1050 (SP β ⁰) ICEBsI:: <i>kan</i> (non-disruptive)
CMJ82	CU1050 (ICEBsI ⁰) (SP β ⁰) <i>amyE</i> ::{ <i>spbK cat</i> }
CMJ98	JH642 <i>trpC2 pheA1</i> ICEBsI ⁰ <i>yolBC98</i> :: <i>spc</i>
CMJ104	PY79 (ICEBsI ⁰) SP β + Δ <i>yonF67</i> :: <i>spc</i> (Johnson and Grossman, 2014)
CMJ114	CU1050 (ICEBsI ⁰) SP β c2 <i>yolBC98</i> :: <i>spc</i>
CMJ116	CU1050 (ICEBsI ⁰) SP β c2 <i>yolBC98</i> :: <i>spc amyE</i> ::{ <i>spbK cat</i> }
CMJ388	PY79 (ICEBsI ⁰) SP β + Δ <i>yonF155</i> :: <i>spc</i> (reverse orientation) (Johnson and Grossman, 2014)
CMJ402	PY79 (ICEBsI ⁰) SP β + Δ <i>yonEF396</i> :: <i>spc</i>
CMJ403	PY79 (ICEBsI ⁰) (SP β ⁰) <i>amyE</i> ::{Pspank(hy)-empty <i>lacI spc</i> } <i>lacA</i> ::{Pspank(hy)- <i>yonE lacI mls</i> }
CMJ405	PY79 (ICEBsI ⁰) (SP β ⁰) <i>amyE</i> ::{Pspank(hy)-empty <i>lacI spc</i> } <i>lacA</i> ::{Pspank(hy)-empty <i>lacI mls</i> }
CMJ431	JH642 <i>trpC2 pheA1 amyE</i> ::{PxyI- <i>rapI xylR cat</i> } Δ (<i>spbK-rapI-phrI</i>)1500:: <i>kan</i>
CMJ434	JH642 <i>trpC2 pheA1 argA85</i> ::Tn917 SP β c2 Δ <i>yonE434</i> :: <i>lox-cat</i>
CMJ440	CU1050 (ICEBsI ⁰) (SP β ⁰) <i>lacA</i> ::{Pspank(hy)- <i>yonE lacI mls</i> }
CMJ443	CU1050 (ICEBsI ⁰) SP β c2 Δ <i>yonE443</i> (unmarked)
CMJ455	CU1050 (ICEBsI ⁰) SP β c2 Δ <i>yonE443</i> (unmarked) <i>yolBC98</i> :: <i>spc</i>
CMJ457	CU1050 (ICEBsI ⁰) SP β c2 Δ <i>yonE443</i> (unmarked) <i>yolBC98</i> :: <i>spc</i> <i>lacA</i> ::{Pspank(hy)- <i>yonE lacI mls</i> }
CMJ472	CU1050 (ICEBsI ⁰) (SP β ⁰) <i>comK</i> :: <i>cat</i>
CMJ616	PY79 (ICEBsI ⁰) (SP β ⁰) <i>amyE</i> ::{Pspank(hy)- <i>yonE lacI spc</i> }
CMJ684	PY79 (ICEBsI ⁰) (SP β ⁰) <i>lacA</i> ::{ <i>spbK kan</i> }
CMJ685	PY79 (ICEBsI ⁰) (SP β ⁰) <i>amyE</i> ::{Pspank(hy)- <i>yonE lacI spc</i> } <i>lacA</i> ::{ <i>spbK kan</i> }
CMJ826	CU1050 SP β c2 <i>yolBC98</i> :: <i>spc</i> ICEBsI:: <i>kan</i> (non-disruptive)
CMJ827	CU1050 (SP β ⁰) ICEBsI:: <i>kan</i> , <i>comK</i> :: <i>cat</i>
CMJ913	CU1050 (SP β ⁰) ICEBsI+ Δ (<i>rapI-phrI</i>)342:: <i>kan</i>

