Regulation of horizontal gene transfer by intercellular peptide signaling in *Bacillus subtilis*

by

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B.S., Microbiology
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ABSTRACT

Horizontal gene transfer plays an important role in bacterial evolution. Although acquisition of foreign DNA can be beneficial to cells, it can also be detrimental. Therefore, cells that possess mechanisms to regulate horizontal gene transfer likely have a competitive advantage. Similarly, several mobile genetic elements possess mechanisms that regulate transfer. This regulation maintains stability and promotes dissemination of the element, thereby ensuring its survival. Elucidating the mechanisms that regulate transfer should provide insights into conditions that favor horizontal gene transfer and bacterial evolution. This thesis describes the regulation of two means of horizontal gene transfer in the gram-positive bacterium *Bacillus subtilis*.

Under certain conditions, *B. subtilis* cells undergo differentiation into competent cells capable of acquiring foreign DNA from the environment. A variety of factors regulate competence development. Initiation of genetic competence is controlled by a transcription factor, ComA, that also activates expression of genes that encode degradative enzymes, antibiotics, and secreted products important for biofilm formation. Three signaling peptides were known to stimulate the activity of ComA. I have characterized a fourth signaling peptide that stimulates the activity of ComA and have shown that intercellular peptide signaling modulates the timing and levels of ComA-dependent gene expression in response to different cellular cues.

B. subtilis cells also contain a mobile genetic element known as ICE*Bs1* (integrative and conjugative element *B. subtilis* #1). ICE*Bs1* is normally integrated in the chromosome of the host cell. Under certain conditions, ICE*Bs1* excises from the chromosome and transfers through a self-encoded conjugative apparatus to recipient cells. Both the global DNA damage response and intercellular peptide signaling regulate excision and transfer of ICE*Bs1*. The global DNA damage response stimulates ICE*Bs1* excision and transfer and likely provides a mechanism for the ICE*Bs1* element to escape a distressed host cell for a more suitable host. Intercellular peptide signaling limits excision and transfer of ICE*Bs1* to conditions when successful dissemination to cells lacking ICE*Bs1* is most likely to occur. The ICE*Bs1*-encoded proteins that regulate excision and transfer in response to intercellular peptide signaling and the global DNA damage response are encoded by other mobile genetic elements, indicating that this may be a conserved mechanism regulating their dissemination.

Thesis Advisor: Alan D. Grossman
Title: Professor of Biology

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My time in Alan's lab has been enriched by the useful collaborations that I have had throughout the years. Cathy Lee has been a wonderful collaborator and a great source of advice, encouragement, and enthusiasm. I also had the opportunity to work with three great undergrads - Kat Garrison, Alisa Lehman, and Rita Monson. I appreciate their hard work in spite of their heavy class loads.

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Thesis plan

Chapter 1 is an introduction to intercellular signaling mechanisms that regulate responses in bacterial cells. This chapter provides a general overview of intercellular signaling and focuses primarily upon regulation of horizontal gene transfer by intercellular signaling. The chapter ends with a discussion of the role that horizontal gene transfer plays in bacterial evolution and the potential benefits provided by regulation.

Chapter 2 describes the identification of a signaling peptide, the PhrK peptide, that stimulates the activity of the transcription factor ComA in *Bacillus subtilis*. I also describe our characterization of the roles that PhrK and other Phr peptides known to stimulate ComA activity play in modulating the timing and level of ComA-dependent gene expression in response to certain biological cues. This work was done in collaboration with Catherine Lee, a senior research scientist in Alan's lab. Catherine performed the *phrC*, *phrF*, and *phrK* microarray and *srfA-lacZ* fusion experiments. I performed the microarray and *pel-lacZ* fusion experiments in *rapF*- and *rapK*-overexpressing cells and the *srfA-lacZ* experiments examining the effects of *rap*mutations in wild-type and mutant strain backgrounds. We expect to submit a manuscript detailing this work to the Journal of Bacteriology.

Chapter 3 was published in the Proceedings of the National Academy of Sciences, volume 102, pages 12554-9, as "Regulation of a *Bacillus subtilis* mobile genetic element by intercellular signaling and the global DNA damage response," by Jennifer M. Auchtung, Catherine A. Lee, Rita E. Monson, Alisa P. Lehman, and Alan D. Grossman. Chapter 3 describes the initial identification and characterization of the mobile genetic element ICE*Bs1* and its regulation by intercellular peptide signaling and the global DNA damage response. Catherine performed the microarray and mating experiments described in the supplementary material, replicates of mating experiments described in the text, and *rapI-lacZ* expression assays. Rita performed the initial

experiments assaying mating into other *Bacillus* species and the *B. subtilis* ICE*Bs1*-cured strain. Alisa assayed ICE*Bs1* transfer into *Listeria monocytogenes* and *rapI-lacZ* expression. I performed the initial microarray experiments, the quantitative excision assays, and the mating experiments into cells lacking *phrI*.

Chapter 4 describes our ongoing work to understand the molecular mechanisms that regulate ICE*Bs1* gene expression, excision, and transfer. In this chapter, I describe the identification of two element encoded proteins, ImmR and ImmA, that regulate ICE*Bs1* gene expression in response to intercellular peptide signaling and the global DNA damage response. This work was also done in collaboration with Catherine Lee. She performed the *lacZ* experiments shown in Fig. 5, as well as replicates of some of the other *lacZ* experiments shown in this chapter. I performed the remaining *lacZ* experiments, the primer extension experiments, the purification of ImmR, the electrophoretic mobility shift assays, and the comparative sequence analysis.

Chapter 5 is a discussion of the work presented in the thesis. This chapter briefly summarizes the work that was done and discusses the potential benefits of mechanisms of regulating horizontal gene transfer. I also describe several questions to be addressed by future research that build upon the insights gained through the work described in this thesis.

Chapter 1: Introduction to intercellular signaling mechanisms that regulate DNA transfer in bacterial cells

Outline:

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I. Regulation by diffusible intercellular signaling molecules in bacteria

Cells sense changes in their environment and respond. Intercellular signaling molecules are one broad class of environmental signals perceived by cells; these signals can be used to coordinate the activities of cells in multicellular organisms and to coordinate the activities of unicellular organisms in multicellular communities (reviewed in 131, 167). Several types of intercellular signaling molecules have been characterized, including surface-associated (82, 172) and diffusible signaling molecules (131, 167). The focus of this work is to characterize responses regulated by diffusible intercellular signaling molecules in the gram-positive bacterium *Bacillus subtilis*.

Early work in a few bacterial species revealed the roles that secreted intercellular signaling molecules play in regulating biological responses. Cells of the gram-positive pathogen *Streptococcus pneumoniae* produce the machinery required to acquire DNA from the environment (become competent for DNA transformation) in response to a threshold concentration of self-produced intercellular signaling peptides (69, 154). This regulation limits acquisition of DNA from the environment to conditions when DNA from other *S. pneumoniae* cells is likely to be abundant, thereby limiting the likelihood of acquiring heterologous DNA that may be detrimental to the cell (reviewed in 141, 155). In the gram-negative squid symbiont *Vibrio fischeri*, exposure to a threshold concentration of self-produced signaling molecules (also known as autoinducers (AIs)) stimulates bioluminescence (47). Bioluminescence is energetically costly to *V. fischeri* cells; regulation by autoinduction limits bioluminescence to conditions where it is required for *V. fischeri* to maintain its symbiotic association with its host (164, and references therein). Secreted sex pheromones produced by the gram-positive pathogen *Enterococcus faecalis* stimulate transfer of conjugal plasmids from plasmid containing cells to

plasmid-less cells (45). *E. faecalis* plasmids utilize this regulation to promote dissemination to new host cells (45). Building upon these early insights, subsequent work has shown that many bacterial species use intercellular signaling molecules to regulate specific responses (reviewed in 54, 104, 167).

In order for cells to respond to intercellular signaling molecules, a threshold concentration of signal must accumulate and be sensed by the cells (reviewed in 54, 167). For many processes regulated by intercellular signaling, this threshold concentration is achieved when cells have reached a certain population density; therefore, this mechanism of regulation is often called quorum sensing as it requires a "quorum" (minimum number) of signal-producing cells to trigger a response (54, 167).

In some cases, quorum sensing is thought to regulate processes, such as acquisition of DNA from the environment, bioluminescence in the light organ of the squid, and biofilm formation, that are more beneficial when a high concentration of closely related bacteria are present, or are more efficiently accomplished by a high concentration of cells than by individual cells (141, 155, 165, 167). In other cases, such as regulation of flagellar gene expression in *V. fischeri* (102) and production of virulence factors in *V. cholerae* (185), quorum sensing inhibits cellular responses once cells have reached a certain concentration. In *V. fischeri*, repression of flagellar gene expression is thought to be important for maintaining its symbiotic association with the host (102). In *V. cholerae*, repression of virulence factors is thought to allow detachment of cells from host tissue, which leads to dissemination of cells to new sites of infection within the host or to release of cells into the environment (185).

