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A conserved anti-repressor controls horizontal gene transfer by proteolysis

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Summary

The mobile genetic element ICE*Bs1* is an integrative and conjugative element (a conjugative transposon) found in the *Bacillus subtilis* chromosome. The SOS response and the RapI-PhrI sensory system activate ICE*Bs1* gene expression, excision, and transfer by inactivating the ICE*Bs1* repressor protein ImmR. Although ImmR is similar to many characterized phage repressors, we found that, unlike these repressors, inactivation of ImmR requires an ICE*Bs1*-encoded anti-repressor ImmA (YdcM). ImmA was needed for the degradation of ImmR in *B. subtilis*. Co-expression of ImmA and ImmR in *E. coli* or co-incubation of purified ImmA and ImmR resulted in site-specific cleavage of ImmR. Homologs of *immR* and *immA* are found in many mobile genetic elements. We found that the ImmA homolog encoded by *B. subtilis* phage ø105 is required for inactivation of the ø105 repressor (an ImmR homolog). ImmA-dependent proteolysis of ImmR repressors may be a conserved mechanism for regulating horizontal gene transfer.

Keywords

Bacillus subtilis; phage ø105; Integrative and conjugative element; conjugative transposon

Introduction

Mobile genetic elements play significant roles in genome plasticity, the spread of antibiotic resistance, and acquisition of new traits (Dobrindt et al., 2004; Frost et al., 2005)}. Mobile genetic elements include bacteriophages, conjugative plasmids, and conjugative transposons (Churchward, 2002; Whittle et al., 2002), also known as integrative and conjugative elements (ICEs) (Burrus *et al.*, 2002b; van der Meer and Sentchilo, 2003). Mobile elements typically have regulatory mechanisms to reduce the burden placed on the host cells while maintaining the potential for the element to spread under specific conditions. A tremendous amount is known about phages and plasmids and mechanisms controlling their transfer. In addition, much is known about enzymes and mechanisms of integration and excision of many phages and conjugative transposons (Churchward, 2002; Mullany *et al.*, 2002). In contrast, less is known about regulation of ICEs and environmental conditions controlling their stability and transfer.

ICEs and putative ICEs are present in many bacteria (Burrus and Waldor, 2004) and are important agents of horizontal gene transfer (Beaber et al., 2002; Rice, 2002; Scott, 2002;

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Whittle et al., 2002). They reside integrated in the chromosome of a host cell and can excise and transfer to recipients through conjugation (mating) where they then integrate into the chromosome of the recipient (Churchward, 2002; Whittle et al., 2002). ICEs typically encode proteins needed for their regulation, integration, excision, and transfer. Many of the characterized ICEs also encode resistance to antibiotics, and transfer of some ICEs is induced in the presence of an antibiotic {(Celli and Trieu-Cuot, 1998; Moon *et al.*, 2005) and references therein}.

ICE*Bs1* (Fig. 1) is an integrative and conjugative element found in the chromosome of *Bacillus subtilis* (Auchtung *et al.*, 2005;Burrus *et al.*, 2002a). It is not known what benefit, if any, is conferred to cells carrying ICE*Bs1*. ICE*Bs1* encodes an integrase and excisionase (Lee *et al.*, 2007), a relaxase needed for DNA transfer, and an origin of transfer, *oriT* (Lee and Grossman, 2007)}. The putative mating machinery of ICE*Bs1* resembles that of the conjugative transposon Tn*916* and many conjugative plasmids from Gram-positive bacteria (Auchtung *et al.*, 2005;Burrus *et al.*, 2002a). The regulatory module at the left end of ICE*Bs1* (Fig. 1) resembles those from many different bacteriophages (Auchtung *et al.*, 2007;Lucchini *et al.*, 1999) and indicates that the mode of regulation is different from that of ICEs that are induced in response to antibiotics. During exponential growth, the ICE*Bs1* gene product ImmR, the immunity repressor, represses transcription of genes needed for excision and transfer (Auchtung et al., 2007). Inactivation of ImmR and de-repression of ICE*Bs1* gene expression, excision, and mating occurs either when cells induce the RapI-dependent sensory response due to crowding by neighboring cells that lack a copy of ICE*Bs1*, or when cells induce the RecA-dependent SOS response to DNA damage (Auchtung et al., 2005).

Many mobile genetic elements are induced by the highly conserved RecA-mediated response to DNA damage, the SOS response {reviewed in (Walker, 1996)}. The best characterized responses involve induction of lysogenic phages. After DNA damage, the host protein RecA is activated by binding to single-stranded DNA. In the paradigmatic example of lambda (and lambdoid phages), activated RecA directly facilitates the autocleavage of the phage repressor cI (Little, 1984; Roberts et al., 1978). An analogous mechanism of autocleavage inactivates the cellular repressor LexA (Little, 1984; Miller et al., 1996), thereby inducing expression of the many genes normally repressed by LexA (Au et al., 2005; Courcelle et al., 2001; Fernandez De Henestrosa et al., 2000; Goranov et al., 2006).

We investigated the mechanism by which ImmR is inactivated under conditions that promote ICE*Bs1* excision and transfer. Although inactivation of ImmR by DNA damage requires RecA, ImmR does not contain some of the key residues known to be required for auto-proteolysis and activated RecA does not directly facilitate the autocleavage of ImmR. Instead, we found that inactivation of ImmR requires the ICE*Bs1*-encoded protein, ImmA (ImmR Antagonist, formerly YdcM), an anti-repressor. ImmR was rapidly degraded in vivo when antagonized by ImmA. ImmA and ImmR interacted directly in a yeast two-hybrid assay. We purified ImmA and found that in vitro, it caused cleavage of purified ImmR. We conclude that ImmA is an anti-repressor that inactivates ImmR, likely through site-specific cleavage that results in ImmR degradation and de-repression of ICE*Bs1* in vivo.

Homologs of *immA* and *immR* are found in many other mobile genetic elements. We found that the ImmA homolog of *B. subtilis* phage ø105 {(ø105)ImmA, formerly Orf2, a.k.a., ø105_33 in NCBI} was required for inactivation of the phage repressor cø105 (an ImmR homolog). ImmA-dependent proteolysis of ImmR likely represents a conserved strategy for regulating transfer of many different mobile genetic elements.

Results

Identification of the anti-repressor gene *immA*

ImmR is the only ICE*Bs1* gene both necessary and sufficient to repress transcription from P*xis*, the promoter upstream of the gene encoding excisionase (Auchtung et al., 2007; Lee et al., 2007). We found that ImmR was not sufficient for induction of P*xis-lacZ* by RapI or the RecA-dependent DNA damage response. In cells cured of ICE*Bs1* (ICE*Bs1*^{0}) that express *immR* from its own promoter at an ectopic site, there was no detectable induction of P*xis-lacZ*, either after addition of mitomycin C (MMC) to induce the *recA*-dependent DNA damage response (Fig. 2A), or after overproduction of the cell-cell sensory regulator RapI (Fig. 2B). In contrast, in cells containing ICE*Bs1*, expression of P*xis-lacZ* was induced after addition of MMC (Fig. 2A) or overproduction of RapI (Fig. 2B), as observed previously (Auchtung et al., 2007). These results indicate that at least one additional ICE*Bs1* gene is required to de-repress expression from P*xis*.

Previously we found that a deletion-derivative of ICE*Bs1* containing only four genes, *int*, *immA* (*ydcM*), *immR*, and *xis*, was stably maintained in the chromosome and could be induced to excise by either the DNA damage response or by overexpression of *rapI* (Lee et al., 2007). Since *int* and *xis* encode the integrase and excisionase respectively (Lee et al., 2007), we suspected that *immA* might encode a regulatory protein required for de-repression. Analysis of ImmA using the conserved domain architecture retrieval tool {C-DART (Geer et al., 2002)} revealed that it has a conserved domain of unknown function (COG2856) that contains the characteristic HEXXH motif found in many zinc-dependent metalloproteases {(Fujimura-Kamada et al., 1997), and references therein}.

