Interactions between mobile genetic elements: An anti-phage gene in an integrative and conjugative element protects host cells from predation by a temperate bacteriophage

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Abstract

Most bacterial genomes contain horizontally acquired and transmissible mobile genetic elements, including temperate bacteriophages and integrative and conjugative elements. Little is known about how these elements interact and co-evolved as parts of their host genomes. In many cases, it is not known what advantages, if any, these elements provide to their bacterial hosts. Most strains of \textit{Bacillus subtilis} contain the temperate phage SPβ and the integrative and conjugative element ICE\textsubscript{Bs1}. Here we show that the presence of ICE\textsubscript{Bs1} in cells protects populations of \textit{B. subtilis} from predation by SPβ, likely providing selective pressure for the maintenance of ICE\textsubscript{Bs1} in \textit{B. subtilis}. A single gene in ICE\textsubscript{Bs1} (\textit{yddK}, now called \textit{spbK} for SPβ killing) was both necessary and sufficient for this protection. \textit{spbK} inhibited production of SPβ, during both activation of a lysogen and following \textit{de novo} infection. We found that expression \textit{spbK}, together with the SPβ gene \textit{yonE} constitutes an abortive infection system that leads to cell death. \textit{spbK} encodes a TIR (Toll-interleukin-1 receptor)-domain protein with similarity to some plant antiviral proteins and animal innate immune signaling proteins. We postulate that many uncharacterized cargo genes in ICEs may confer selective advantage to cells by protecting against other mobile elements.

Author summary

Chromosomes from virtually all organisms contain genes that were horizontally acquired. In bacteria, many of the horizontally acquired genes are located in mobile genetic elements, elements that promote their own transfer from one cell to another. These elements include viruses and conjugative elements that are parts of the host genome and they can contain genes involved in metabolism, pathogenesis, symbiosis, and antibiotic resistances. Interactions between these elements are poorly understood. Furthermore, the majority of these elements confer no obvious benefit to host cells. We found that the presence of an
integrative and conjugative element (ICE) in a bacterial genome protects host cells from predation by a bacteriophage (virus). There is a single gene in the integrative and conjugative element that confers this protection, and one gene in the bacteriophage that likely works together with the ICE gene. When expressed at the same time, these two genes cause cell death, before functional viruses can be made and released to kill other cells. We postulate that other ICEs may confer selective advantage to their host 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, temperate 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 [1–4]. 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 and segregated to daughter cells along with the host genome [5–7]. Under certain conditions, or stochastically, an ICE can excise from the chromosome and be transferred 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 [6]. For example, the ICE Tn916 was discovered because it confers tetracycline resistance to host cells and can move between cells via conjugation [8,9]. Likewise, many other ICEs were identified because they carry genes that confer specific phenotypes including: antibiotic resistance [10–15], pathogenesis [16], symbiosis [17], and metabolic functions [18–21].

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 [22,23] and the temperate phage SPβ [24]. B. subtilis strains also contain several defective mobile genetic elements [25–27].

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 [22,28]. 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 [22]. 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 [29] and because it is regulated by cell-cell signaling [22]. At the time of its discovery, it was not known if ICEBs1 conferred a beneficial phenotype to its host.

SPβ is a temperate phage found in the chromosome of many isolates of B. subtilis. Historically, SPβ was thought to be a defective phage [reviewed in [30]]. When strains cured of SPβ (SPβ0) were isolated, it became clear that it is functional [31], and cured strains are used to grow the phage. A widely used strain missing SPβ is also missing ICEBs1 [32,33].
In strains lysogenic for SPβ, the phage is integrated in spsM, near the terminus of replication [32,34,35]. SPβ contains genes needed for production of and resistance to the peptide antibiotic sublancin [35,36], 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, SPβ gene expression is induced and the phage excises from the host chromosome. In cells capable of producing phage particles, the activated phage enters the lytic cycle, produces progeny phage, and causes cell lysis and release of phage particles.