Alternatively, it has been argued that regulation by intercellular signaling molecules is not a mechanism by which cells assess the concentration of other cells around them, but rather a

mechanism to sense the amount of diffusion and mixing within a cell's environment (135). However, several lines of evidence indicate that intercellular signaling molecules can serve multiple purposes, including providing information about signal diffusion and fidelity (40), spatial relationships between cells (48), the presence of other bacterial species (176) or host cells (20, 29), and the population density of cells. Therefore, it is likely that intercellular signaling may serve more than one purpose under a given condition. For example, a high concentration of cells likely experiences limited diffusion of signaling molecules (140).

Although intercellular signaling molecules may serve alternative purposes under different conditions, several general questions relate to the investigation of processes regulated by intercellular signaling. Some of these questions include:

What types of signals are produced?

How are signals sensed and how is this information translated into a cellular response?

What types of responses are regulated?

The next few sections of this introduction will provide an overview of the three most extensively characterized types of intercellular signaling systems and the processes they regulate. Since the remaining chapters of this thesis focus on how intercellular signaling regulates horizontal gene transfer in *B. subtilis*, I have provided more detailed descriptions of intercellular signaling systems that regulate DNA transfer. I also describe mechanisms that are known to interfere with intercellular signaling. Following these sections, I briefly review the molecular mechanisms that mediate horizontal gene transfer and the role that horizontal gene transfer is thought to play in bacterial evolution.

II. Overview of bacterial intercellular signaling systems

A. Peptide signaling in Gram-positive bacteria. Intercellular peptide signaling regulates a variety of processes in gram-positive bacteria (reviewed in 104, 146, 167). Intercellular signaling peptides share the common features that they are relatively small (5-38 amino acids), synthesized ribosomally, and processed from larger precursor peptides or proteins. Multiple mechanisms for production and response to intercellular signaling peptides have been characterized.

1. Production of intercellular signaling peptides. After translation, intercellular signaling peptides may be additionally modified through the addition of side groups, such as the addition of isoprenyl groups to the ComX peptide in *B. subtilis* (4) and thiolactone or lactone ring formation in the *Staphylococcus aureus* and *S. intermedius* autoinducing peptides (AIPs) (reviewed in 115). In both *B. subtilis* and *S. aureus*, production of the mature, modified peptides requires a protein that is encoded upstream of the precursor signaling peptide; it is thought that this protein functions in modification of the peptides (4, 8, 115). It is currently not clear how these peptides are exported. Other peptides, such as the competence stimulating peptide (CSP) of *S. pneumoniae*, are exported and processed by a dedicated transporter but do not undergo additional post-translational modifications (69, 78, 169).

Some lantibiotics, such as nisin, salivaricin A, subtilin, and cytolysin, act as intercellular signaling molecules (reviewed in 21, 85, 146). Lantibiotics are lanthionine-containing peptide antibiotics that are produced from precursor peptides and undergo extensive post-translational processing to produce the mature lantibiotic (reviewed in 21). Post-translational modification is mediated by the products of the lantibiotic biosynthesis clusters (21, 28, 83, 85). Precursor

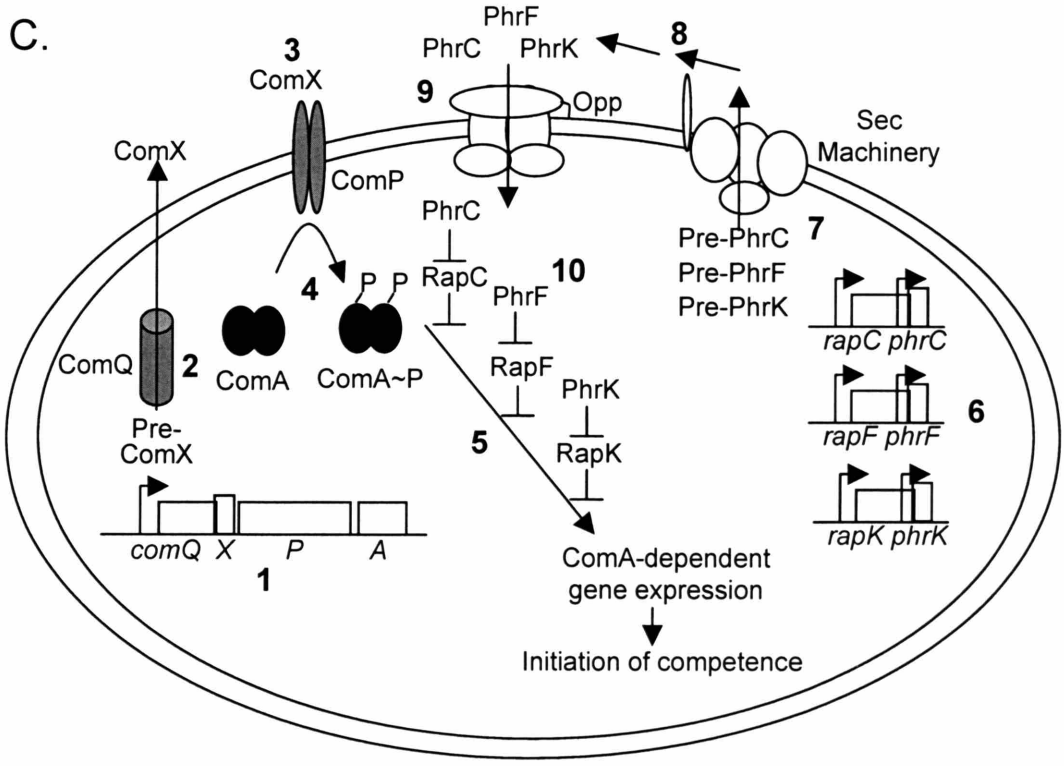
A. **ComX signaling peptides produced by *B. subtilis* strains**

Strain:	*B. subtilis* group[1]	Peptide[2]	Modification
168[3]	*B. subtilis* 168	ADPITRQWGD	farnesyl[4,5]
		DPITRQWGD	farnesyl[4,5]
RO-C-2	*B. mojavensis*	TREWDG	farnesyl[4]
RO-E-2	*B. subtilis* W23	GIFWEQ	geranyl[4]
RS-B-1	*B. subtilis* W23	MMDWHY	unknown[6]
		MDWHY	unknown[6]
		DWHY	unknown[6]
RO-H-1	*B. mojavensis*	MLDWKY	unknown[5,6]
		LDWKY	unknown[5,6]
		DWKY	unknown[5,6]
RO-B-2	*B. mojavensis*	YTNGNWVPS	geranyl[4,5]
		TNGNWVPS	geranyl[4,5]

B. **Phr signaling peptides that regulate the activity of ComA**

Phr	Peptide	Target(s)
PhrC (CSF)[1]	ERGMT[2]	RapC, RapB, unknown[3]
PhrF	QRGMI[4]	RapF[5]
PhrK	ERPVG[6]	RapK[7]

C.



D.

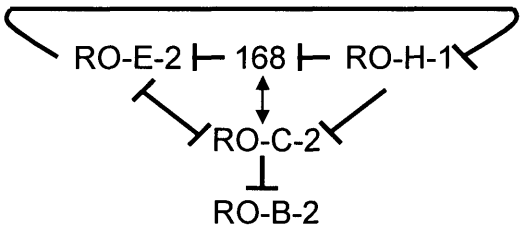


Figure 1: Regulation of competence development by intercellular signaling in *B. subtilis*.
A. ComX signaling peptides produced by *B. subtilis* strains. Several ComX pherotypes were described by Ansaldi *et al.* (4). This table is modified from (4).

1. Several *B. subtilis* groups were described by Roberts and Cohan (136).
2. Peptide sequence was determined through mass spectrometry of the purified or partially purified peptide(s). For some *B. subtilis* strains, multiple peptides that differ in length at the N-terminus elute with the active fraction of the peptide (4, 103).
3. JH642, the lab strain used in the experiments described in Chapter 2-4, is a derivative of 168.
4. Modification is predicted to be either a farnesyl or geranyl group based on its mass (~206 and ~136 Daltons respectively (4, 103)). The modification is thought to be an isoprenoid due to the presence of an isoprenyl binding domain in ComQ, the protein required for production of mature ComX (8).
5. Modified peptide was shown to be synthesized from isoprenylated precursor (4).
6. Mass of modification is not consistent with addition of a simple isoprenoid and therefore cannot be predicted (4).

B. Phr signaling peptides that regulate the activity of ComA. Previous research and the work presented in Chapter 2 show that these Phrs and their cognate Rap proteins regulate ComA-dependent gene expression.
   1. The mature PhrC peptide is also known as competence and sporulation factor (CSF) (142).
   2. Active form of the PhrC peptide purified from culture supernatant (142).
   3. PhrC affects the activity of three proteins. PhrC stimulates ComA activity by antagonizing RapC, stimulates sporulation by antagonizing RapB, and inhibits ComA activity by interacting with an unknown protein (92, 126, 142).
   4. Form of the PhrF peptide shown to be active *in vitro* (14).
   5. RapF was shown to be the target of PhrF in (14).
   6. Mature form of the PhrK peptide predicted based on its similarity to other Phrs (90).
   7. RapK is shown to be the target of PhrK in Chapter 2.