We found that *immA*, the gene immediately downstream from *immR* (Fig. 1), was needed for de-repression of P*xis-lacZ*. An *immA* null mutant was unable to de-repress expression of P*xis-lacZ* in response to MMC (Fig. 2C) or overproduction of RapI (Fig. 2D). This defect in induction was rescued by providing wild type *immA* from a heterologous promoter (P*spank-immA*) at an ectopic site in the chromosome (Fig. 2C, D), indicating that the defect in induction was due to loss of *immA* and not to unintended secondary effects.

We also found that expression of *immA* and *immR* together from their normal promoter, in the absence of any other ICE*Bs1* genes, was sufficient for normal repression and de-repression of P*xis-lacZ* (Fig. 2A, B). Transcription of P*xis-lacZ* was strongly de-repressed following addition of MMC (Fig. 2A) or overproduction of RapI (Fig. 2B). This is in marked contrast to the lack of de-repression in cells expressing only *immR* and not *immA* (Fig. 2A, B). These results indicate that of all the ICE*Bs1* genes, *immA* and *immR* together are sufficient for ICE*Bs1* induction in response to DNA damage or overproduction of RapI (also an ICE*Bs1* gene product).

ImmA contains an HEXXH motif that is required for proteolytic activity of many zinc-dependent metalloproteases. We constructed an *immAH75A* mutation (altering the first histidine in this motif) and found that it caused an *immA* null phenotype. That is, in cells containing wild type *immR* and the *immAH75A* allele, there was no de-repression of P*xis-lacZ* by either MMC or overproduction of RapI (data not shown).

Taken together, all the genetic data indicate that ImmA is an anti-repressor required for de-repression of ICE*Bs1* in response to DNA damage or RapI and the first histidine in the HEXXH motif found in many metalloproteases is somehow important for ImmA function (see below).

ImmR and ImmA homologs encoded by other mobile genetic elements

immR and *immA* are encoded with the integrase gene in a so-called lysogeny module at the left end of ICE*Bs1* (Fig. 1). Lysogeny modules are found in many temperate (lysogenic) phage and encode genes that promote integration and suppress lytic phage growth. Several phages of low G+C-content gram- positive bacteria contain modules with homologs of *int*, *immR* and *immA* (Lucchini et al., 1999).

Homologs of ImmA and ImmR are readily identifiable through comparative sequence analyses and over 80% of these homologs are encoded in known or putative mobile genetic elements (Table 2). The genes most similar to *immR* and *immA* from ICE*Bs1* are found in a putative mobile element in the genome of a vancomycin resistant *Enterococcus faecalis* (Paulsen et al., 2003). ImmA and ImmR homologs are also present in many phages that are induced by the global DNA damage response. It is generally assumed that induction is caused by RecA-stimulated auto-proteolysis of the phage repressor, although the mechanism has not been characterized for elements containing ImmA homologs. Some of these mobile genetic elements (ø105, *skin*, and λBa04) also contain homologs of RapI and PhrI (Auchtung et al., 2005). Based on the conservation of ImmA and ImmR, the conservation of RecA, and the prevalence of lysogenic phages and other mobile elements that are induced by the RecA-dependent DNA damage response, we suspect that the function of the anti-repressor ImmA represents a conserved mechanism for inactivating repressor proteins and stimulating horizontal gene transfer.

The ImmA-like protein from the *B. subtilis* phage ø105 is required for inactivation of the phage repressor in response to DNA damage

We investigated the roles of the *immR*- and *immA*-like genes encoded by the *B. subtilis* bacteriophage ø105 (*c*ø105 and *orf2*, respectively) in regulating phage gene expression. The organization of these ø105 genes is similar to that of their counterparts in ICE*Bs1*. *c*ø105, *orf2*, and *int* are in a putative operon that is transcribed divergently from other phage genes (Fig. 3A) (Van Kaer et al., 1987). cø105 is the phage repressor, and it regulates transcription of itself and the divergent *orf4* (Van Kaer et al., 1987). ø105 lysogens are induced by the *recA*-dependent DNA damage response (Love and Yasbin, 1984).

We constructed a fusion of the promoter for *orf4* to *lacZ* (P*orf4-lacZ*) and analyzed repression and de-repression during DNA damage induced by MMC. Consistent with previous findings (Van Kaer et al., 1987), P*orf4-lacZ* was expressed at very low levels in ø105 lysogens (Fig. 3B). Expression was significantly induced shortly after treatment of cells with MMC (Fig. 3B). Expression of P*orf4-lacZ* was high in non-lysogenic cells lacking ø105 and was not further induced by addition of MMC (Fig 3B).

The high level of expression of P*orf4-lacZ* in non-lysogenic cells was significantly reduced by expression of *c*ø105 (Fig. 3B), indicating that the ø105 repressor, in the absence of any other phage gene products, is sufficient to repress transcription from P*orf4*. However, in cells in which *c*ø105 was the only phage gene expressed, P*orf4-lacZ* was not de-repressed in response to DNA damage induced by addition of MMC (Fig. 3B), indicating that at least one other ø105 gene is needed for induction by DNA damage.

We found that de-repression of P*orf4-lacZ* during the DNA damage response required the ImmA homolog Orf2. Concomitant expression of *c*ø105 and *orf2* {(ø105)*immA*} allowed efficient de-repression of P*orf4-lacZ* after addition of MMC (Fig. 3B). Based on these results, we conclude that the ø105 repressor and (ø105)ImmA function analogously to ImmR and ImmA of ICE*Bs1*. It is likely that other ImmR and ImmA homologs similarly regulate the activities of their respective mobile elements.

ImmA is required for degradation of ImmR

To explore the mechanism of ImmA-mediated inactivation of ICE*Bs1* ImmR in vivo, we monitored the fate of ImmR under conditions that induce ICE*Bs1* gene expression, excision, and mating. We found that under these conditions, ImmR is proteolyzed in vivo in an ImmA-dependent manner.

We measured the stability of ImmR in vivo in pulse-chase experiments in ICE*Bs1*^{0} cells that expressed *immR* and *immA* ectopically and expressed *rapI* under control of a xylose-inducible promoter (P*xyl-rapI*). We monitored the amount of radioactively labeled ImmR by immunoprecipitation at various times before and after production of RapI. ImmR was stable in the absence of induction of *rapI* (Fig. 4A; lanes 1-4). However, 10-20 minutes after xylose was added to cultures to induce RapI overproduction, pulse-labeled ImmR was degraded (Fig. 4A, lanes 5-8). These results demonstrate that *rapI* overexpression promotes degradation of ImmR in cells that also express ImmA.

We also assessed ImmR levels using Western blots. We found that one hour after treatment with MMC (Fig. 4B) or overexpression of *rapI* (Fig. 4C), the level of ImmR protein was greatly reduced. This decrease occurred in cells that contained ICE*Bs1* as well as in ICE*Bs1*^{0} cells that expressed *immR* and *immA* (Fig. 4B, C). In contrast, in the absence of *immA*, ImmR was stable, even under inducing conditions (Fig. 4B, C), indicating that the reduced level of ImmR was dependent on the presence of ImmA.

Whereas *immA* was required for degradation of ImmR under both conditions tested, *recA* was only required for ImmA-dependent ImmR degradation during the SOS response (Fig. 4D) and not after overproduction of RapI (Fig. 4E). *rapI* was not required for ImmA-dependent degradation of ImmR during the SOS response (Fig. 4B). Similarly, previous studies showed that either activated RecA or RapI induce ICE*Bs1* gene expression, excision and transfer (Auchtung et al., 2005). Taken together, our results demonstrate that RecA and RapI function independently to cause ImmA-dependent degradation of ImmR and subsequent de-repression of ICE*Bs1* gene expression, excision, and transfer.