We found that the presence of ICEBs1 in B. subtilis inhibited production of active SPβ, both when the phage was activated from the lysogenic state and during de novo infection. The ICEBs1 gene spbK, although dispensable for conjugation, was necessary and sufficient for the inhibition of SPβ. The spbK gene product contains a Toll/Interleukin-1 Receptor (TIR) domain that was needed for function. The anti-SPβ phenotype (abortive infection) caused by spbK was dependent on the SPβ gene yonE. We found that yonE was essential for SPβ 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 ICEBs1 or SPβ genes. The presence of ICEBs1 in cells prevented the spread of SPβ, thereby protecting nearby B. subtilis cells from infection and allowing the population to continue growing. This phenotype likely provides strong selective pressure to maintain ICEBs1 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**

**ICEBs1 prevents SPβ from forming plaques**

ICEBs1 was not known to confer phenotypes to B. subtilis, aside from those directly related to conjugation. However, the left end of ICEBs1 (Fig 1) encodes a phage-like repressor ImmR
[28], anti-repressor ImmA [37], and recombinase Int [38]. In addition, the strain CU1050, that is cured of and often used to grow the temperate subtilis phage SPβ [30,31], still contains the defective prophage PBSX and skin but is cured of ICEBsI [33]. This information led us to wonder if there might be some interaction between ICEBsI and SPβ. We tested if the presence of ICEBsI in B. subtilis altered the ability of SPβ to make plaques. We mixed SPβ with two B. subtilis strains, one that was missing ICEBsI (ICEBsI0) and that has been used as an indicator strain for SPβ strain (CU1050) [32,33] and an isogenic derivative that contained ICEBsI (CMJ81; ICEBsI+). SPβ formed plaques on a lawn of the ICEBsI0 strain (Fig 2A), but not on a lawn of the isogenic ICEBsI+ strain (Fig 2B), even when 100-fold more plaque forming units (PFUs) were mixed with cells (Fig 2C). Based on these results, we conclude that the presence of ICEBsI inhibited 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 3A and 3B). We mixed ~10^5 PFUs of SPβ with ~10^7 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 3A). 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 3A). 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 [24].

Cells with ICEBsI that were exposed to SPβ 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 3A). Furthermore, the number of phage produced per initial infective center was ~2.2 ± 0.4 (Fig 3A). 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 did not support propagation of phage in the lytic cycle.

**ICEBs1 has little or no effect on entry of phage into cells**

The ICEBs1-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 ICEBs1 was causing a block in phage entry, then there should be a corresponding reduction in the frequency of lysogen formation. We used SPß that contained spc, conferring resistance to spectinomycin, to measure the frequency of lysogenization. Cells with or without ICEBs1 were mixed with SPß::spc98 (MOI ~ 0.001), unbound phage

![Fig 3. Production of SPß during the lytic cycle is reduced in cells containing ICEBs1 or spbK. A. Effect of ICEBs1 on single-round infection of B. subtilis cultures. SPß 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, ICEBs10 (CU1050); diamonds, ICEBs1+ (CMJ81). B. Effect of spbK on single-round infection of B. subtilis cultures. SPß null strains were grown and infected with SPß as in 2A. crosses, ICEBs1+ delrapI-phrI (CMJ913); squares, ICEBs1+ delspbK-rapI-phrI (CMJ914); triangles, ICEBs1+ amyE::spbK (CMJ82). C. Effect of ICEBs1 and spbK on spontaneous phage production. Strains carrying wild type SPß lysogens and different ICEBs1 variants were grown in rich medium; ICEBs10 (JMA222), ICEBs1+ (AG174), ICEBs1+ delrapI-phrI (IBN342), ICEBs1+ delspbK-rapI-phrI (CAL1500), ICEBs1+ amyE::spbK (CMJ74). Supernatant was collected from each culture during exponential growth 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.

https://doi.org/10.1371/journal.pgen.1010065.g003
were washed off, and cells were spread on plates containing spectinomycin to select for lysogens. The lysogenization frequency of cells without ICEBs1 (CMJ472) was ~1% (1.1x10^{-2} ± 0.46x10^{-2}), or approximately one lysogen per 100 initial phage. Similarly, the lysogenization frequency of ICEBs1+ cells (CMJ827) was ~0.4% (4.3x10^{-3} ± 1.3x10^{-3}), or about 40% of that of the ICEBs10 cells. These results indicate that ICEBs1 has a relatively minor (if any) effect on lysogenization frequency and that the anti-SPβ phenotype conferred by ICEBs1 was not due to a block in adhesion or entry of the phage.