C. Intercellular peptide signaling mechanisms regulating competence development in *B. subtilis*.
   1. The ComX signaling peptide is encoded together with a protein required for its production, ComQ, and the two-component signal transduction system, ComP-ComA, that regulates competence development in response to peptide signaling.
   2. After transcription and translation, pre-ComX is exported and modified. This is dependent upon ComQ, which likely modifies pre-ComX.
   3. ComX interacts extracellularly with ComP and stimulates its kinase activity.
   4. ComP activates ComA through phosphorylation.
   5. ComA activates expression of several genes, including *comS*, which encodes a protein required to initiate competence development.
   6. PhrC, PhrF, and PhrK are encoded together with their cognate Rap proteins.
   7. The pre-Phr peptides are thought to be exported through the host secretion (Sec) machinery.
   8. Pre-Phrs are thought to be processed by signal peptidases associated with the Sec machinery and to undergo at least one more processing step that is mediated by an unknown protease or proteases.
   9. Mature Phr peptides are imported into the cell through the oligopeptide permease (Opp).
   10. PhrC, PhrF, and PhrK stimulate ComA-dependent gene expression by antagonizing the activities of their cognate Rap proteins. Regulation by these Raps and Phrs is described in Chapter 2.

D. Synergistic and antagonistic relationships observed between different ComX pheromone variants. Ansaldi *et al.* characterized the ability of ComX variants to inhibit and activate signaling through other ComP-ComA signaling pathways. This figure is modified from (4).

lantibiotics are exported through dedicated exporters that cleave their N-terminal leader sequences; these exporters are also encoded in the lantibiotic biosynthesis clusters (28, 83, 85). For the *E. faecalis* cytolysin, an additional cleavage step occurs through the action of an extracellular protease that is encoded in the cytolysin biosynthesis cluster (28).

Alternatively, some precursor peptides and proteins are secreted through the cellular secretion (Sec) machinery and undergo processing via signal peptidases (reviewed in 18, 90). These peptides then undergo additional processing to generate the active forms of the peptides (reviewed in 18, 90). This class of signaling peptides includes the Phr signaling peptides produced by *B. subtilis*; processes regulated by Phr peptide signaling in *B. subtilis* are the focus of the remaining chapters of this thesis.

2. Response to intercellular signaling peptides. Intercellular signaling peptides can be divided into two classes, those peptides that interact extracellularly with their receptor (Figs. 1 and 2) (reviewed in 28, 72, 84, 91, 115, 146)) and those peptides that are imported and act intracellularly with their receptors (Figs. 3 and 4) (reviewed in 18, 90).

a. Receptors that interact extracellularly with signaling peptides. Two types of receptors that interact extracellularly with signaling peptides have been identified: receptor histidine kinases, which serve as receptors for most of the extracellular signaling peptides, and the two-protein signal transduction system that mediates response to cytolysin signaling in *E. faecalis*.

i. Receptor histidine kinases. One of the major types of signal transduction systems found in bacterial cells is known as two-component signal transduction and is composed of a histidine kinase and its cognate response regulator protein (reviewed in 13, 145). Receptor histidine kinases autophosphorylate on a conserved histidine residue and donate phosphate to response regulator proteins, which are often transcription factors activated by phosphorylation (13, 145).

Histidine kinases can also function as phosphatases and signaling molecules can affect the kinase or phosphatase activity of the histidine kinase (13, 145). Response regulators are often encoded together in an operon with the histidine kinase that modifies their activity (13, 145).

Extracellular signaling peptides are typically thought to stimulate the kinase activity of their partner receptor histidine kinases, which leads to activation of downstream response regulators (Figs. 1 and 2) (72, 91, 115, 146). These response regulators have been shown to regulate transcription of genes involved in a variety of responses. In *S. aureus*, intercellular peptide signaling stimulates the production of virulence factors; intercellular peptide signaling is thought to be one of several signals that modulates the timing and levels of expression of genes encoding virulence factors to allow optimal functioning of *S. aureus* in a variety of niches within the host (115). In *B. subtilis* and *Streptococcus* species, extracellular signaling peptides stimulate production of proteins required for competence for DNA transformation. This regulation is discussed in later sections.

Several lantibiotics activate responses through two-component signal transduction pathways (83, 84, 161). In response to the presence of lantibiotics, histidine kinases stimulate the activity of their partner response regulator proteins, which results in increased transcription of the lantibiotic biosynthesis and immunity genes (83, 84, 161). This regulation increases production of the lantibiotic at high population density and also increases production of immunity proteins that protect the producing cells from the detrimental effects of high lantibiotic concentration. Thus far, lantibiotics have not been shown to regulate the expression of genes in addition to those involved in production and immunity to the lantibiotic, so it is not clear whether additional processes are regulated by lantibiotic signaling.

A. **Competence stimulating peptides (CSPs) produced by *S. pneumoniae***

comC allele	CSP sequence:
comC1	EMRLSKFFRDFILQRKK
comC2	EMRISRIILDFLFLRKK

B.

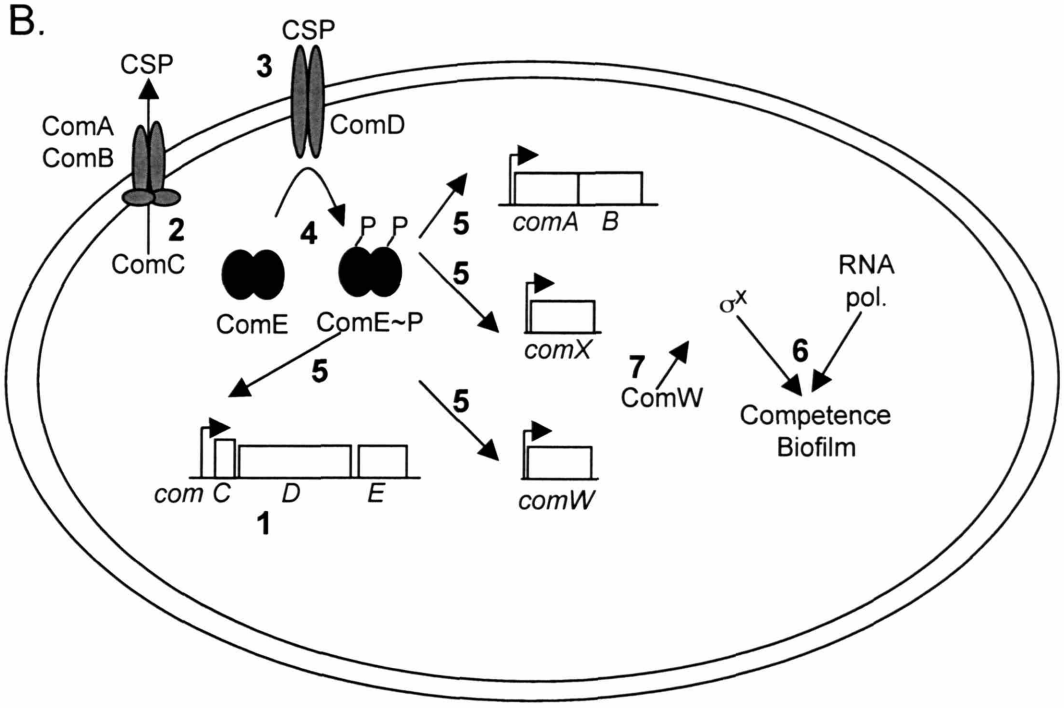


Figure 2. Regulation of competence development by intercellular peptide signaling in *S. pneumoniae*

A. Competence stimulating peptides produced by *S. pneumoniae*. Two pherotypes of CSP produced by several strains of *S. pneumoniae* were identified by Pozzi *et al.* (132).

B. Intercellular peptide signaling mechanism regulating competence development in *S. pneumoniae*.
   1. The CSP precursor (ComC) is encoded together with the two-component signal transduction system (ComD-ComE) that activates competence.
   2. The precursor ComC peptide is exported and processed by the ComA and ComB proteins.
   3. CSP interacts with ComD and stimulates its kinase activity.
   4. ComD activates ComE through phosphorylation.
   5. ComE activates the expression of *comAB, comX*, and *comW*.
   6. *comX* encodes the alternative sigma factor σ^X which binds to RNA polymerase and transcribes genes required for competence development.
   7. ComW promotes competence development by stabilizing and activating σ^X.

ii. *E. faecalis* cytolysin response. A second type of two-protein signaling system perceives the lantibiotic cytolysin in *E. faecalis*. Cytolysin is composed of a large and small subunit. The small subunit functions as a signaling molecule; the large subunit forms a complex with the small subunit that inactivates it. The small subunit of cytolysin is sensed by the CylR1 protein, which is thought to be an intermembrane protein that modulates the activity of a DNA binding protein, CylR2 (reviewed in 28). In the absence of the small subunit of cytolysin, CylR1/CylR2 acts as a transcriptional repressor of the cytolysin structural, biosynthetic, and immunity genes (28, 65). In the presence of the small subunit of cytolysin, the expression of these genes is de-repressed (28, 29, 65). It is currently not known how CylR1 perceives the cytolysin signal and modulates the activity of CylR2.