ImmA interacts directly with ImmR

We hypothesized that ImmA-dependent degradation of ImmR might involve direct interaction between the two proteins. Using a yeast two-hybrid system, we found that ImmA and ImmR interact. We constructed fusion proteins of ImmR to the Gal4 activation domain and ImmA to the Gal4 DNA binding domain. We introduced these fusions into *Saccharomyces cerevisiae* cells that had *ADE2*, a gene required for adenine synthesis, under control of a Gal4-activated promoter (James et al., 1996). Growth of these cells on medium lacking adenine requires an interaction between ImmR and ImmA to unite the two domains of Gal4 and to activate transcription of *ADE2*. Cells that contained both the ImmR and ImmA fusion proteins were able to grow on medium lacking adenine (data not shown). This result indicates that ImmR and ImmA can interact, and based on the phenotypes of *immR* and *immA* mutants, we suspect that these proteins interact in *B. subtilis*.

Several phage repressors act as dimers or multimers to regulate gene expression {e.g., (Little, 1984; Walker, 1996)}. We detected self-interaction between ImmR fusion proteins (but not ImmA), indicating that ImmR likely forms a dimer or higher order multimer to regulate gene expression.

ImmA causes degradation of ImmR in *E. coli*

To further explore the action of ImmA outside of *B. subtilis*, we assayed ImmR degradation in *E. coli*. Using Western blots, we observed a shortened form of ImmR when N-terminally

His-tagged ImmR (His6-ImmR) and ImmA were co-expressed in *E. coli* (Fig. 5A). This fragment was not detected in the absence of ImmA, and the relative abundance of the fragment was increased by concurrent expression of RapI (Fig. 5A). Mass analysis of the His-tagged ImmR fragment (Fig. 5C) indicated that in *E. coli*, ImmR is cleaved between F95 and M96 (Fig. 5D) in an ImmA-dependent fashion.

Purified ImmA causes cleavage of ImmR in vitro

To determine its mechanism of action, we purified ImmA and tested its activity in vitro. We overproduced and purified His6-ImmR and untagged ImmA from *E. coli*. Incubation of purified ImmA with His6-ImmR led to cleavage of ImmR (Fig. 5B). There was no detectable cleavage of His6-ImmR in the absence of ImmA (Fig. 5B). To be sure that the proteolytic activity observed in vitro was due to ImmA and not a contaminant, we also purified and tested ImmA(H75A) mutant protein. His6-ImmR was not cleaved in vitro during incubation with ImmA(H75A). Previously we had purified a C-terminally His-tagged ImmR (Auchtung et al., 2007). This protein was also cleaved in vitro when incubated with ImmA, but remained stable during incubation alone or with ImmA(H75A) (data not shown).

Although we did not detect autocleavage of ImmR in vitro in incubations containing ImmR alone (above), repressors from phages λ, 434, and P22 cleave themselves in vitro when incubated with activated RecA or without RecA at elevated pH. We conducted additional tests for autocleavage of ImmR by incubating ImmR-His6 at pH's in the range from 4 to 11 at 30°, 37°, or 42° for ~19 hrs and ~188 hrs. We observed no evidence of specific autocleavage (data not shown).

We determined the masses of ImmR fragments from the ImmA-dependent cleavage reactions by mass spectrometry (MALDI-TOF). In reactions with His6-ImmR and ImmA, we detected masses corresponding to two fragments of ImmR (Fig. 5C), consistent with site-specific cleavage between F95 and M96 of ImmR (Fig. 5D). The mass of the larger fragment matched that of the His6-ImmR fragment from ImmA-dependent cleavage in *E. coli* (Fig. 5B, C). In the presence of ImmA(H75A), we only detected full length His6-ImmR (Fig. 5C). In reactions with C-terminally tagged ImmR-His6 and ImmA, we also detected mass peaks consistent with site-specific cleavage between F95 and M96 (Fig. 5C). Taken together, our results indicate that ImmR is cut in an ImmA-dependent manner at a single site, between F95 and M96 (Fig. 5D).

Discussion

Using in vivo and in vitro analyses, we found that ImmA is an anti-repressor that most likely functions as a site-specific protease to cleave and inactivate the repressor ImmR thereby causing de-repression of the mobile genetic element ICE*Bs1*. ImmA mediates ICE*Bs1* induction in response to cues of DNA damage and cell-cell signaling. ImmA homologs are widely conserved in mobile genetic elements and the mechanism of action of members of this anti-repressor family appears to be quite different from that of other characterized anti-repressors involved in horizontal gene transfer.

SOS-induced auto-proteolysis of repressors

A well understood mechanism of de-repression of mobile genetic elements is the RecA-stimulated autocleavage of phage repressors in response to DNA damage. RecA bound to single-stranded DNA stimulates autocleavage of the repressors of phages λ, 434, and P22 {e.g., (Little, 1984; Roberts and Roberts, 1975; Roberts et al., 1978; Sauer et al., 1982)}. RecA also appears to facilitate autocleavage of repressors of two conjugative transposons SXT and ICE*St1* (Beaber et al., 2004; Bellanger et al., 2007). In addition, RecA facilitates

autocleavage of the cellular SOS repressor, LexA (Little, 1984; Miller et al., 1996). In all cases, autocleavage depends on the presence of conserved catalytic residues in the C terminus of the repressor (Slilaty and Little, 1987).

Proteolysis mediated by the anti-repressor ImmA

Destruction of the ICE*Bs1* repressor ImmR does not resemble auto-proteolysis of previously characterized repressors. ImmR does not contain the conserved residues necessary for autocleavage of LexA and phage repressors. The cleavage site in ImmR is between a phenylalanine and methionine, whereas the autocleavage site of LexA and similar repressors is typically between an alanine and glycine. We were unable to detect specific autocleavage of purified ImmR under conditions suitable for in vitro autocleavage of characterized phage repressors (Little, 1984; Slilaty et al., 1986).

Unlike previously characterized phage repressors, cleavage of ImmR in vivo and in vitro requires the anti-repressor ImmA and there is no evidence to suggest that ImmR is capable of cleaving itself. The simplest conclusion from our data is that the anti-repressor ImmA is a site-specific protease required for inactivation of the repressor ImmR.

The mechanism(s) by which RapI and RecA stimulate proteolysis of ImmR by ImmA is unknown. RapI and RecA may increase synthesis, stability, or specific activity of ImmA. Expression of *immA* from the IPTG-inducible promoter P*spank* did not de-repress P*xis-lacZ* unless RapI was overproduced or DNA damage was induced (Fig. 2C,D). Therefore, RapI and RecA likely affect the anti-repressor ImmA at a level other than transcription initiation.

Anti-repressors and horizontal gene transfer

Several anti-repressors from mobile genetic elements have been characterized, but none are known to cause degradation of the target repressor. Many anti-repressors function by direct interaction with their cognate repressor. For example, Tum, Coi, E, and Ant, antirepressors from bacteriophage 186, P1, P4, and P22, respectively, all form complexes with their respective repressors and inhibit the ability of these repressors to bind DNA (Heinzel et al., 1992; Liu et al., 1998; Shearwin et al., 1998; Susskind and Youderian, 1983). The satellite phage RS1 encodes RstC, an antirepressor that promotes aggregation of the RstR repressor of the CTX phage (Davis et al., 2002). In contrast, the λ Cro protein binds directly to DNA and competes with the λ CI repressor for its binding sites (Ptashne, 1992), and references therein).

Conservation of ImmA and ImmR

ImmR and ImmA homologs reside in other known or putative mobile genetic elements (Table 2). We found that the ImmR and ImmA homologs encoded by the *B. subtilis* bacteriophage ø105 (cø105 and (ø105)ImmA, respectively) function as a repressor and anti-repressor to regulate expression of genes in ø105. Thus, we propose that these proteins represent a conserved strategy for regulating the activity of many mobile genetic elements.