**ICEBs1 reduces the number of phage released by SPβ lysogens**

We found that the presence of ICEBs1 in an SPβ 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 ICEBs1 had approximately 100-fold more PFUs/ml than cultures of an ICE+ lysogen (Fig 3C). Together our results demonstrate that ICEBs1 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 ICEBs1 prevented production of SPβ following induction of a temperature sensitive lysogen. We grew strains with a temperature sensitive SPβ lysogen (SPβc2) in rich medium, induced the lysogen by heat shock, and measured phage release. Phage production was reduced by ~1,000-fold in cells with ICEBs1 compared to cells without (Fig 3D). 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 ICEBs1 (CMJ114). Based on these results we conclude that ICEBs1 was probably not preventing induction of SPβ but rather was inhibiting production of active phage particles post-induction.

**The ICEBs1 gene spbK is necessary and sufficient to inhibit SPβ**

We were interested in determining which ICEBs1 gene was responsible for the inhibition of SPβ. Most ICEBs1 genes are repressed when ICEBs1 is integrated in the host genome. Because the inhibition of SPβ did not appear to depend on activation of ICEBs1, we focused on the handful of ICEBs1 genes that are constitutively expressed, including genes toward the left and right ends of the element (Fig 1). Preliminary experiments led us to focus on spbK (formerly yddK). These experiments included testing for the presence of SPβ in the culture supernatant from lysogens, essentially as described above, with various regions of ICEBs1 deleted. Most deletion mutants tested had been described previously [22,39–41]. The preliminary results indicated that strains in which spbK was intact, including ΔcwT and ΔrapI-yddM, inhibited phage release. In contrast, strains in which spbK had been deleted, including ΔconG-yddM, ΔydcB-yddM, ΔnicK-yddM, and ΔydcQ-yddM [39], did not inhibit phage production. Based on these results, we inferred that spbK was likely needed for ICEBs1-mediated inhibition of spontaneous release of SPβ from a lysogen and tested this directly. We used three different assays to test the effects of spbK on SPβ. In all three assays, we compared three B. subtilis strains: an ICEBs1+ strain with spbK (A(rapI-phrI)::kan, Fig 1C), an ICEBs1+ strain lacking spbK (A(spbK-phrI)::kan, Fig 1D), and an ICEBs10 strain expressing spbK from its own promoter at an ectopic locus (ICEBs10 amyE::[spbK kan], Fig 1E).

We measured: 1) the appearance of infective centers following a single round of infection with SPβ (Fig 3A and 3B); 2) the number of phage spontaneously released from an SPβ lysogen (Fig 3C); and 3) the number of phage produced after induction of a temperature sensitive SPβ lysogen (Fig 3D). In all cases, we found that spbK was necessary for ICEBs1 to inhibit the formation of infective centers and the production of phage, and that ICEBs1+ ΔspbK strains were
indistinguishable from strains entirely lacking ICEBs1. Furthermore, ectopic expression of spbK was sufficient to inhibit phage production in the absence of ICEBs1 in all three assays.

Expression of the SPß gene yonE inhibits acquisition of ICEBs1 and this inhibition is dependent on the ICEBs1 gene spbK

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

In previous work [42], we used Tn-seq to identify genes in recipients that affected the efficiency of stable acquisition of ICEBs1 in conjugation. Briefly, a library of random transposon insertions in a strain that is an SPß lysogen and cured of ICEBs1 was used as the recipient in conjugation. We selected for transconjugants that had acquired ICEBs1. Insertion mutations that cause a decrease in acquisition of ICEBs1 were underrepresented in transconjugants relative to controls. We found that insertions in some position in the SPß gene yonF were underrepresented, indicating that these insertion mutations reduced the ability of would-be recipients to stably acquire ICEBs1 from donors. Because the frequency of insertions in other positions in yonF was unaltered in transconjugants [42], and because neither yonF nor yonE is normally expressed in SPß lysogens, the phenotype could not be due to loss of yonF. We hypothesized that the reduction in acquisition of ICEBs1 might be due to inappropriate expression of yonE, the gene immediately downstream of yonF, likely transcribed from the promoter for the antibiotic resistance gene (spc) in the transposon. We therefore tested directly the effects of inappropriate expression of yonE on acquisition of ICEBs1.