As with other lantibiotics, cytolysin signaling stimulates production of proteins involved in production and immunity to cytolysin (reviewed in 28). The large subunit of cytolysin is bound preferentially to host cell tissues, thereby liberating the small subunit to act as an intercellular signaling molecule when *E. faecalis* cells are associated with the host (29). This regulation functions to increase production of cytolysin, which is also a virulence factor capable of lysing host cells, when *E. faecalis* cells are present in host tissues (29).

b. Intracellular receptors. For peptides that act intracellularly, a relatively sequence independent peptide transporter known as the oligopeptide permease mediates uptake of imported intercellular signaling peptides (Figs. 1, 3 and 4) (18, 90). The oligopeptide permease is a member of the ATPase-Binding Cassette (ABC) transporter family of intermembrane transporters and is normally composed of five subunits; these include the peptide binding protein (OppA), a lipoprotein anchored to the extracellular face of the membrane, two transmembrane

domain proteins (OppB and OppC) which form the peptide transport channel, and two ATP binding proteins (OppD and OppF) that are associated with the cytoplasmic face of the membrane and hydrolyze ATP to drive transport (reviewed in 75, 152). Once inside the cell, these signaling peptides interact with at least two types of regulatory proteins. These regulatory proteins will be discussed in more detail in later sections. Peptides that act intracellularly have been shown to regulate mechanisms of horizontal gene transfer in *E. faecalis* (Fig. 3) and *B. subtilis* (Fig. 4). Regulation by intercellular peptide signaling in *E. faecalis* is discussed in a later section. Regulation by intercellular Phr peptide signaling in *B. subtilis* is discussed in a later section and is also the subject of Chapters 2-5.

B. *N*-acyl homoserine lactone (HSL) signaling in Gram-negative bacteria. Many species of gram-negative bacteria produce a variety of *N*-acyl-HSL molecules (also known as autoinducers (AIs)) (reviewed in 104, 167). These molecules vary based on the length, substitution, and saturation of their acyl side chains (reviewed in 54, 167). These differences in structure impart specificity to the signaling molecules, although some species of bacteria do utilize the same *N*-acyl-HSL (56).

1. Production of *N*-acyl HSLs. *N*-acyl-HSLs are primarily synthesized by members of the LuxI family of synthetases, which catalyze the acylation of *S*-adenosylmethionine (SAM) by an acyl-acyl carrier protein (Fig. 5) (56, 168). LuxI is the name of the first characterized *N*-acyl HSL synthetase from *V. fischeri*. However, two additional types of *N*-acyl HSL synthetases have been identified: the LuxM/AinS family of proteins from *V. harveyi* and *V. fischeri* (Fig. 6) (60, 68, and references therein), and the HdtS protein from *Pseudomonas fluorescens* (89). AinS (and presumably LuxM) uses a similar mechanism for AI synthesis as LuxI, although acyl-acyl CoA can also serve as an acyl chain donor (68). The mechanism of *N*-acyl HSL synthesis by HdtS

A. **Intercellular signaling peptides that regulate pCF10 transfer**

Peptide	Gene	Sequence	Function
cCF10	*ccfA*	LVTLVFV	Promotes pCF10 transfer
iCF10	*prgA*	AITLIFI	Antagonizes cCF10

B.

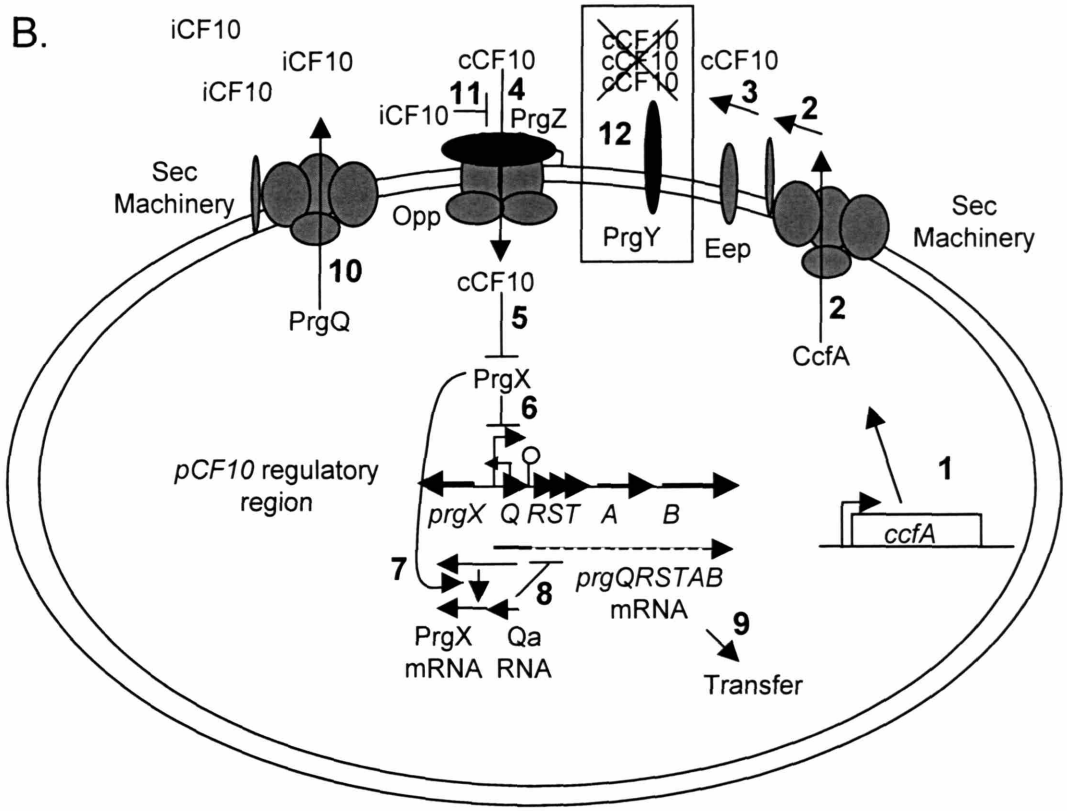


Figure 3. Regulation of pCF10 transfer by intercellular peptide signaling in *E. faecalis*.

A. Intercellular signaling peptides that regulate pCF10 transfer. The genes that encode the peptides that promote and inhibit pCF10 transfer, as well as the amino acid sequences of the mature forms of these peptides are identified.

B. Intercellular peptide signaling mechanisms regulating pCF10 transfer in *E. faecalis*.
   1. The chromosomally-encoded CcfA protein contains the cCF10 sequence in the leader peptide of this lipoprotein.
   2. CcfA is secreted by the host Sec machinery and processed by the associated Type II signal peptidase.
   3. The leader peptide containing cCF10 undergoes at least one additional processing step, likely mediated by the Eep protease.
   4. cCF10 is imported into the cell through the oligopeptide permease containing the plasmid-encoded peptide binding protein PrgZ.
   5. cCF10 interferes with dimerization of PrgX, thereby inhibiting its ability to bind DNA.
   6. In the absence of peptide, PrgX represses transcription from the *prgQ* promoter.

7. PrgX also promotes its own expression in the absence of peptide, likely by stimulating processing of the precursor RNA containing the *prgX* mRNA and the regulatory Qa RNA.
8. Qa RNA inhibits synthesis of full length *prgQRSTAB* transcript from the *prgQ* promoter.
9. Proteins required for conjugal transfer are encoded on the full length *prgQRSTAB* transcript. When cCF10 is present, these proteins are produced and transfer can occur.
10. The inhibitory peptide precursor (PrgQ) is exported through the Sec machinery and likely processed by the signal peptidase.
11. iCF10 inhibits cCF10 signaling, likely by competing for binding to PrgZ.
12. A second plasmid-encoded protein, PrgQ, limits extracellular accumulation of cCF10 through an unknown mechanism.