ICE*Bs1* differs from previously characterized DNA damage-inducible mobile genetic elements in that it requires the anti-repressor ImmA for inactivation of the repressor ImmR, and in that excision and mating occur in response to cues from cell-cell signaling as well as cues of DNA damage. RapI and RecA function independently of each other to mediate cues from these different pathways and stimulate transfer of ICE*Bs1* by stimulating ImmA-mediated proteolysis of ImmR. ImmA homologs in other mobile genetic elements may be capable of responding to more than one signal, thereby allowing multiple conditions to affect horizontal gene transfer.

Experimental Procedures

Media and growth conditions

B. subtilis cells were grown at 37°C with aeration in S7 minimal salts medium {(Vasantha and Freese, 1980), except that 50 mM MOPS was used instead of 100} supplemented with 1% glucose or arabinose, 0.1% glutamate, 40 μg/ml tryptophan and phenylalanine, and 120 μg/ml threonine. Arabinose was used when *rapI* was expressed from P*xyl*. When appropriate, antibiotics were used at the following concentrations: chloramphenicol (5 μg/ml); kanamycin (5 μg/ml); tetracycline (10 μg/ml); spectinomycin (100 μg/ml); and erythromycin (0.5 μg/ml) and lincomycin (12.5 μg/ml) together to select for macrolide-lincosamide-streptogramin B (MLS) resistance. IPTG and MMC (Sigma) were used at final concentrations of 1 mM and 1 μg/ml respectively.

S. cerevisiae cells were grown at 30°C in yeast peptone dextrose medium or synthetic complete medium lacking uracil and leucine or lacking uracil, leucine, and adenine (Sambrook and Russell, 2001).

E. coli cells were grown in LB at 30°C or 37°C. When appropriate, ampicillin (100-200 μg/ml) and or chloramphenicol (15 μg/ml) were added. To induce gene expression in BL21-AI cells (Invitrogen), growth medium was supplemented with 1-4mM IPTG and 0.2% arabinose.

Strains and alleles

B. subtilis strains used in this study are listed in Table 1. Standard techniques were used for cloning and strain construction (Harwood and Cutting, 1990;Sambrook and Russell, 2001). The ICE*Bs1*^{0} strain, and the Δ*int*205::*cat*, *amyE*::{(P*spank*(*hy*)-*rapI*) *spc*}, *cgeD*::{(P*spank*(*hy*)-*rapI*) *kan*}, *cgeD*::{(P*immR*-*immR*) *kan*}, *thrC*::{(P*xis*-*lacZ*Ω343) *mls*}, Δ*recA260*::*cat*-*mls*, and Δ*(rapI phrI)*::*kan* alleles were previously described (Auchtung *et al.*, 2005;Auchtung *et al.*, 2007). (Note: *mls* is the same as the previously used *erm*).

The promoter for *orf4* from *B. subtilis* phage ø105 was fused to *lacZ* (P*orf4*-*lacZ*) by cloning from 298 bp upstream of the *orf4* start codon to 252 bp into *orf4* upstream of the promoterless *lacZ* in the vector pDG793 (Guerout-Fleury et al., 1996). This promoter fragment contains all six immunity repressor binding sites known to control expression of *orf4* (Van Kaer et al., 1989). This fusion was integrated into *thrC* by homologous recombination.

immR and *immA* from ICE*Bs1* were cloned together under control of their own promoter into the integration vector pMMB124 (Auchtung et al., 2007). Sequences extended from 268 bp upstream of the *immR* start codon to 2 bp downstream of the *immA* stop codon. *c*ø105 was cloned with its own promoter into pMMB124. Sequences extended from 293 bp upstream of the *c*ø105 start codon to 24 bp downstream of the stop codon. Each clone was introduced into the *B. subtilis* chromosome at *cgeD* by homologous recombination.

LacI-repressible, IPTG-inducible copies of *immA* and *immA*(ϕ105) were generated by cloning from 24 bp upstream of *immA* to 2 bp downstream of its stop codon and from 28 bp upstream of *immA*(ϕ 105) to 29 bp downstream of its stop codon downstream of P*spank* in pDR110 (Rokop et al., 2004), followed by integration of these plasmids at *amyE* by homologous recombination. P*spank*-*immAH75A* was constructed using Quikchange (Invitrogen) site-directed mutagenesis of a plasmid containing P*spank*-*immA*. The xylose-inducible copy of *rapI* was constructed by M. Berkmen and was created by cloning *rapI* downstream of P*xyl* in vector pDR160, (from D. Rudner) followed by integration of the plasmid at *amyE* by homologous recombination.

Δ*immA* Δ*int*::*cat* was generated through a combination of splicing by overlap extension (SOE) and long-flanking homology PCR. This construct creates a deletion of *immA* (first 3 codons at 5′ end joined to last two codons at 3′ end) linked to a replacement of +53 to +1097 of *int* with the chloramphenicol resistance gene from pGEM*cat* (Youngman et al., 1989). This *int* deletion removes the same sequence as Δ*int205*::*cat* (Auchtung et al., 2005).

The state of ImmR in *E. coli* was monitored in strains BOSE799 (BL21-AI pBOSE794), BOSE817 (BL21-AI pBOSE794, pBOSE801), and BOSE819 (BL21-AI pBOSE792, pBOSE801). pBOSE792 expresses immR with the N-terminal tag MGSSH_6SSGLVPRGSH from the T7 promoter in pET14b (Novagen). pBOSE794 expresses the aforementioned N-terminally tagged ImmR and untagged ImmA from the T7 promoter in pET14b. pBOSE801 expresses untagged RapI from pBAD33 (Guzman et al., 1995), with an exogenous rbs incorporated during cloning.

Proteins for in vitro assays were purified from the following strains. N-terminally His-tagged ImmR was purified from BOSE798 (BL21-AI pBOSE792); pBOSE792 is described above. C-terminally His-tagged ImmR was purified from JMA622 (BL21-AI pJMA605); pJMA605 was previously described (Auchtung et al., 2007). ImmA was purified from BOSE848 (BL21-AI pBOSE831). pBOSE831 expresses untagged immA from the T7 promoter in pSA27 (Duncan et al., 1996). In pBOSE831, the codon for V14 was mutated (gtg to gta) by Quikchange (Stratagene) to prevent translation from starting at this site. ImmA(H75A) was purified from BOSE847 (BL21-AI pBOSE841). pBOSE841 was made by Quikchange site-directed mutagenesis of H75 to A in the immA encoded by pBOSE831.

Yeast two-hybrid assays

Plasmids encoding ImmR and ImmA fused to the Gal4 DNA binding domain (Gal4-BD) or Gal4 activation domain (Gal4-AD) were generated by cloning the coding sequence of *immR* or *immA* in the same reading frame as the upstream Gal4-AD coding sequence in plasmid pGAD-c1 (James et al., 1996) or the upstream Gal4-BD coding sequence in plasmid pGBDu-c3 (James et al., 1996). Plasmids containing the ImmR and ImmA Gal4 binding and activation domain fusions were transformed into *S. cerevisae* strain PJ69-4A (*trp-901 leu2-3 ura3-52 his3-200 gal4*Δ *gal80*Δ *LYS2*::(*GALl-HIS3*) *GAL2-ADE2 met2*::(*GAL7-lacZ*) (James et al., 1996) selecting for growth on synthetic complete medium lacking uracil and leucine. Six transformants were purified to single colonies on medium without uracil and leucine, then repurified and tested on the same medium without and with adenine. A pair of proteins were designated as interacting if all 6 transformants grew on medium lacking adenine. Similar results were observed with 6 transformants from each of three independent transformation experiments.

Pulse-chase experiments

Cells were grown in defined minimal medium containing arabinose to an OD600 ~ 0.5, at which point ^{35}S-labeled methionine (1,100 mCi/mmol) was added to the culture to a final concentration of between 30 - 60 μCi/ml. Cells were incubated with label for 1 min and an aliquot was removed (T=0 sample). Unlabeled methionine (50 μM final concentration) and xylose (2% final concentration to induce expression from P*xyl*) were added. Samples were collected at 5, 10, and 20 minutes as indicated. All samples were frozen immediately on dry ice and stored at -80°C.