We found that inappropriate expression of yonE reduced the ability of cells to stably acquire a copy of ICEBs1. We made a series of mutations in SPß (Fig 4A) and tested these for effects on the ability of cells to act as ICEBs1 recipients during conjugation. We found that an insertion of spc into a deletion of yonF (ΔyonF::spc) reduced acquisition of ICEBs1 only when spc was co-directional with yonE. Furthermore, deletion of yonE in this context eliminated the defect in acquisition of ICEBs1 (Fig 4B). In the absence of all other SPß genes, expression of yonE from the IPTG-inducible promoter Pspank(hy) was sufficient to inhibit acquisition of ICEBs1 (Fig 4B). We conclude that yonE in SPß is both necessary and sufficient to cause the decrease in stable acquisition of ICEBs1. Results presented below demonstrate that when ICEBs1 is transferred to cells expressing yonE, those nascent transconjugants die. It is for this reason that there are no stable transconjugants recovered.

The decreased acquisition of ICEBs1 by recipients expressing yonE was dependent on the ICEBs1 gene spbK. We tested strains expressing yonE for the ability to acquire ICEBs1 that was missing spbK (ICEBs1 ΔspbK), and found that they all acquired the ΔspbK element at the same frequency as wild type recipients not expressing yonE (Fig 4B, right end of panel). From these results we conclude that expression of yonE caused a defect in the stable acquisition of ICEBs1 and that this defect was dependent on the presence of spbK in the incoming ICEBs1. We note that loss of spbK caused no reduction in conjugation efficiency (Fig 4B), demonstrating that it is dispensable for conjugation. We also note that the presence or absence of wild type SPß had no detectable effect on conjugation efficiency (Fig 4B).

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 5).
Fig 4. Expression of yonE in recipients reduces acquisition of ICEBs1 via conjugation. A. Map of the operon in SPβ 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 SPβ (CMJ48) within the same experiment, (average 9.7 x 10^{-4} ± 1.3 x 10^{-3} transconjugants/donor). The ΔspbK donor (CMJ431) carries an ICEBs1 in which spbK-rapl-phr1 have been deleted. The recipient with the promoter Pspank(hy) 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).

https://doi.org/10.1371/journal.pgen.1010065.g004
This caused a rapid growth arrest as measured by optical density (Fig 5A) and an ~1000-fold drop in viability as measured by plating for CFUs on LB plates made with Noble agar (Fig 5B; see below). 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 5A and 5B). Together, our results indicate that co-expression of yonE and spbK is detrimental to cell growth. In an SPβ lysogen that also contains ICEBs1, spbK, is expressed, but yonE is not. yonE would be expressed only if SPβ were activated, or upon infection of non-lysogens.

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) (S1 Fig), 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 (ΔyonE443) in a temperature-sensitive SPβ lysogen. We found that cultures of this inducible ΔyonE lysogen cleared comparably to a yonE+ strain following a shift to high temperature (Fig 6A), demonstrating that yonE is not needed for induction of SPβ from a lysogenic state, nor is it needed to cause host cell lysis.

Despite the fact that the ΔyonE host cells lysed, there were no detectable viable phage (< 10 PFUs/ml) produced by the mutant lysogen (Fig 6B, first two columns). This defect in phage production was partially complemented by expression of yonE from an ectopic chromosomal locus. These ΔyonE phage (recovered from the complemented strain) were capable of forming plaques on an indicator strain that also expressed yonE (Fig 6B, last three columns). Although
Fig 6. *yonE* is needed for production of infectious phage. A. Strains carrying a temperature-sensitive SPβ lysogen with a wild type *yonE* allele (black bars, CMJ114) or Δ*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 SPβ lysogen with a wild type *yonE* allele (CMJ114), Δ*yonE* (CMJ455), or a Δ*yonE* allele with *yonE* complemented from the chromosome (lacA::[Pspank(hy)-*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::[Pspank(hy)-*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.
a small number of phage produced by a ΔyonE 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 SPβ.