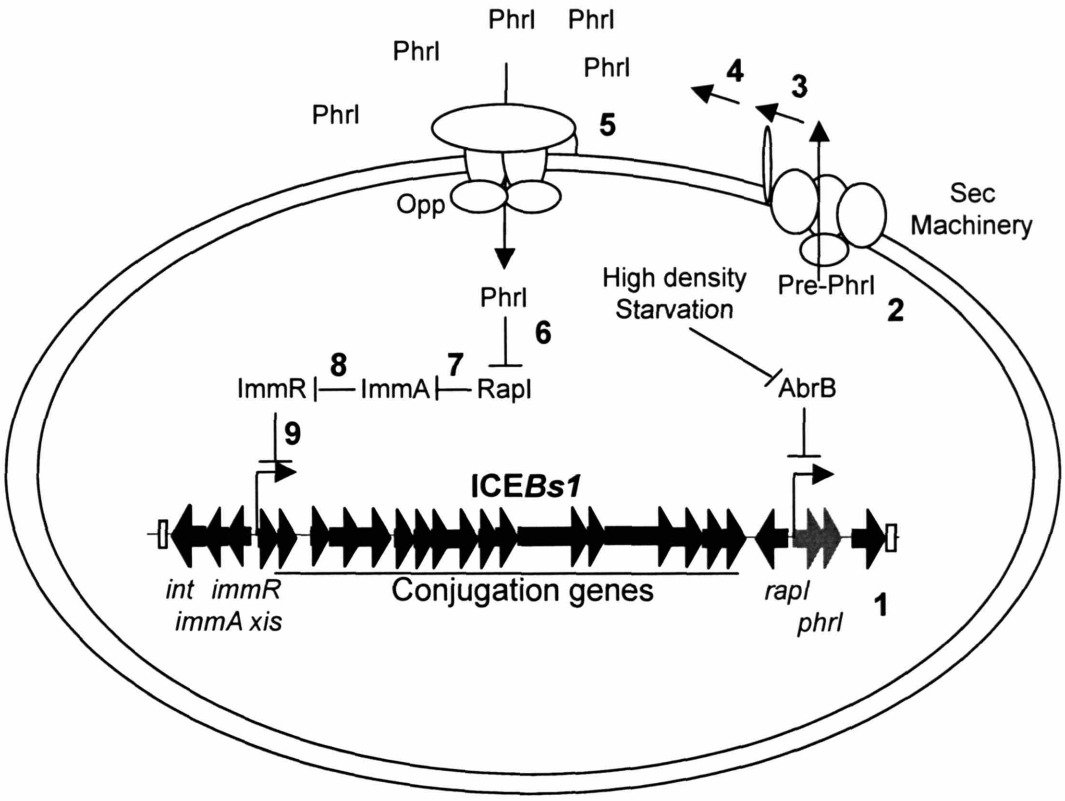


Figure 4. Regulation of ICE*Bs1* transfer by intercellular peptide signaling in *B. subtilis*. The intercellular peptide signaling mechanisms regulating transfer of ICE*Bs1* are described in Chapters 3 and 4.

1. RapI and PhrI are encoded together in the ICE*Bs1* element.
2. AbrB represses transcription of *rapI*. Transcription of AbrB is repressed under conditions of high cell density and starvation.
3. Pre-PhrI is secreted, likely by the host Sec machinery.
4. PhrI is processed to its mature form, likely by a signal peptidase and another unknown protease.
5. The PhrI peptide is imported through Opp.
6. The PhrI peptide antagonizes the activity of RapI.
7. RapI stimulates the activity of ImmA, the immunity repressor antagonist, through an unknown mechanism.
8. ImmA antagonizes the activity of the immunity repressor (ImmR).
9. ImmR binds to the *xis* promoter region and represses transcription of genes required for excision and transfer.

is unknown; subsequent work has also cast doubt on whether HdtS is an *N*-acyl HSL synthetase (34). Short chain *N*-acyl-HSLs are thought to diffuse freely through the membrane (54), but export of some longer chain *N*-acyl-HSLs is dependent on active transport (123).

2. Response to *N*-acyl HSLs. The majority of characterized *N*-acyl HSLs are sensed intracellularly by members of the LuxR family of proteins (Fig. 5) (reviewed in 54, 120, 167). *N*-acyl HSLs diffuse into the cell and interact with their cognate LuxR proteins (54, 120, 167). Several LuxR family members are activators of transcription that stimulate expression of genes in response to binding of their cognate signals (54, 120, 167). In *V. fischeri*, LuxR bound to its signaling molecule, *N*-3-oxo-hexanoyl-*L*-HSL, activates transcription of genes required for bioluminescence; as described above, this limits the energetically costly process of bioluminescence in free-living cells and promotes maintenance of cells in the symbiotic host (164, and references therein). In *Agrobacterium tumefaciens*, TraR, a LuxR homolog, activates expression of genes involved in transfer of a conjugative plasmid when bound to *N*-3-oxo-octanoyl-*L*-HSL. This regulation is described in more detail in a later section. *luxI* and *luxR* homologs are often encoded together in a single operon that is positively auto-regulated by the LuxR homolog (54).

Other LuxR family members, such as EsaR from *Pantoea stewartii* and SmaR from *Serratia* sp ATCC39006, are transcriptional repressors that bind to DNA in the absence of their cognate autoinducer and dissociate from DNA when the autoinducer molecule is bound (50, 107). In *Serratia* sp ATCC39006, SmaR represses expression of genes involved in the production of antibiotics and degradative enzymes (50). In *P. stewartii*, EsaR represses transcription of genes involved in capsular polysaccharide synthesis (107). Both the degradative enzymes produced by

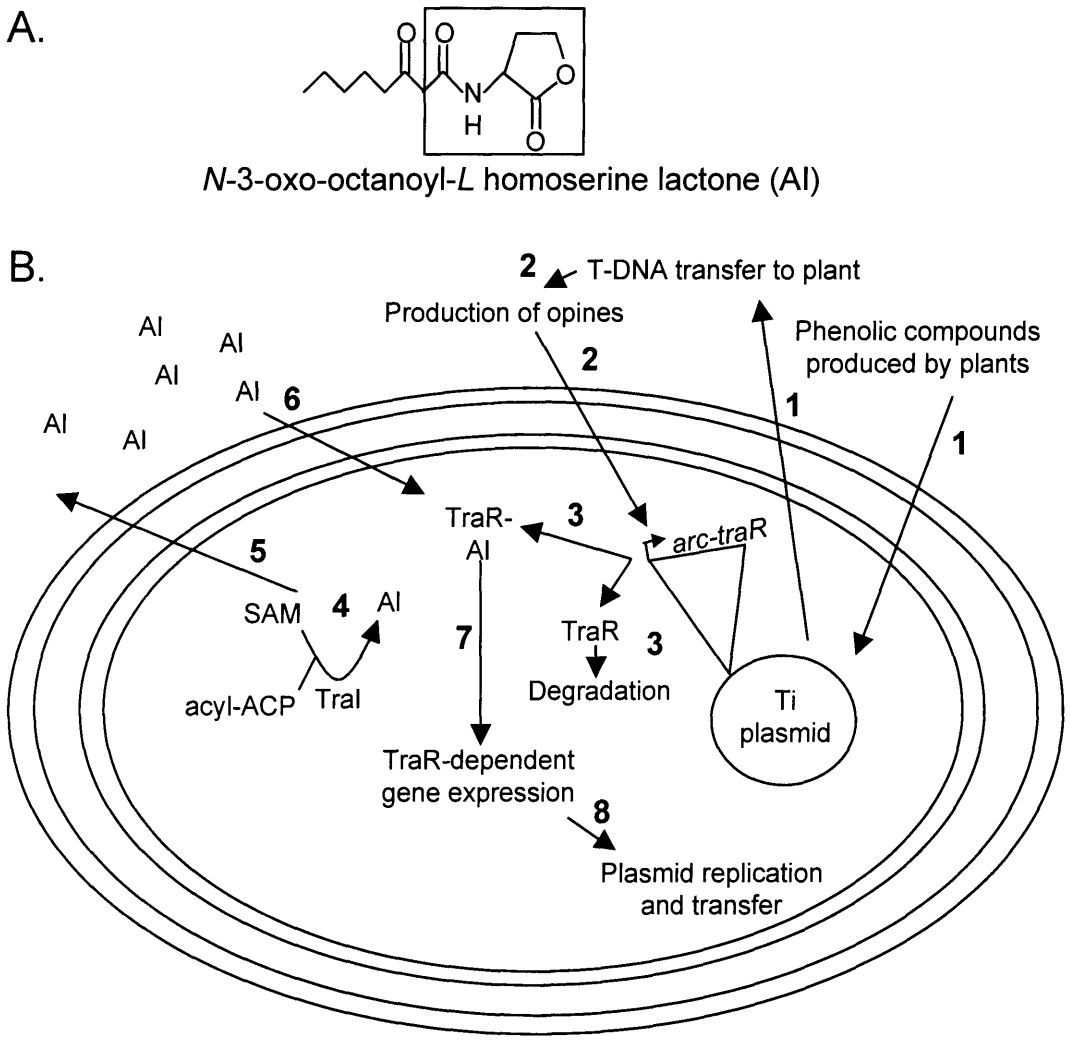


Figure 5. Regulation of Ti plasmid transfer in *A. tumefaciens*.

A. Structure of the *N*-acyl HSL signaling molecule (*N*-3-oxo-octanoyl-*L*-HSL) produced by the plasmid-encoded TraI protein. The homoserine lactone backbone is indicated by a box.