Samples were thawed and centrifuged to recover the cells. Cells were resuspended in buffer (10 mM Tris 1 mM EDTA) containing 20 mg/ml lysozyme and 50 μg/ml PMSF and incubated on ice for 30 min. Cell pellets were resuspended in a volume of buffer proportionate to the OD600 at which they were recovered in order to normalize the

concentration of proteins in the sample. SDS was added to 1%, then cells were lysed by heating at 100°C for 10 min. Twenty volumes of KI buffer (50 mM Tris 150 mM NaCL 1 mM EDTA 0.5% Triton X-100) containing 25 μg/ml PMSF were mixed with the cell lysates to reduce the SDS concentration to 0.05%. Samples were then centrifuged at 4°C for 15 min. The supernatant was removed and incubated overnight in the presence of rabbit polyclonal anti-ImmR sera (Covance) at 4°C (~1:300 dilution). Bound protein was separated from the supernatant by affinity purification with protein A sepharose. Bound protein was washed six times with KI Buffer containing PMSF. Proteins were eluted at 70°C for 15 min. in 2X SDS Buffer (0.2 M DTT 4% SDS 100 mM Tris-HCl pH 6.8 20% glycerol 0.005% bromophenol blue). The immunoprecipitated samples were analyzed by SDS-PAGE on 15% gels followed by electro-blotting to PVDF membranes. Radioactivity was detected by phosphorimaging using the Typhoon imager 9400 (Amersham Biosciences).

Western Blots

B. subtilis cells were grown in defined minimal glucose medium to OD600 ~ 0.5. Cultures were split and inducer (either MMC or IPTG) was added to half of the cultures. Samples were collected 60 minutes after addition of inducer from induced and uninduced cultures. Cells were pelleted by centrifugation, washed with TN Buffer (50 mM Tris 300 mM NaCl, pH8), and stored at −20°C.

Cell pellets were thawed on ice, resuspended in an appropriate volume of Buffer (10 mM Tris 10 mM EDTA, pH 8) containing 0.05 mg/ml lysozyme and 1 mM AEBSF, and incubated at 37°C for 20 min. Pellets were resuspended in a volume of buffer proportionate to the OD600 at which they were recovered in order to normalize the concentration of proteins in the sample. SDS-Loading Buffer was then added to samples, which were heated at 100°C for 10 min. followed by centrifugation to remove insoluble material.

Proteins were separated by SDS-PAGE on 15% gels and transferred to PolyScreen PVDF membrane (Perkin Elmer) using the Trans-blot semi-dry electro-blot transfer apparatus (BioRad). Membranes were blocked in 0.2% I-Block (Tropix) in TBST (50 mM Tris 200 mM NaCl 0.05% Tween-20 pH 8) either at room temperature for 1 hr. or overnight at 4°C. Membranes were incubated in 1:10,000 anti-ImmR rabbit polyclonal antisera in 0.2% I-Block TBST for 1 hr. at room temperature, washed several times in TBST, incubated in 1:3,000 goat anti-rabbit IgG-HRP conjugate (BioRad) for 1 hour at room temperature, and washed several times in TBST. Signals were detected using Western Lightning chemiluminescence reagents (Perkin-Elmer) followed by exposure to Kodak Biomax Light film.

The amount of ImmR was consistently higher in cells that expressed *immR* ectopically from its native promoter than in ICE*Bs1*$^+$ cells, and this made the pulse-chase experiments technically easier. We suspect that this increased production of ImmR is due to the loss of a potential ImmR binding site upstream of the promoter that might be involved in auto-repression (Auchtung et al., 2007). Increased production of ImmR is likely not due to the absence of another protein in ICE*Bs1* because levels of ImmR protein in cells lacking most of the genes in ICE*Bs1* (Δ*xis-yddM*) and expressing *immR, immA,* and *int* from its native promoter were similar to ICE*Bs1*$^+$ cells (data not shown).

β-galactosidase assays

β-galactosidase specific activity was assayed as described (Jaacks et al., 1989). Specific activity was calculated relative to the optical density at 600 nm of the samples and is plotted relative to the time of treatment as indicated. Results shown are from a single experiment and are representative of results obtained in at least two independent experiments.

Comparative sequence analysis

Potential homologs of ImmR and ImmA were identified by a combination of protein BLAST (Altschul et al., 1997) and C-DART (Geer et al., 2002). More specifically, protein-protein BLAST was used to identify potential homologs of ImmR or ImmA that shared significant sequence identity and similarity. The adjacent or nearby genes were then analyzed using BLAST 2 (Tatusova and Madden, 1999) or C-DART to identify those proteins that had either significant sequence identity and similarity to ImmR or ImmA or contained the conserved domains found in ImmR (XRE family helix-turn-helix) or ImmA (COG2856). When pairs of *immR*- and *immA*-like genes were identified, the genes in the surrounding area were analyzed to determine if they encoded proteins likely indicative of a mobile genetic element or remnant (e.g., putative transposases, integrases, or other phage-related proteins). Many proteins were found to be ImmR- and ImmA-like and over 80% of these appear to be in putative mobile elements or remnants of mobile elements (Table 2).

ImmR cleavage in *E. coli*

ImmR in *E. coli* was monitored by Western blots of samples from BOSE799, BOSE817, and BOSE819. Strains were grown in shaking LB plus antibiotic(s) at 37°C to an OD600 of 0.45-0.65 and then induced with 4mM IPTG and 0.2% arabinose. Growth was continued for 2 hours, cells were pelleted, and pellets were stored at -20°C. For Western blots, cells were resuspended into TN buffer, mixed with 5x SDS sample buffer, boiled, and run on SDS-PAGE. Western blots to detect ImmR were performed as described above. His6-ImmR and the His-tagged ImmR fragment were purified from BOSE817 pellets using the lysis and chromatography procedures for purification of His6-ImmR from BOSE798, described below.

Protein purification

N-terminally His-tagged ImmR was purified from BOSE798. Cells were grown with shaking in LB at 37°C to OD600~ 0.5 and induced with 1mM IPTG and 0.2% arabinose. Approximately 4 hours after induction, cells were pelleted, and pellets were stored at -20°C. Cells were lysed by inversion at room temperature in a lysis buffer (43.4mM Tris, 263mM NaCl, 1x Cellytic, 196μg/ml lysozyme, 4.9μg/ml DnaseI, 9.8mM imidazole pH8). The lysate was centrifuged at 17,000 rcf, and His-tagged protein was purified from the supernatant by Ni-NTA (Qiagen) affinity chromatography using TN buffer supplemented with increasing concentrations of imidazole (10mM-1M). Eluted protein was dialyzed against TN buffer, then mixed with an equal volume of glycerol, and stored at 4°C or -80°C.

ImmA and ImmA(H75A) were purified from BOSE848 and BOSE847, respectively. Cells were grown shaking at 30°C overnight in LB with 100μg/ml Amp, 4mM IPTG, and 0.2% arabinose. Cells were pelleted by centrifugation, and pellets were stored at -20°C. Pellets were resuspended in 50mM Tris, 50mM NaCl, 10mM EDTA, 20mM DTT, pH8 and lysed by sonication on ice. Upon centrifugation, ImmA was found in inclusion bodies in the pellet. Inclusion bodies were isolated (Martin and Schmid, 2003), and protein solubilized in 100mM Tris, 6M GdmCl, 1mM EDTA, 50mM reduced glutathione, pH8.5 was stored at 4°C. ImmA was refolded immediately before use in assays by 11-fold dilution on ice into refolding buffer (50mM Tris, 10mM DTT, 5%glycerol, 100μM $ZnCl_2$, 0.8M L-arginine, pH7). Diluted samples were centrifuged at 16,100 rcf at 4°C for 20 min. Protein in the supernatant was used for in vitro assays.