CMJ914	CU1050 (SPB ⁰) ICEBsI+ Δ (<i>spbK-rapI-phrI</i>)1500:: <i>kan</i>
CMJ917	CU1050 SPBc2 ICEBsI+ Δ (<i>rapI-phrI</i>)342:: <i>kan</i> , <i>yolBC98</i> :: <i>spc</i>
CMJ918	CU1050 SPBc2 ICEBsI+ Δ (<i>spbK-rapI-phrI</i>)1500:: <i>kan</i> , <i>yolBC98</i> :: <i>spc</i>
CU1050	ICEBsI ⁰ SPB ⁰ <i>metA thrC leu codY sup-3 (trnS-lys)</i> (Georgopoulos, 1969; Johnson and Grossman, 2016)
IRN342	<i>trpC2 pheA1</i> Δ (<i>rapI-phrI</i>)342:: <i>kan</i> (Auchtung <i>et al.</i> , 2005)
JMA222	<i>trpC2 pheA1</i> ICEBsI ⁰ (Auchtung <i>et al.</i> , 2005)
KM250	<i>trpC2 pheA1</i> Δ (<i>rapI-phrI</i>)342:: <i>kan amyE</i> ::{Pxyl- <i>rapI cat</i> } (Menard and Grossman, 2013)
PY79	ICEBsI ⁰ SPB ⁰ (Youngman <i>et al.</i> , 1984)

Table 2. Yeast strains used.

yeast strain*	genotype
CMJ620	<i>S. cerevisiae</i> mat a ura3-52 leu2-3 his3 trp1 dph2Δ::HIS3 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL-lacZ pCJ113 pCJ107
CMJ621	<i>S. cerevisiae</i> mat a ura3-52 leu2-3 his3 trp1 dph2Δ::HIS3 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL-lacZ pCJ113 pCJ108
CMJ622	<i>S. cerevisiae</i> mat a ura3-52 leu2-3 his3 trp1 dph2Δ::HIS3 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL-lacZ pCJ113 pCJ109
CMJ626	<i>S. cerevisiae</i> mat a ura3-52 leu2-3 his3 trp1 dph2Δ::HIS3 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL-lacZ pCJ114 pCJ107
CMJ627	<i>S. cerevisiae</i> mat a ura3-52 leu2-3 his3 trp1 dph2Δ::HIS3 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL-lacZ pCJ114 pCJ108
CMJ628	<i>S. cerevisiae</i> mat a ura3-52 leu2-3 his3 trp1 dph2Δ::HIS3 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL-lacZ pCJ114 pCJ109
CMJ632	<i>S. cerevisiae</i> mat a ura3-52 leu2-3 his3 trp1 dph2Δ::HIS3 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL-lacZ pCJ115 pCJ107
CMJ633	<i>S. cerevisiae</i> mat a ura3-52 leu2-3 his3 trp1 dph2Δ::HIS3 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL-lacZ pCJ115 pCJ108
CMJ634	<i>S. cerevisiae</i> mat a ura3-52 leu2-3 his3 trp1 dph2Δ::HIS3 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL-lacZ pCJ115 pCJ109

* all yeast strains were derived from PJ69-4A (James *et al.*, 1996)

Figures

Fig. 1. Map of ICEBs1 and some mutants.

A. A linear map of ICEBs1 is shown. Genes are indicated as filled boxes with arrowheads at the ends indicating the direction of transcription. *spbK* is shown as an open arrow. The attachment sites *attL* and *attR* mark the junctions between ICEBs1 and chromosomal sequences. Below the map are shown the ICEBs1 mutants used in this work. The regions of ICEBs1 that are present in each construct are shown as bars beneath the map. **B.** ICEBs1::*kan*. The open triangle indicates that this construct contains a *kan* gene inserted in the intergenic region between *rapI* and *yddM*. **C.** ICEBs1 Δ (*rapI-phrI*). **D.** ICEBs1 Δ (*spbK-phrI*). **E.** ICEBs1⁰ *spbK*⁺. Many of these derivatives of ICEBs1 are used in multiple strains as indicated in Table 1.

Fig. 2. Production of SPβ during the lytic cycle is reduced in cells containing ICEBsI or spbK.