B. Intercellular signaling mechanism regulating Ti plasmid transfer.
   1. In response to phenolic compounds produced by plant cells, transfer of T-DNA to plant cells is stimulated.
   2. T-DNA incorporates in the chromosome of the plant cell and causes it to produce specific opines. Plant-produced opines stimulate expression of *traR* and genes required for opine catabolism.
   3. TraR either binds to its cognate *N*-acyl HSL (AI) or is degraded.
   4. TraI synthesizes AI from SAM and an acyl chain donated by acyl-acyl carrier protein (acyl-ACP).
   5. AI diffuses out of the cell.
   6. Once a threshold concentration is reached, AI diffuses into the cell and binds to TraR.
   7. TraR bound to AI (TraR-AI) activates gene expression.
   8. TraR-dependent gene expression leads to plasmid replication and conjugal transfer.

Serratia sp ATCC39006 and the capsular polysaccharides produced by *P. stewartii* act as virulence factors for these phytopathogenic species of bacteria (50, 107). It is thought that quorum sensing-dependent regulation of virulence factor production allows the bacteria to escape detection by host defense mechanisms until they have reached a sufficient concentration to overwhelm host defenses (165).

A second type of *N*-acyl HSL receptor is present in *V. harveyi* and *V. cholerae*. This receptor is a membrane-bound histidine kinase that acts as a phosphatase in response to a threshold extracellular concentration of *N*-acyl HSL (Fig. 6) (105, and references therein). In the absence of signal, the histidine kinase stimulates phosphorylation of a downstream response regulator (105, and references therein). This response regulator activates transcription of several small regulatory RNAs, which inhibit translation of a second transcriptional regulator (95). This second transcriptional regulator activates the expression of genes involved in bioluminescence and inhibits the expression of genes involved in virulence (Type III secretion) in *V. harveyi*, and inhibits the expression of genes involved virulence and biofilm formation in *V. cholerae* (74, 105, 108, 184). Potential benefits of these regulatory mechanisms were described in earlier sections.

C. Autoinducer-2 (AI-2) signaling in bacteria. AI-2 was initially identified as a signaling molecule in the gram-negative bacterium *V. harveyi*, but subsequent work has shown that many species of bacteria, both gram-positive and gram-negative, produce this signal (reviewed in 149, 167, 176).

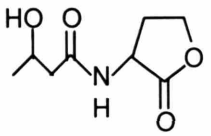
1. Production of AI-2. AI-2 is formed from derivatives of the molecule 4,5-dihydroxy-2,3-pentanedione (DPD), which spontaneously cyclizes to the known active forms of AI-2: (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate, which binds to the *V. harveyi* AI-2

receptor and stimulates a response (24, 106), and (2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran which binds to the *Salmonella typhimurium* AI-2 binding protein and is imported into the cell (106). Interestingly, bacteria that sense particular AI-2s can detect these molecules in the mixture of epimeric forms derived from DPD produced by other bacteria (106, 175, 176). This has led to the idea that AI-2 acts as an interspecies chemical signal (reviewed in 167, 176).

However, AI-2 may not act as intercellular signaling molecules in all bacteria that produce this collection of molecules. AI-2 production requires the activity of the LuxS protein, an *S*-ribosylhomocysteine cleavage enzyme that is involved in the conversion of the toxic SAM byproduct, *S*-adenosylhomocysteine, to homocysteine (reviewed in 174). This pathway appears to be the only pathway for detoxification of *S*-adenosylhomocysteine present in many species of bacteria (167, 174). Therefore, production of AI-2 molecules by some bacterial species may result solely as a byproduct of metabolism and may not play a role in intercellular signaling.

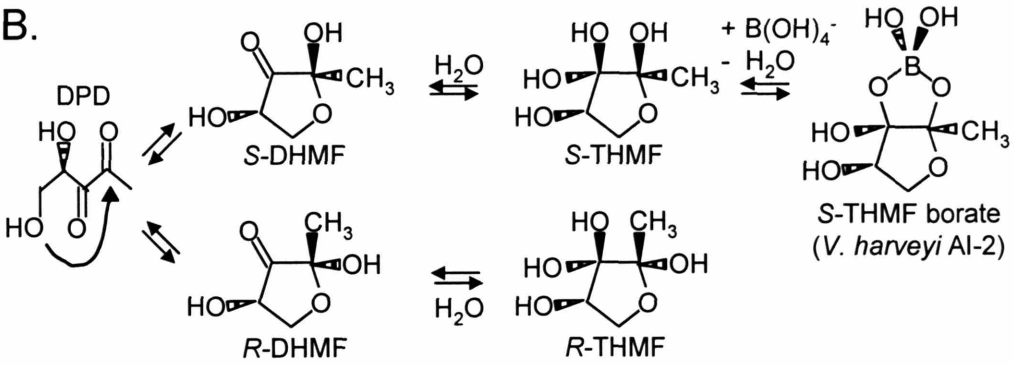
2. Response to AI-2. Two response pathways for AI-2 have been characterized in detail. One type of pathway, which functions in *V. harveyi* and *V. cholerae*, utilizes a periplasmic binding protein that binds to AI-2 and a receptor histidine kinase that acts as a phosphatase in response to AI-2 binding (Fig. 6) (24, 95, 105, and references therein). This pathway acts in parallel to the *N*-acyl HSL signaling pathway in both *V. harveyi* and *V. cholerae* and causes dephosphorylation of the same downstream response regulator. In both organisms, the activity of both pathways is needed for full activation of the quorum response. It is thought that *N*-acyl HSL signaling provides information about the concentration of cells of the same species while AI-2 signaling provides information about the overall concentration of bacterial cells in the population (105, 108).

A.



N-3-hydroxy-butanoyl-*L* homoserine lactone (HAI-1)

B.



C.

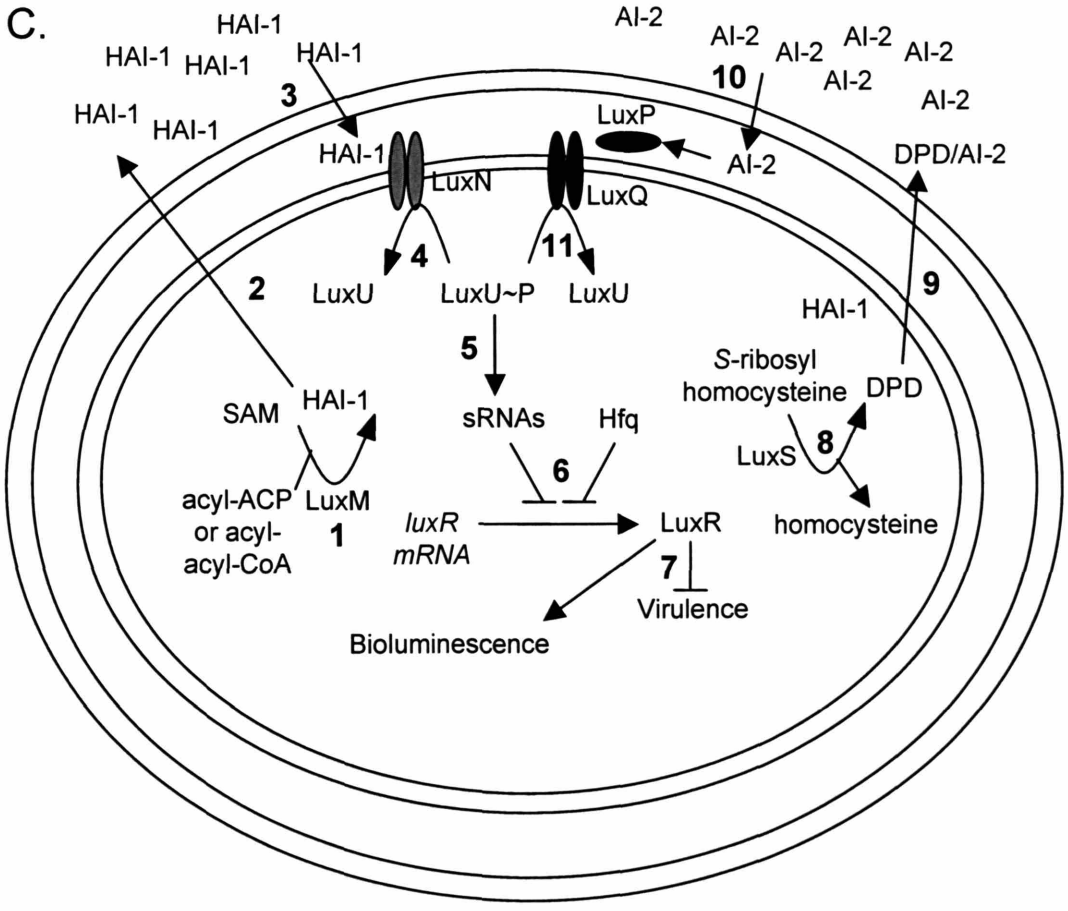


Figure 6. Regulation of bioluminescence and virulence by *N*-acyl HSL and AI-2 signaling in *V. harveyi.*

A. Structure of the *N*-acyl HSL signaling molecule (*N*-3-hydroxy-butanoyl-*L*-HSL) produced by the LuxM protein.

B. Production of AI-2 signals from 4,5-dihydroxy-2,3-pentanedione (DPD). The breakdown of *S*-ribosylhomocysteine by the LuxS protein produces homocysteine and DPD. This figure was modified from (106).
   1. DPD undergoes spontaneous cyclization to generate two epimeric forms: (2*S*, 4*S*)-2,4-dihydroxy-2-methyldihydrofuran-3-one (*S*-DHMF) and (2*R*, 4*R*)-2,4-dihydroxy-2-methyldihydrofuran-3-one (*R*-DHMF).
   2. These epimeric forms are hydrated to form (2*S*, 4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*S*-TMHF) and (2*R*, 4*R*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-TMHF). *R*-TMHF is the *S. typhimurium* AI-2 signal.
   3. *S*-TMHF borate ((2*S*, 4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran borate), the *V.* harveyi AI-2 signal, is formed by dehydration and addition of borate to *S*-TMHF.