In vitro assays

Cleavage of ImmR was assayed in vitro by incubating 17μM His6-ImmR in vitro overnight at 37°C alone, with 11μM or 22μM ImmA, or with 18μM ImmA(H75A), as indicated. The

final reaction buffer was 43.8mM Tris, 6.44mM DTT, 19.22% glycerol, 48mM NaCl, 64.4 μM $ZnCl_2$, 0.516M L-arginine, 0.22M GdmCl, 36.4 μM EDTA, 1.82mM GSH, pH~7.3. Reactions were mixed with 5x sample buffer, boiled, and run on SDS-PAGE. Gels were stained with Coomassie brilliant blue.

Mass analysis

In vitro reactions and His-tagged protein purified from BOSE817 were analyzed by mass spectometry (MALDI-TOF) by the MIT Biopolymers Laboratory. Expected masses were calculated using the “compute pI/MW tool” on the Expasy proteomics server {http://us.expasy.org/tools/pi_tool.html}.

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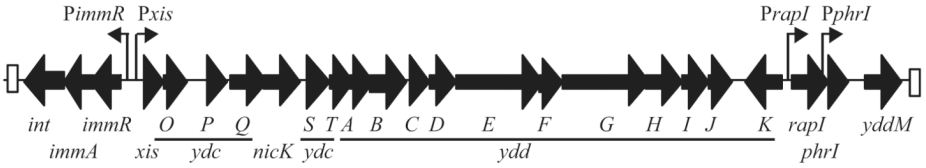


Figure 1. Map of ICE*Bs1*

Each of the 24 genes encoded by ICE*Bs1* is indicated by the thick black arrows oriented in the direction of transcription. Gene names are indicated under each arrow. Single letter designations are given for the genes of unknown function (*ydcO-ydcT; yddA-yddK; yddM*). Thin arrows indicate the positions of the characterized promoters. White boxes denote the ends of the element.

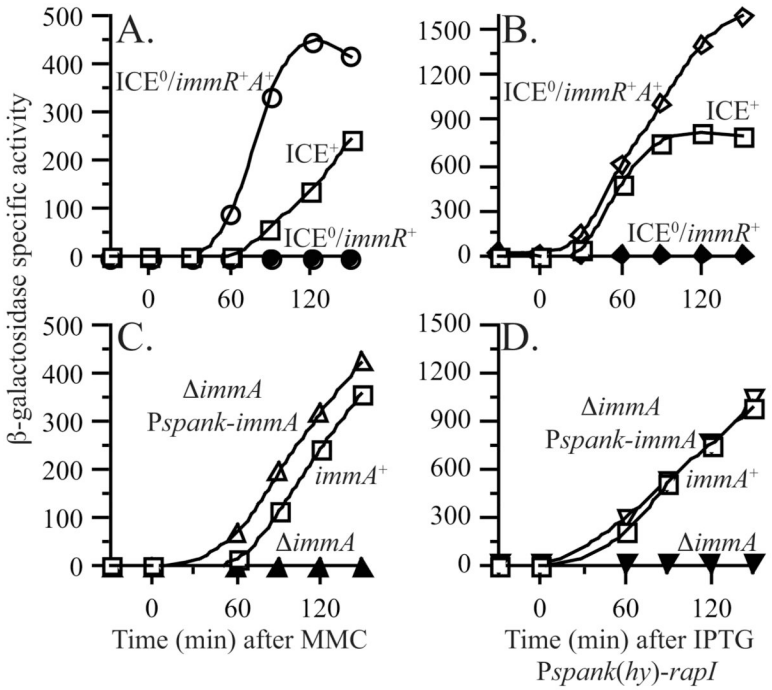


Figure 2. ImmA is required for de-repression of P*xis-lacZ*

Expression of P*xis-lacZ* was monitored in cells grown in defined minimal medium and treated at time 0 with either MMC to induce the SOS response (A & C), or with IPTG to induce expression of P*spank*(*hy*)-*rapI* (B & D). β-galactosidase specific activity is plotted as a function of time after the indicated treatment.

A. Effects of MMC on expression of P*xis-lacZ*. ICE*Bs1*$^{+}$ cells (JMA201, □, ICE^{+}); ICE*Bs1*^{0} cells expressing only *immR* from its own promoter (JMA421, ●, ICE^{0}/*immR*$^{+}$); ICE*Bs1*^{0} cells co-expressing *immR* and *immA* from the *immR* promoter (JMA436, ○, ICE^{0}/*immR*$^{+}$*A*$^{+}$). Data for JMA201 were published previously (Auchtung et al., 2007) and are re-plotted for comparison.

B. Effects of overproducing RapI on expression of P*xis-lacZ*. ICE*Bs1*$^{+}$ (KLG126, □, ICE^{+}); ICE*Bs1*^{0} cells expressing *immR* (JMA444, ◆, ICE^{0}/*immR*$^{+}$); ICE*Bs1*^{0} cells expressing both *immR* and *immA* (JMA446, ◊, ICE^{0}/*immR*$^{+}$*A*$^{+}$).

C-D. Effects of ImmA on expression of P*xis-lacZ* induced with MMC (C) or RapI (D). All cells contained the Δ*int*::*cat* mutation and the indicated *immA* allele. **C.** *immA*$^{+}$ (CAL16, □); Δ*immA* (JMA726, σ); Δ*immA* P*spank-immA* (JMA840, △). IPTG was present throughout growth of JMA840. **D.** *immA*$^{+}$ (JMA836, □); Δ*immA* (JMA838, τ); Δ*immA* P*spank-immA* (JMA842, ∇).

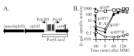


Figure 3. The bacteriophage ø105 homolog of ImmA, (ø105)ImmA, is needed for de-repression of phage gene expression in response to DNA damage

A. Diagram of the region of ø105 that contains *immA*(ø105) {aka *orf2*}, *c*ø105, and *orf4*. Genes are indicated by thick black arrows, with the name of each gene indicated above. Thin arrows indicate the positions of the *orf4* and *c*ø105 promoters (Van Kaer et al., 1987). Black rectangles indicate the positions of the six ø105 repressor bindings sites, three upstream from and adjacent to the leftward promoter P*c*ø105, two upstream from and adjacent to the rightward promoter P*orf4*, and one internal to *orf4* (Van Kaer et al., 1989). The white box underneath the map indicates the region of the *orf4* promoter cloned upstream of *lacZ* that was used to assay gene expression in B.

B. P*orf4-lacZ* expression was monitored in a ø105 lysogen (BOSE447, ◆, ø105^{+}), and in cells lacking ø105 that were otherwise wild-type (BOSE446, □, ø105^{0}), expressed the ø105 repressor from its native promoter (BOSE451, ○, ø105^{0}/R^{+}), or expressed the ø105 repressor from its native promoter and also expressed ImmA(ø105) from the IPTG-inducible promoter P*spank* (BOSE567, △, ø105^{0}/R^{+}A^{+}). Cells were grown in minimal medium containing IPTG and were treated with mitomycin C at OD600 ~ 0.5. Samples were collected at the times indicated and β-galactosidase specific activity was determined.