To determine if yonE is needed to form a lysogen, we made a stock of spc-marked ΔyonE phage by growing the ΔyonE mutant on a B. subtilis strain ectopically expressing yonE. The frequency of lysogenization of spc-marked yonE+ and ΔyonE 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 SPβ extends from yonG to yonZ, indicating that these genes may encode conserved phage functions [43]. The phage E3 from Geobacillus encodes a putative portal protein (accession number AJA41333) that is 25% identical to YonE [44]. 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 [45]. 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 SPβ 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 [46] 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) (**S2A** Fig). 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 [47]. In plants they mediate disease resistance, often in response to infectious agents [48]. Some pathogenic bacteria encode TIR domain proteins that interact with eukaryotic host TIR domain proteins to modulate the host immune response [49]. Many non-pathogenic bacteria also contain TIR domain proteins and it is thought that the TIR domains mediate protein-protein interaction [50]. 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 [51].

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. ICEBs1 and SPβ), predicted to encode a TIR domain. Using a yeast two-hybrid assay (Methods), we found that full-length SpbK multimerizes in vivo (**S2B** Fig). 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 (**S2B** Fig). We also tested for, but were unable to detect, interaction between SpbK and YonE, indicating that if these two proteins interact, that interaction was not detectable with the yeast two-hybrid system that we used (Methods).

**ICEBs1 protects B. subtilis populations from attack by SPβ**

As described above, when SPβ undergoes lysogenic to lytic conversion, SPβ lysogens that contain ICEBs1 die without significant production of progeny phage. De novo infection of non-lysogens that contain ICEBs1 also die without producing progeny phage. We found that in a
population of cells, this abortive infection system in \textit{ICEBs1} protected cells from killing by SPβ. We grew SPβ-cured strains of \textit{B. subtilis} that either contained or did not contain \textit{ICEBs1}, infected the cultures with SPβ 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 7). We suspected that use of SPβ that is capable of making lysogens could mask possible effects on cell death and would be measuring possible protection of the population by \textit{ICEBs1} and by the formation of lysogens (which are themselves immune to superinfection, see below). Therefore, we first analyzed a clear plaque mutant (incapable of making lysogens; Methods) to eliminate possible effects of lysogeny. We then measured effects of \textit{ICEBs1} on phage that could form lysogens.

![Fig 7. ICEBs1 protects \textit{B. subtilis} populations against SPβ.](https://doi.org/10.1371/journal.pgen.1010065.g007)
When cultures of an ICEBs1<sup>+</sup> strain were infected with a clear plaque mutant of SPβ (MOI ~0.01) 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 7A) and an approximately 5,000-fold decrease in CFUs (Fig 7B). During this time (1.5 hrs) the concentration of phage in the culture (Fig 7C) surpassed the concentration of cells (Fig 7B).

In contrast, when an ICEBs1+ strain was infected with the clear plaque mutant of SPβ (MOI ~0.01), cell growth was indistinguishable from an uninfected culture as measured by both the optical density (Fig 7D) and the number of CFUs (Fig 7E). The population of phage in the culture generally decreased to below the initial inoculum (Fig 7F). These results indicate that the presence of ICEBs1 is beneficial to the population of cells even though individual infected cells may not survive.

Experiments described above were done with an SPβ mutant that was unable to form lysogens. We repeated these experiments using SPβ::spc, that, other than the spc insertion is wild type and able to form lysogens. Lysogens were detected as spectinomycin-resistant colonies.

When cells without ICEBs1 were infected with SPβ::spc (MOI ~0.01), there was a 10-fold drop in both the optical density of the culture (Fig 7A) and the number of CFUs (Fig 7B). During the experiment, many cells became lysogenized with SPβ. Lysogens are then protected from killing by new SPβ infection [24]. These lysogens continued to grow, and after about five hours the population of cells had increased and virtually all cells were SPβ lysogens (Fig 7B).

Although wt SPβ killed only ~90% of the cells, (compared to >99.9% killing by the clear plaque mutant), wt SPβ became established in the entire outgrown population.