C. Intercellular signaling mechanisms regulating bioluminescence and virulence in *V. harveyi*. This figure was modified from (167).
   1. LuxM synthesizes the *V. harveyi* autoinducer-1 (HAI-1, *N*-3-hydroxy-butanoyl-*L*-HSL) from SAM and an acyl chain donated by acyl-ACP or acyl-acyl-CoA.
   2. HAI-1 diffuses out of the cell.
   3. Once HAI-1 has accumulated to a threshold concentration, it diffuses into the periplasmic space and interacts with its receptor the histidine kinase LuxN.
   4. HAI-1 signaling stimulates the phosphatase activity of LuxN and results in dephosphorylation of the response regulator LuxU.
   5. Phosphorylated LuxU activates expression of several small regulatory RNAs (sRNAs).
   6. The sRNAs bind to the Hfq protein and destabilize the *luxR* mRNA, thereby inhibiting LuxR protein synthesis.
   7. LuxR activates expression of genes required for bioluminescence and inhibits expression of virulence genes.
   8. The LuxS protein breaks down *S*-ribosylhomocysteine into homocysteine and DPD.
   9. DPD diffuses through the cell membrane and undergoes the spontaneous rearrangements described in part B to form AI-2.
   10. Once a threshold concentration of AI-2 accumulates, it diffuses into the periplasmic space and interacts with the AI-2 binding protein LuxP.
   11. The LuxP-AI-2 complex stimulates the phosphatase activity of the histidine kinase LuxQ.
   12. LuxQ also dephosphorylates the LuxU response regulator.

The second type of AI-2 response pathway is encoded by *S. typhimurium* and *Escherichia coli* and utilizes a periplasmic AI-2 binding protein and an ABC transporter for uptake of AI-2 (150, 151, 177). Once AI-2 has been imported, it is phosphorylated by a dedicated kinase and likely metabolized (150, 177). It is thought that the phosphorylated AI-2 interacts with the transcriptional repressor of the AI-2 uptake and metabolism machinery to inhibit its DNA binding activity (150, 177). Thus far, the only genes known to be regulated by this system are responsible for the uptake and metabolism of AI-2 (150, 151, 177). It is possible that this system has evolved to utilize AI-2 as a nutrient source (174) or alternatively as a means to interfere with AI-2 signaling in other organisms (175).

III. Regulation of horizontal gene transfer by intercellular signaling molecules

Intercellular signaling is known to regulate many different processes (reviewed in 54, 104, 167). Several of these processes, such as production of antibiotics, virulence factors, and bioluminescence, have been discussed in previous sections. The following sections will focus on intercellular signaling mechanisms that regulate horizontal gene transfer and will highlight the similarities and differences that exist among these regulatory pathways.

A. Transfer of *A. tumefaciens* conjugal plasmids. In the gram-negative α-proteobacterium *A. tumefaciens*, intercellular signaling through acyl-HSL quorum sensing molecules regulates transfer of the Ti conjugal plasmid (reviewed in 15, 114, 165, 173). Although Ti plasmid transfer is the prototypical model for regulation of conjugal plasmid transfer by quorum sensing in gram-negative bacteria, recent work has shown that quorum sensing also regulates conjugal plasmid transfer in other α-proteobacteria (37, 73, 158). However, I will focus on the regulation of Ti

plasmid transfer, as the molecular details of this system have been characterized most extensively.

A. tumefaciens is a soil microbe that is capable of forming crown gall tumors in plants; this is dependent upon the presence of the conjugal Ti plasmid (15, 114, 165, 186). The Ti plasmid encodes two sets of DNA transfer systems, one that is required for intercellular transfer of the entire plasmid from one bacterial cell to another (reviewed in 15, 186), and a second conjugation-like Type IV secretion system that transfers a segment of oncogenic DNA (T-DNA) from the plasmid into plant cells (reviewed in 15, 25). Although quorum sensing is not directly involved in regulating T-DNA transfer, quorum sensing does promote increased Ti plasmid copy number (97, 121) and transfer of Ti plasmids to other bacteria present in the tumor (15, 186). These two mechanisms are thought to increase virulence of *A. tumefaciens* by increasing the amount of T-DNA present in the tumor (121).

Virulence gene expression is stimulated and T-DNA is transferred to plant cells in response to phenolic compounds released by plant cells; this leads to formation of a crown gall tumor (reviewed in 15, 186). In addition to genes that promote plant cell proliferation, the T-DNA also encodes genes that are involved in the production of opines (reviewed in 15, 186). In response to plant cell-produced opines, expression of Ti plasmid-encoded genes involved in opine catabolism is induced (Fig. 5) (186). Expression of *traR*, which is a member of the LuxR-type family of quorum sensing regulators, is also activated in response to opines (57, 130). TraR responds to the *N*-acyl-HSL (*N*-3-oxo-octanoyl-*L*-HSL) produced by the TraI protein (79). When bound to its cognate acyl-HSL, TraR activates expression of genes involved in the conjugal transfer of the Ti plasmid (55, 101). Interestingly, the presence of its cognate acyl-HSL

is required for the proper folding of TraR, which is rapidly degraded in the absence of this signal (183, 187).

As *A. tumefaciens* cells enter stationary phase, AttM, a chromosomally-encoded protein, is produced. AttM is an acyl-homoserine lactonase and its activity results in the inhibition of Ti plasmid transfer and acyl-HSL signaling that occurs in *A. tumefaciens* cells in stationary phase (180, 181, and references therein). It is thought that AttM may be needed to promote signal turnover and reset the quorum sensing system (181).

B. Transfer of *E. faecalis* conjugal plasmids. The gram-positive bacterium *E. faecalis* is an important reservoir of mobile genetic elements. Although often a commensal member of the human gastrointestinal tract, *E. faecalis* is also an opportunistic pathogen that is responsible for a large fraction of hospital-acquired infections (reviewed in 59). Mobile genetic elements have likely played a role in the evolution of pathogenic *E. faecalis* as several proteins involved in pathogenicity are encoded on mobile genetic elements (113, 122, 139). In addition to proteins involved in pathogenicity, many *E. faecalis* mobile elements encode resistance to antibiotics. Although the presence of virulence factors and antibiotic resistance genes is not unique to *E. faecalis*, mobile genetic elements do appear to be overrepresented in *E. faecalis*. Recent sequencing of the *E. faecalis* pathogenic strain V583 revealed that approximately 25% of the genome is composed of known and putative mobile genetic elements, many of which encode potential virulence factors and antibiotic resistance proteins (122). In contrast, mobile genetic elements compose less than 5% of the genome in both *E. coli* strain K12 and *B. subtilis* 168 (116).

Intercellular peptide signaling regulates transfer of several *E. faecalis* conjugative plasmids (reviewed in 18, 27). Transfer of these plasmids is stimulated by a peptide, referred to as a

pheromone, produced by a potential recipient cell that lacks that plasmid (18). Several pheromone-responsive plasmids have been identified (44-46). Regulation of the transfer of these plasmids has been characterized in various levels of molecular detail, and many aspects of the regulation are similar among the different plasmids (reviewed in 18, 27). I will focus on the regulation of the pCF10 plasmid, as it is one of the most extensively characterized (Fig. 3).

Transfer of pCF10 is stimulated by the pheromone cCF10, a seven amino acid peptide that is derived from the signal sequence of a chromosomally-encoded lipoprotein, CcfA (5, 109). CcfA is thought to be secreted through the cellular secretion (Sec) machinery and cleaved by a Type II signal protease, thereby liberating the 22 amino acid signal sequence which contains cCF10 (5). cCF10 is then thought to undergo additional processing steps, including a step that involves Eep, a predicted integral membrane zinc metalloprotease that is required for production of active cCF10 (1, 2, 5). After processing, cCF10 accumulates extracellularly. A fraction of active cCF10, as well as a significant fraction of immature cCF10, remains associated with the cell wall; additional cCF10 accumulates in the extracellular milieu (17). Although it is known that extracellular cCF10 can induce expression of transfer functions in broth culture (44), it is thought that wall associated cCF10 may be important for contact-dependent activation of conjugation, which might occur in surface-associated communities (17, 18). Although this hypothesis has not been tested, *E. faecalis* is known to form biofilms (67, 153, and references therein).

cCF10 acts intracellularly to regulate expression of conjugation functions following import into the cell through the oligopeptide permease (Fig. 3) (96). Although the chromosomally-encoded oligopeptide permease is able to transport cCF10 into the cell, transport is ~10 times more efficient when the chromosomally encoded peptide-binding protein, OppA, is replaced by the plasmid-encoded peptide binding protein, PrgZ (96).