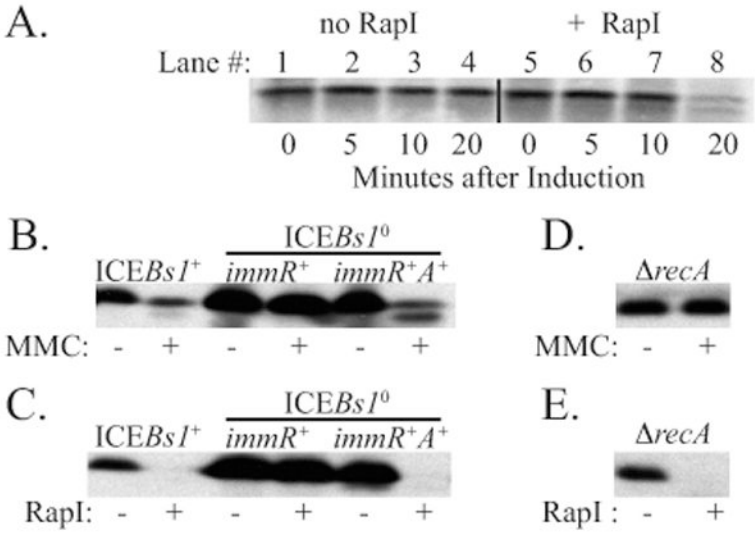


Figure 4. ImmA promotes degradation of ImmR in vivo

A. ImmR stability was monitored through pulse-chase experiments. ICE*Bs1*^{0} cells that co-expressed *immR* and *immA* from their native promoter and were otherwise wild-type (JMA436, Lanes 1-4) or expressed *rapI* from a xylose-inducible promoter (CAL746, Lanes 5-8) were grown in defined minimal medium lacking xylose. At OD600 ~ 0.5, ^{35}S-met was added to the cultures. One minute later >1000-fold excess unlabeled methionine was added along with xylose to induce expression of *rapI*. Samples were collected just prior to addition of unlabeled methionine and xylose, and 5, 10, and 20 minutes after addition. Samples were immunoprecipitated with anti-ImmR antibodies and analyzed by polyacrylamide gel electrophoresis followed by phosphorimaging.

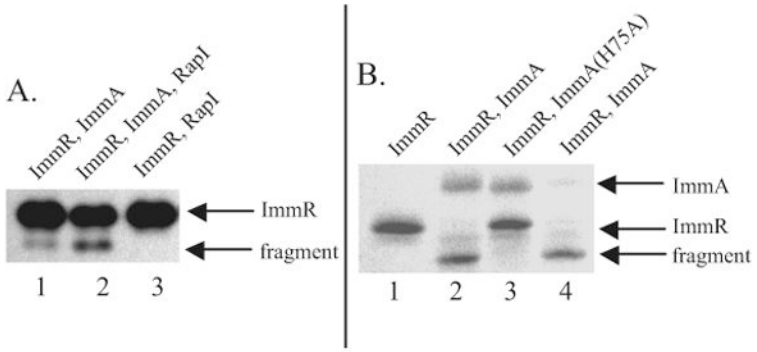
B-E. ImmR levels were monitored with Western blots using anti-ImmR antibodies. Cultures were grown in minimal medium to an OD600 ~ 0.5 and split in two. Cells were untreated (-) (B-E), or treated with MMC to induce the SOS response (+) (B, D), or treated with IPTG to overproduce RapI (+) (C, E). Samples were collected 60 minutes after the indicated treatment.

B. ICE*Bs1*$^+$ (JMA201); ICE*Bs1*^{0} P*immR-immR* (JMA421); ICE*Bs1*^{0} P*immR-immR immA* (JMA436)

C. All three strains contained P*spank*(*hy*)-*rapI*. ICE*Bs1*$^+$ (KLG126); ICE*Bs1*^{0} P*immR-immR* (JMA444); ICE*Bs1*^{0} P*immR-immR immA* (JMA446)

D. ICE*Bs1*$^+$ Δ*recA* (IRN444)

E. ICE*Bs1*$^+$ P*spank*(*hy*)-*rapI* Δ*recA* (CAL92).



C.

system	ImmR	ImmA	RapI	ImmR molecular mass expected (Da)	ImmR molecular mass observed (Da)
in vitro	His6-ImmR	ImmA	-	12961.3 3738.3	12960.9 3741.6
in vitro	His6-ImmR	ImmA(H75A)	-	16681.6	16682.2
in vitro	ImmR-His6	ImmA	-	11060.4 4803.4	11060.3 4806.7
E. coli	His6-ImmR	ImmA	RapI	12961.3	12961.8

D.