When cells with ICEBs1 were infected with SPβ::spc (MOI ~0.01), cell growth continued and there was no obvious drop in optical density (Fig 7D) nor in the number of CFUs (Fig 7E). Five hours after the initial infection, the number of phage in the culture was below the initial inoculum (Fig 7E) and the number of SPβ lysogens remained at approximately 10<sup>4</sup>–10<sup>5</sup> per ml (Fig 7F), a relatively small fraction of the total number of cells.

Together, these results indicate that the presence of ICEBs1 in cells limits phage production, thereby protecting a population of cells from predation by SPβ. The presence of ICEBs1 does not limit initial lysogenization. However, because ICEBs1 limits the production of new phage, the number of lysogens is limited by the initial inoculum of phage. In this way, ICEBs1 protects the population from killing by SPβ, and secondarily, prevents SPβ from invading all the cells, thereby preventing lysogens taking over the population.

**Discussion**

Results presented here demonstrate that ICEBs1 encodes an anti-phage system that inhibits production of the phage SPβ. This inhibition occurs upon de novo infection by SPβ and also upon induction of an SPβ lysogen. There is little or no direct effect on the formation of lysogens. The ICEBs1 gene <i>spbK</i> is both necessary and sufficient to inhibit SPβ: deleting <i>spbK</i> from ICEBs1 abolishes the phenotype, and expressing <i>spbK</i> in a strain missing ICEBs1 fully inhibits SPβ. Expression of <i>yonE</i> apparently triggers this anti-phage response, and co-expression of <i>spbK</i> and <i>yonE</i> in a strain that otherwise lacks ICEBs1 and SPβ rapidly kills the cells. There are multiple possibilities for how YonE triggers this killing. It could directly interact with SpbK and the two proteins together, perhaps with other host products, and could disrupt an essential host function. YonE could somehow modify SpbK, perhaps covalently, or by stabilizing it, thereby activating it. Alternatively, YonE could modify an essential host product, making cells susceptible to SpbK. Of course, it is possible that SpbK makes cells susceptible to YonE, and that cells with ICEBs1 are ‘primed’ to be killed when <i>yonE</i> is expressed.
Whatever the mechanism, we conclude that ICEBs1 encodes an abortive infection system that protects its host from predation by SPβ. Below, we briefly describe the yonE and spbK gene products and TIR-domains, comment generally on abortive infection systems, and conclude with general thoughts about cargo genes in ICEs.

**Genes involved in protection against SPβ**

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 [52]. In some bacterial pathogens, TIR-domain proteins modulate the host immune response [50,53–55]. Recent work has implicated some bacterial TIR-domain proteins as being essential components of a class of anti-phage defense systems (“Thoeris”) [51]. 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 (ThrA) in proximity to (typically multiple) genes encoding TIR-domain proteins (ThrB) [51]. 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 SPβ, 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.

Many isolates of *B. subtilis* have both ICEBs1 and SPβ. These elements likely co-evolved and it is possible that the spbK-mediated abortive infection is specific to SPβ. However, we suspect that spbK-mediated abortive infection might respond to other phages, perhaps those with yonE orthologs.

**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 [56]. 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 [6]. 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 [29,57]. 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 [58,59]. *B. subtilis* cells were grown in LB or S7_50 defined minimal medium with 0.1% glutamate [60] 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-ß-D-thiogalactopyranoside (IPTG), and the Pxyl promoter was activated with 1% (w/v) xylose, typically in cells grown in arabinose.

**Strains and alleles**

*B. subtilis* strains and genotypes are listed in Table 1. Specific alleles are described below. All *B. subtilis* strains are derived from parent AG174 (JH642), unless otherwise indicated.

**SPB::spc.** *spc* (spectinomycin resistance) was inserted between *yolB* and *yolC* in SPB. *spc* was amplified by PCR, and Gibson assembly [63] was used to join this fragment to genomic sequences containing the apparent bidirectional terminator located between the convergently transcribed genes *yolB* and *yolC* [64] 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 SPB::spc98, or SPB::spc.

**Clear plaque mutant of SPB.** SPB typically makes turbid plaques, characteristic of temperate phages. In the course of determining the titre of a stock of SPB::spc, we noticed a plaque that appeared clear. We picked this plaque and designated the phage SPB::spc-clear. We grew the phage and then tested for the ability to form lysogens by infecting cells and selecting for spectinomycin resistance. Cells infected with the SPB::spc phage readily formed lysogens. We did not detect any lysogens (spectinomycin resistance) from cells infected with SPB::spc-clear. In addition, all plaques observed were clear, verifying that this phage was indeed unable to form lysogens.