A. Effect of ICEBsI on single-round infection of *B. subtilis* cultures. SPβ null strains were grown in rich medium, infected with phage (MOI = 0.01) and then diluted in fresh medium. The number of infective centers in the culture was tracked over time using strain CU1050 as the indicator (methods). circles, ICEBsI⁰ (CU1050); diamonds, ICEBsI⁺ (CMJ81). **B.** Effect of *spbK* on single-round infection of *B. subtilis* cultures. SPβ null strains were grown and infected with SPβ as in 2A. crosses, ICEBsI⁺ Δ*rapI-phrI* (CMJ913); squares, ICEBsI⁺ Δ(*spbK-rapI-phrI*) (CMJ914); triangles, ICEBsI⁰ *amyE::spbK* (CMJ82).

C. Effect of ICEBsI and *spbK* on spontaneous phage production. Strains carrying wild type SPβ lysogens and different ICEBsI variants were grown in rich medium; ICEBsI⁰ (JMA222), ICEBsI⁺ (AG174), ICEBsI⁺ Δ*rapI-phrI* (IRN342), ICEBsI⁺ Δ(*spbK-rapI-phrI*) (CAL1500), ICEBsI⁰ *amyE::spbK* (CMJ74). Supernatant was collected from each culture during exponential growth and used as a phage source in a plaque assay (methods). **D.** Effect of ICEBsI and *spbK* on phage production after induction of a lysogen. Strains carrying ts SPβ lysogens and different ICEBsI variants were grown in rich medium; ICEBsI⁰ (CMJ114), ICEBsI⁺ (CMJ826), ICEBsI⁺ Δ*rapI-phrI* (CMJ917), ICEBsI⁺ Δ(*spbK-rapI-phrI*) (CMJ918), ICEBsI⁰ *amyE::spbK* (CMJ116). SPβ lysogens were induced by a heat shock during exponential growth and supernatants were collected and used as a phage source in a plaque assay (methods). For C and D the Y axis shows the number of PFU/ml of culture divided by the OD600 of the culture.

Fig. 3. Expression of *yonE* in recipients reduces acquisition of ICEBs1 via conjugation.

A. Map of the operon in SP β that contains *yonF* and *yonE*, and relevant mutations. Genes are shown as arrows. *yonF* and *yonE* are indicated by arrows filled with a mottled pattern. *spc* is shown as an open arrow. Promoters are shown as bent arrows. The allele and the recipient strain carrying that allele are indicated.

B. The relative conjugation frequencies are shown, normalized to the conjugation frequency between a donor carrying a wild type ICEBs1 (KM250) and a recipient with a wild type SP β (CMJ48) within the same experiment, (average $9.7 \times 10^{-4} \pm 1.3 \times 10^{-3}$ transconjugants/donor). The $\Delta spbK$ donor (CMJ431) carries an ICEBs1 in which *spbK-rapI-phrI* have been deleted. The recipient with the Pspank(hy) promoter and no *yonE* allele is CMJ405. Other recipient strain numbers are indicated in panel A. Each experiment was repeated ≥ 3 times. Asterisks indicate that the conjugation frequency with the given recipient is significantly different than that with the wild type control ($p < 0.05$, t-test).

Fig. 4. Co-expression of *spbK* and *yonE* kills cells and results in a growth defect.

Strains null for *ICEBsI* and *SPB* (closed circles, PY79), expressing *yonE* (closed triangles, *amyE*::Phy-*yonE*, CMJ616), expressing *spbK* (open circles, *lacA*::*spbK*, CMJ684), or both *yonE* and *spbK* (open triangles, CMJ685) were grown in minimal medium. *yonE* expression was induced by the addition of 1 mM IPTG and the culture turbidity (A), and cell viability (B) were followed over time. T = 0 samples were collected immediately prior to induction with IPTG. Cell viability was measured as the number of colony forming units per ml, measured by plating for CFUs on LB plates made with Noble agar. Experiment was repeated 3 times.

Fig 5. *yonE* is needed for production of infectious phage.

A. Strains carrying a temperature-sensitive SP β lysogen with a wild type *yonE* allele (black bars, CMJ114) or $\Delta yonE$ (white bars, CMJ455) were cultured in rich medium, then heat shocked for 20 minutes (methods). The Y axis shows the average and standard deviation of the OD600 from each culture immediately before and 70 minutes (± 5 minutes) after the heat shock. Each experiment was repeated ≥ 3 times.