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  1  (Helix-turn-Helix domain)
 62 Leu Gly Lys Asp Glu Val Ser Lys Lys Asn
 72 Glu Thr Asp Leu Leu Asn Lys Thr Ile Asn
 82 Glu Ala Ile Gln Glu Leu Lys Asp Glu Asp
 92 Thr Leu Leu Phe/Met Asn Asp Gly Glu Phe
102 Asp Glu Glu Thr Ala Arg Leu Val Lys Lys
112 Ala Leu Lys Asn Gly Ile Lys Phe Ile Asp
122 Leu Lys Lys Lys Glu
```

Figure 5. ImmA-mediated cleavage of ImmR in *E. coli* and in vitro

A. ICE*Bs1* proteins were overexpressed in *E. coli* and lysates were analyzed by Western blot using anti-ImmR antibodies. Lysates were collected two hours after induction from cells that overexpressed His6-ImmR with ImmA (BOSE799, lane 1), with ImmA and RapI (BOSE817, lane 2), or with RapI (BOSE819, lane 3).

B. His6-ImmR (17μM) was incubated in vitro overnight at 37°C: alone (lane 1); with 22μM ImmA (lane 2); with 18μM ImmA(H75A) (lane 3); or with 11μM ImmA (lane 4). Reactions were run on SDS-PAGE and stained with Coomassie brilliant blue. Bands representing ImmA, His6-ImmR, and the ImmR fragment are indicated.

C. Products of ImmR cleavage assays in vitro and in *E. coli* were analyzed by MALDI-TOF. The form of the ImmA, ImmR, and RapI present in each reaction is indicated. Experimentally determined masses are shown alongside expected masses for the cleavage occurring between F95 and M96 of ImmR. The smaller ImmR fragment was detected from the in vitro reactions by not from *E. coli*.

D. The cleavage site in ImmR. The helix-turn-helix domain, contained in the first 61 N-terminal residues, is the presumed DNA binding domain that is similar to that of other phage-like repressors. The amino acid sequence from residue 62 to the C-terminus is shown with the cleavage site between F95 and M96 marked with a slash.

Table 1

B. subtilis strains

Strain	Relevant genotype
1L11	ø105 lysogen
BOSE447	ø105 lysogen (ø105^{+}) *thrC*::{(P*orf4-lacZ*) *mls*}
BOSE446	*thrC*::{(P*orf4-lacZ*) *mls*}
BOSE451	*cgeD*::{(P*c*ø105-*c*ø105) *kan*} *thrC*::{(P*orf4-lacZ*) *mls*}
BOSE567	*cgeD*::{(P*c*ø105-*c*ø105) *kan*} *amyE*::{(P*spank-orf2*) *spc*} *thrC*::{(P*orf4-lacZ*) *mls*}
CAL16	Δ*int*205::*cat thrC*::{(P*xis-lacZ*Ω343) *mls*}
CAL92	*amyE*::{(P*spank*(*hy*)-*rapI*) *spc*} Δ(*rapI phrI*)::*kan* Δ*recA260:cat mls*
CAL746	ICE*Bs1*^{0}*cgeD*::{(P*immR-immR immA*) *kan*} *amyE*::{(P*xyl-rapI*) *spc*} *thrC*::{(P*xis-lac*Ω343) *mls*}
IRN342	Δ(*rapI phrI*)::*kan*
IRN444	Δ*recA260*::*cat mls*
JMA168	*amyE*::{(P*spank*(*hy*)-*rapI*) *spc*} Δ(*rapI phrI*)::*kan*
JMA201	*thrC*::{(P*xis-lacZ*Ω343) *mls*}
JMA421	ICE*Bs1*^{0}*cgeD*::{(P*immR-immR*) *kan*} *thrC*::{(P*xis-lacZ*Ω343) *mls*}
JMA436	ICE*Bs1*^{0}*cgeD*::{(P*immR-immR immA*) *kan*} *thrC*::{(P*xis-lacZ*Ω343) *mls*}
JMA444	ICE*Bs1*^{0}*cgeD*::{(P*immR-immR*) *kan*} *amyE*::{(P*spank*(*hy*)-*rapI*) *spc*} *thrC*::{(P*xis-lacZ*Ω343) *mls*}
JMA446	ICE*Bs1*^{0}*cgeD*::{(P*immR-immR immA*) *kan*} *amyE*::{(P*spank*(*hy*)-*rapI*) *spc*} *thrC*::{(P*xis-lacZ*Ω343) *mls*}
JMA726	(Δ*immA*720 Δ*int*::*cat*) *thrC*::{(P*xis-lacZ*Ω343) *mls*}
JMA836	Δ*int*205::*cat cgeD*::{(P*spank*(*hy*)-*rapI*) *kan*} *thrC*::{(P*xis-lacZ*Ω343) *mls*}
JMA838	(Δ*immA*720 Δ*int*::*cat*) *cgeD*::{(P*spank*(*hy*)-*rapI*) *kan*} *thrC*::{(P*xis-lacZ*Ω343) *mls*}
JMA840	(Δ*immA*720 Δ*int*::*cat*) *amyE*::{(P*spank-immA*Ω218) *spc*} *thrC*::{(P*xis-lacZ*Ω343) *mls*}
JMA842	(Δ*immA*720 Δ*int*::*cat*) *cgeD*::{(P*spank*(*hy*)-*rapI*) *kan*} *amyE*::{(P*spank-immA*Ω218) *spc*} *thrC*::{(P*xis-lacZ*Ω343) *mls*}
JMA895	ICE*Bs1*^{0}*lacA*::{(P*immR-immR immA*) *tet*} *thrC*::{(P*xis-lacZ*Ω343) *mls*}
JMA900	ICE*Bs1*^{0}*lacA*::{(P*immR-immR immAH75A*) *tet*} *thrC*::{(P*xis-lacZ*Ω343) *mls*}
KLG126	*amyE*::{(P*spank*(*hy*)-*rapI*) *spc*} *thrC*::{(P*xis-lacZ*Ω343) *mls*}

*All *B. subtilis* strains are derived from AG174 (JH642) and contain *trpC2* and *pheA1* except for 1L11 and BOSE447, which are derivatives of *B. subtilis* 168 and contain *trpC2*. 1L11 was obtained from the *Bacillus* Genetic Stock Center.

Table 2

ImmR and ImmA homologs in mobile and putative mobile genetic element

Organism	Element	ImmR[1] (%Id/%Sim)	ImmA[2] (%Id/%Sim)	Din[3]	Reference[4]
Known mobile elements					
Bacillus clarkii	BCJA1c phage	Gp5 (29/48)	Gp4 (39/59)	nd	(Kropinski et al., 2005)
B. subtilis	ø105 phage	ø105 repressor (24/50)	ImmA(ϕ105) (32/53)	Yes	(McVeigh and Yasbin, 1996)
B. subtilis	PBSX phage	Xre (34/50)	XkdA (N.S.)	Yes	(McDonnell et al., 1994)
B. subtilis	*skin* element[5]	YqaE (34/52)	YqaB (N.S.)	No	(Krogh et al., 1996)
B. thuringiensis	MZTP02 phage	AAX62112.1 (N.S.)	AAX62113.1 (34/50)	Yes	(Liao et al., 2007)
Listeria monocytogenes	A118 phage	Gp36 (33/62)	Gp35 (N.S.)	Yes	(Loessner et al., 2000)
Geobacillus	GBSV1 phage	Gp48 (31/52)	Gp47 36/51)	nd	--
Geobacillus thermodenitrifica ns	defective prophage	GTNG_2860 (23/44)	GTNG_2861 (38/53)	nd	(Feng et al., 2007)
Streptococcus pneumoniae	MM1 phage	CI (25/52)	Orf2 (N.S.)	Yes	(Obregon et al., 2003)
S. thermophilus	ϕO1205 phage	Orf4 (35/43)	Orf3 (N.S.)	Yes	(Stanley et al., 1997)
Putative mobile elements[6]					
Actinobacillus pleuropneumonia serovar 1	--	Aple02002129 (35/54)	Aple02002131 (25/43)	nd	--
Bacillus anthracis	λBa04	BA3829 (30/52)	BA3830 (33/52)	nd	(Read et al., 2003)
B. cereus subsp. cytotoxis NVH391-98	--	Bcer98DRAFT_3608 (33/60)	Bcer98DRAFT 3609 (28/53)	nd	--
B. halodurans C-125	--	BH3549 (22/42)	BH3550 (38/57)	nd	(Canchaya et al., 2003)
B. weihensteph-anensis KBAB4	--	BcerKBAB4DRAFT 1206 (32/54)	BcerKBAB4DRAF T 1207 (34/51)	nd	--

Organism	Element	ImmR[1] (%Id/%Sim)	ImmA[2] (%Id/%Sim)	Din[3]	Reference[4]
B. weihensteph-anensis KBAB4	--	BcerKBAB4DRAFT 3005 (31/53)	BcerKBAB4DRAFT _3006 (32/53)	nd	--
C. perfringens ATCC13124	--	CPF_1021 (30/48)	CPF_1020 (33/53)	nd	(Myers et al., 2006)
C. perfringens ATCC 13124	--	CPF_1604 (34/52)	CPF1607 (33/58)	nd	--
Clostridium sp. OhILAs	--	ClosDRAFT_0334 (22/49)	ClosDRAFT_0333 (30/52)	nd	--
Desulfitobacterium hafniense DCB-2	--	DhafDRAFT_2630 (22/40)	DhafDRAFT_2631 (N.S.)	nd	--
Desulfotomaculum reducens MI-1	--	Dred_0020 (30/48)	Dred_0019 (31/50)	nd	--
Enterococcus faecalis V583	--	EF2544 (45/68)	EF2545 (42/64)	nd	(Paulsen et al., 2003)
E. faecium DO ctg653	--	EfaeDRAFT_2195 (29/47)	EfaeDRAFT_2196 (N.S.)	nd	--
Lactobacillus reuteri F275	--	Lreu_1144 (33/61)	Lreu_1145 (26/45)	nd	--
Listeria innocua CLIP 11262	--	Lin1762 (31/52)	Lin1763 (28/47)	nd	(Glaser et al., 2001)
L. innocua CLIP 11262	--	Lin1234 (32/56)	Lin1233 (35/55)	nd	(Glaser et al., 2001)
L. monocytogenes 1/2a F6854	--	LMOf6854_2699 (36/55)	LMOf6854_2699.1 (29/52)	nd	(Rasko et al., 2004)
Staphylococcus haemolyticus JSC1435	--	SH1805 (26/55)	SH1806 (N.S.)	nd	--
Thermoanaerobacter tencongensis	--	TTE2125 (26/49)	TTE2126 (26/45)	nd	(Bao et al., 2002)

[1] All ImmR-like proteins contain a predicted phage repressor helix-turn-helix motif identified by C-DART (Geer et al., 2002). For those proteins that share significant sequence identity with ImmR, the % amino acid identity and similarity is reported. The protein that does not share significant sequence identity with ImmR (not significant, N.S.) was identified due to the presence of a protein that shares sequence identity with ImmA.

[2] All ImmA-like proteins contain predicted Zinc metalloprotease motifs identified by C-DART (Geer et al., 2002). For those proteins that share significant sequence identity with ImmA, the % amino acid identity and similarity is reported. Proteins that do not share significant sequence identity with ImmA (not significant, N.S.) were identified due to the presence of a protein that shares sequence identity with ImmR.

[3] Din indicates damage inducible. Yes, no, or not determined (nd).

[4] Reference is given for the DNA sequence. When no reference is indicated (--), data were retrieved from unpublished sequences deposited in NCBI.

[5] *skin* is a defective prophage.

[6] Putative mobile genetic elements were identified based on the presence of multiple genes predicted to encode proteins homologous to those found in bacteriophage, transposons, or conjugative elements.