**Δ(spbK-rapI-phrI)1500::kan.** The region of ICEBs1 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 [22]. The linkage between ΔspbK and Δ(rapI-phrI)342::kan was used to transfer the ΔspbK allele as needed.

**ΔyonEF396::spc.** A deletion-insertion that replaces *yonE* and *yonF* with a co-directional *spc* insertion.

**ΔyonE443.** The unmarked ΔyonE443 allele was made by replacing *yonE* with the *cat* gene, flanked by *lox* sites (CMJ434). The Cre recombinase, expressed from the temperature-sensitive...
## Table 1. *B. subtilis* strains used.

| Strain        | Genotype (reference)                                                                 |
|---------------|-------------------------------------------------------------------------------------|
| AG174         | trpC2 pheA1 a.k.a., JH642 \[27\]                                                   |
| CAL321        | trpC2 pheA1 \(\Delta\) (rapI-yddM) J318::kan \[39\]                              |
| CAL322        | trpC2 pheA1 \(\Delta\) (yddG-yddM) J319::kan \[39\]                              |
| CAL323        | trpC2 pheA1 \(\Delta\) (yddB-yddM) J320::kan \[39\]                              |
| CAL347        | trpC2 pheA1 \(\Delta\) (ydcR-yddM) J347::kan \[39\]                              |
| CAL348        | trpC2 pheA1 \(\Delta\) (ydcQ-yddM) J348::kan \[39\]                              |
| CAL1500       | trpC2 pheA1 \(\Delta\) (spbK-rapI-phiR) J1500::kan                                |
| CMJ48         | PY79 (ICEBs1\(^{\Delta}\)) SPβ+ amyE\(\Delta\) [Pspk (hy)-empty lacI spc]       |
| CMJ74         | trpC2 pheA1 ICEBs1\(^{\Delta}\) amyE\(\Delta\) [spbK cat]                       |
| CMJ81         | CU1050 (SPβ\(^{\Delta}\)) ICEBs1::kan (non-disruptive); note: made by crossing donor JMA448 with recipient CU1050 |
| CMJ82         | CU1050 (ICEBs1\(^{\Delta}\)) (SPβ\(^{\Delta}\)) amyE\(\Delta\) [spbK cat]     |
| CMJ98         | trpC2 pheA1 ICEBs1\(^{\Delta}\) yolBC98::spc                                       |
| CMJ104        | PY79 (ICEBs1\(^{\Delta}\)) SPβ+ ΔyonF67::spc \[42\]                             |
| CMJ114        | CU1050 (ICEBs1\(^{\Delta}\)) SPβc2 yolBC98::spc                                 |
| CMJ116        | CU1050 (ICEBs1\(^{\Delta}\)) SPβc2 yolBC98::spc amyE\(\Delta\) [spbK cat]      |
| CMJ388        | PY79 (ICEBs1\(^{\Delta}\)) SPβ+ ΔyonF155::spc (reverse orientation) \[42\]       |
| CMJ402        | PY79 (ICEBs1\(^{\Delta}\)) SPβ+ ΔyonEF396::spc                                   |
| CMJ403        | PY79 (ICEBs1\(^{\Delta}\)) (SPβ\(^{\Delta}\)) amyE\(\Delta\) [Pspk (hy)-empty lacI spc] lacA\(\Delta\) [Pspk (hy)-yonE lacI mls] |
| CMJ405        | PY79 (ICEBs1\(^{\Delta}\)) (SPβ\(^{\Delta}\)) amyE\(\Delta\) [Pspk (hy)-empty lacI spc] lacA\(\Delta\) [Pspk (hy)-yonE lacI mls] |
| CMJ431        | trpC2 pheA1 amyE\(\Delta\) [Psi+rapI sylR cat] Δ(spβK-rapI-phiR) J1500::kan     |
| CMJ434        | trpC2 pheA1 argA85::Tn917 SPβc2 ΔyonE434::lox::cat                               |
| CMJ440        | CU1050 (ICEBs1\(^{\Delta}\)) (SPβ\(^{\Delta}\)) lacA\(\Delta\) [Pspk (hy)-yonE lacI mls] |
| CMJ443        | CU1050 (ICEBs1\(^{\Delta}\)) SPβc2 ΔyonE443 (unmarked)                          |
| CMJ455        | CU1050 (ICEBs1\(^{\Delta}\)) SPβc2 ΔyonE443 (unmarked) yolBC98::spc             |