B. Phage were prepared by culturing strains with a temperature sensitive SP β lysogen with a wild type *yonE* allele (CMJ114), $\Delta yonE$ (CMJ455), or a $\Delta yonE$ allele with *yonE* complemented from the chromosome (*lacA::Phy-yonE*, CMJ457) to an OD600 of approximately 0.4, and then heat shocking the cultures and collecting phage (methods). Lysates were then spread on lawns of the indicator strain CU1050 or an indicator with *lacA::Phy-yonE* (CMJ440) and incubated overnight to allow plaque formation. The Y-axis shows the average and standard deviation of the number of infectious phage /ml, normalized by dividing by the OD600 of the culture at the time of heat shock. ND = not detected. Each experiment was repeated ≥ 3 times.

Fig. 6. ICEBs1 protects *B. subtilis* populations against SPβ.

A, B, C, SPβ⁰ ICEBs1⁰ (CU1050) and **D, E, F,** SPβ⁰ ICEBs1⁺ (CMJ81) were grown in rich medium, infected with no phage (filled squares), SPβ clear plaque (open triangles), or SPβ::spc (open circles) at an MOI of approximately 1:100 and diluted in fresh medium to an OD600 of 0.01. Culture turbidity (**A, D**), CFUs/ml (**B, E**), and the number of infectious phage per ml of culture (**C, D**) were tracked over time. Additionally, for cultures infected with SPβ::spc, the number of lysogenized cells were tracked over time (red open circles).

Fig. S1. *ICEBsI* prevents plaque formation by SP β . Various numbers of PFUs of SP β were mixed with the indicated strains, plated, incubated overnight and checked for the presence of plaques (methods). **A.** Approximately 100 PFUs of SP β were mixed with the indicator strain CU1050. **B.** Approximately 100 PFUs of SP β mixed with strain CMJ81 (the indicator strain CU1050 carrying *ICEBsI*). **C.** Approximately 10^4 PFU of SP β mixed with strain CMJ81 (the indicator strain CU1050 carrying *ICEBsI*).

In preliminary experiments, we found that *ICEBsI* did not inhibit plaque formation by *B. subtilis* phages ϕ 29, ϕ 105, SP01, SP02, SPP1 or SP16. We conclude that the inhibitory effects of *ICEBsI* are largely specific to SP β .

Fig. S2. Co-expression of *spbK* and *yonE* results in a sensitivity to standard bacteriological agar. Strains null for *ICEBs1* and *SPB* (PY79), expressing *yonE* (*amyE*::Phy-*yonE*, CMJ616), expressing *spbK* (*lacA*::*spbK*, CMJ684), or both *yonE* and *spbK* (CMJ685) were grown in minimal medium in the absence of IPTG. At an OD600 of 0.2, cultures were plated for CFUs on LB plates made with standard bacteriological agar (black bars) or on LB plates made with more rigorously purified Noble agar (white bars). Plating efficiency measured as CFUs/ml normalized to OD600.

Fig. S3. SpbK contains a TIR domain that mediates self-interaction.

A. Map of the SpbK peptide sequence showing the location of the TIR domain in black.

B. Yeast two-hybrid screen of SpbK fragments. Yeast strains carrying full length SpbK (Full), SpbK amino acids 1-104 (N-term) or SpbK amino acids 97-266 (TIR) bound to the *GAL4* DNA binding domain (DBD, Y-axis) and/or the *GAL4* activation domain (AD, X-axis) were spotted on medium selective for interaction between the bait and prey peptides and incubated at 30° C to allow for growth (methods). The following combinations were tested: AD-SpbK + DBD-SpbK (CMJ620), AD-N-term + DBD-SpbK (CMJ621), AD-TIR + DBD-SpbK (CMJ622), AD-SpbK + DBD-N-term (CMJ626), AD-N-term + DBD- N-term (CMJ627), AD-TIR + DBD- N-term (CMJ628), AD-SpbK + DBD-TIR (CMJ632), AD-N-term + DBD-TIR (CMJ633), AD-TIR + DBD-TIR (CMJ634).