| CMJ457        | CU1050 (ICEBs1\(^{\Delta}\)) SPβc2 ΔyonE443 (unmarked) yolBC98::spc lacA\(\Delta\) [Pspk (hy)-yonE lacI mls] |
| CMJ472        | CU1050 (ICEBs1\(^{\Delta}\)) (SPβ\(^{\Delta}\)) comK::cat                      |
| CMJ616        | PY79 (ICEBs1\(^{\Delta}\)) (SPβ\(^{\Delta}\)) amyE\(\Delta\) [Pspk (hy)-yonE lacI spc]      |
| CMJ684        | PY79 (ICEBs1\(^{\Delta}\)) (SPβ\(^{\Delta}\)) lacA\(\Delta\) [spbK kan]         |
| CMJ685        | PY79 (ICEBs1\(^{\Delta}\)) (SPβ\(^{\Delta}\)) amyE\(\Delta\) [Pspk (hy)-yonE lacI spc] lacA\(\Delta\) [spbK kan] |
| CMJ826        | CU1050 SPβc2 yolBC98::spc ICEBs1::kan (non-disruptive)                            |
| CMJ827        | CU1050 (SPβ\(^{\Delta}\)) ICEBs1::kan, comK::cat                                |
| CMJ913        | CU1050 (SPβ\(^{\Delta}\)) ICEBs1+ Δ(rapI-phiR) J342::kan                        |
| CMJ914        | CU1050 (SPβ\(^{\Delta}\)) ICEBs1+ Δ(spβK-rapI-phiR) J1500::kan                   |
| CMJ917        | CU1050 SPβc2 ICEBs1+ Δ(rapI-phiR) J342::kan, yolBC98::spc                        |
| CMJ918        | CU1050 SPβc2 ICEBs1+ Δ(spβK-rapI-phiR) J1500::kan, yolBC98::spc                  |
| CU1050        | ICEBs1\(^{\Delta}\) SPβ\(^{\Delta}\) meta thrC leu codY sup-3 (trnS-lys) \[31,33\]; (CMJ28) |
| IRN342        | trpC2 pheA1 Δ(rapI-phiR) J342::kan \[22\]                                       |
| JMA222        | trpC2 pheA1 ICEBs1\(^{\Delta}\) \[22\]                                           |
| JMA448        | trpC2 pheA1 ICEBs1::kan amyE\(\Delta\) [Pspk (hy)-rapI spc] \[22\] note: used as donor with recipient CU1050 to create CMJ81 |
| KM250         | trpC2 pheA1 Δ(rapI-phiR) J342::kan amyE\(\Delta\) [Psi+rapI cat] \[61\]            |
| PY79          | ICEBs1\(^{\Delta}\) SPβ\(^{\Delta}\) \[62\]                                      |

https://doi.org/10.1371/journal.pgen.1010065.t001
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 [42,65], resulting in strain CMJ443.

**Overexpression of yonE.** The yonE coding sequence was cloned into a plasmid containing the IPTG-inducible Pspank(hy) promoter [66], 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 ICEBs1 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 [67] (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 10^5 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. 10^4 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

Yeast strains are listed in Table 2 and were derived from PJ69-4A [59]. The yeast two hybrid strains and vectors used in this study have been previously described [59]. 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 [58] 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 [38,42]. 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 Spizizen's 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) [68] 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.
Supporting information

S1 Fig. Co-expression of spbK and yonE results in a sensitivity to standard bacteriological agar. Strains null for ICEBs1 and SPβ (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.

(TIF)

S2 Fig. 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).

(TIF)

Acknowledgments

We thank Mary Anderson and Josh Jones for helpful discussions and comments on the manuscript, and Eleina England for early experiments confirming interactions between SPβ and ICEBs1.

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