Once inside the cell, cCF10 interacts with the regulatory protein PrgX to promote induction of transfer functions (86). In the absence of cCF10, PrgX represses transcription of the *prgQ* promoter and promotes its own expression (9, 86, and references therein). The *prgQ* promoter directs transcription of *prgQ*, which encodes a peptide (iCF10) that interferes with cCF10 signaling, and several genes encoding proteins involved in transfer (11). The *prgX* promoter directs transcription of *prgX* and of a regulatory RNA, Qa (9). PrgX activity appears to be important for processing of this precursor RNA into the mRNA containing *prgX* and the Qa regulatory RNA (86). In the absence of pheromone, *prgQ* is transcribed at low levels due to the repression of PrgX. However, the transcript that is produced terminates after *prgQ* due to the action of the Qa regulatory RNA (9, and references therein). This keeps transfer functions from being expressed in the absence of pheromone. Once cCF10 enters the cell, it binds to PrgX and disrupts its dimerization, which causes it to dissociate from DNA (86). The levels of *prgQ* transcripts then rise above the levels of *prgX* transcripts, effectively titrating out regulatory Qa RNA and allowing for production of full length transcripts (9, 86).

Two mechanisms limit self-induction of plasmid-containing strains. One involves the plasmid encoded inhibitory peptide iCF10 (encoded by *prgQ*), a seven amino acid peptide that is processed as part of the signal sequence of the larger PrgQ pro-peptide and is thought to inhibit induction through direct competition with cCF10, possibly at the level of binding for PrgZ (18, 112). The second mechanism utilizes the intermembrane protein PrgY, which limits the release of mature cCF10 into the extracellular milieu as well as decreasing the amount of cCF10 associated with the cell wall through an unknown mechanism (17, 19). These two mechanisms are thought to maintain the levels of cCF10 and iCF10 at a ratio that prevents self-induction.

However, this balance is disrupted when the levels of cCF10 increase due to the presence of plasmid-less cells, and induction of plasmid transfer ensues (19, 20).

Although this regulation prevents self-induction of plasmid-containing cells in broth culture, recent work has indicated that some self-induction of plasmid-containing cells occurs in the presence of human plasma and that this is due to the titration of iCF10 away from the cells (20, 76). Self-induction in the presence of plasma provides a mechanism to coordinate the expression of virulence genes with presence in a human host, as the aggregation substance that is activated by cCF10 signaling and mediates cell-to-cell contact during conjugation is also a potent virulence factor (76).

C. Comparison of conjugal plasmid transfer regulation in *A. tumefaciens* and *E. faecalis*. Although there are some similarities between the regulatory strategies at work in *A. tumefaciens* and *E. faecalis* conjugal plasmid transfer, there are also several differences. Activation of both conjugation systems in host tissues is thought to increase virulence of the respective organisms. For *A. tumefaciens*, this is thought to be due to increased copies of T-DNA present in cells within the crown gall tumor. For *E. faecalis*, this is thought to be due to expression of the aggregation substance protein. Both signaling molecules are also involved in regulating replication of their respective plasmids: *A. tumefaciens* signaling increases plasmid copy number (97, 121) and the *E. faecalis* cCF10 pheromone is involved in plasmid maintenance through an uncharacterized mechanism (20). A primary difference between the activation of conjugal plasmid transfer in *A. tumefaciens* and *E. faecalis* is the source of the signaling molecule. In *A. tumefaciens*, a plasmid-encoded signal promotes transfer when the concentration of plasmid-containing cells is high. In *E. faecalis*, the stimulating signal encoded in the

chromosome is produced by plasmid-less cells to stimulate transfer from plasmid-containing cells. In addition, *E. faecalis* plasmids encode multiple regulators that prevent self-induction.

However, an α-proteobacterial conjugation system that is regulated more similarly to that of *E. faecalis* was recently identified in a *Rhizobium leguminosarum* bv. *viciae* strain carrying the pRL1J1 plasmid (37). In this plasmid system, as in *A. tumefaciens*, plasmid transfer is stimulated by the plasmid-encoded TraR bound to its cognate acyl-HSL, *N*-3-oxo-octanoyl-*L*-HSL, which is produced by the plasmid-encoded TraI signal synthetase. However, instead of responding to opine signals produced by plant cells, transcription of *traR* is positively regulated by a second plasmid-encoded TraR-like protein, BisR, that requires binding to *N*-(3-hydroxy-7-cis-tetradecenoyl)-1-HSL for activation. *N*-(3-hydroxy-7-cis-tetradecenoyl)-1-HSL is produced by a chromosomally-encoded signal synthetase, CinI. Since BisR also represses transcription of *cinI*, plasmid-containing cells do not express *cinI* nor produce *N*-(3-hydroxy-7-cis-tetradecenoyl)-l-HSL. Therefore, BisR is not able to activate transcription of *traR* in plasmid-containing cells. However, *R. leguminosarum* strains that lack pRL1J1 also lack BisR; these strains express *cinI* and produce *N*-(3-hydroxy-7-cis-tetradecenoyl)-l-HSL. When plasmid-containing cells are surrounded by plasmid-less *R. leguminosarum*, the *N*-(3-hydroxy-7-cis-tetradecenoyl)-l-HSL produced by plasmid-less cells stimulates BisR-dependent activation of *traR* transcription and subsequent transfer of the pRL1J1 plasmid from donor cells. Therefore, the regulatory strategies that govern conjugal plasmid transfer by *E. faecalis* and *R. leguminosarum* are similar, despite widely divergent molecular components. This convergent evolution indicates the relative importance of restricting the energy-intensive process of conjugation to times when it is most likely to result in productive dissemination of plasmids to plasmid-less cells.

D. Competence in *Streptococcus* species. In several species of *Streptococcus* the ability to take up exogenous DNA from the environment is activated by signaling through a quorum sensing peptide, known as competence stimulating peptide (CSP, 70-72, 98). Early work, which helped to substantiate the role of DNA as genetic material, also demonstrated the role that competence played in transfer of genes involved in extracellular polysaccharide capsule synthesis in *S. pneumoniae* (7). Further research has detected evidence of intra-specific transfer, likely due in part to competence, of genes encoding penicillin binding proteins and capsular polysaccharides (30), fluroquinolone resistance and the targets of fluoroquinolones (144, and references therein), proteins involved in virulence (42), and the gene encoding the competence stimulating peptide (CSP) and its receptor (71).

In addition to its role in regulating competence development, CSP-dependent quorum sensing also activates biofilm development (99, 128, 148) and the production of bacteriocins (64, 87, 162). Some of the bacteriocins produced by *Streptococci* in response to CSP lyse closely related *Streptococcus* species, and are thought to be important in liberating DNA to be acquired by competent cells (64, 87, 162). As many *Streptococcus* species grow together as mixed species biofilms in dental plaque, coordinate regulation of bacteriocin production, biofilm development, and competence by CSP is thought to provide the opportunity for genetic exchange to occur efficiently in the natural environment of *Streptococci* (87, 148).

The molecular details of the response to CSP have been elucidated (Fig. 2) (reviewed in 72, 148]. Although the following description provides an overview of the CSP response in *S. pneumoniae*, similar pathways function in other species that produce CSP (71, 98). CSP is an unmodified peptide of 17 amino acids that is encoded by the *comC* gene (69, 132). At least two different CSP molecules are produced by different *S. pneumoniae* strains; these peptides differ at

8 amino acids and are specific for variants of their receptor, the histidine kinase ComD (80). Interaction of CSP with ComD is thought to stimulate autophosphorylation of ComD, followed by transfer of phosphate to its cognate response regulator ComE, which activates ComE for DNA binding (70, 127). ComE activates transcription of its own locus (*comCDE*) (169), the *comAB* locus, encoding a transporter required for secretion and processing of CSP (78, 169), *comX*, encoding an alternative sigma factor (σ^X) that when bound to RNA polymerase transcribes several of the genes involved in competence (94, 100), and *comW*, encoding a protein required for stabilization and activation of σ^X (147).

E. Competence in *B. subtilis*. In the gram-positive soil bacterium, *B. subtilis*, a network of regulatory proteins and intercellular signaling peptides regulates genetic competence (reviewed in 63, 66). This regulatory network integrates multiple signals to determine whether conditions favor differentiation into competent cells or alternative forms of development, such as sporulation.

One signal that regulates competence development is population density (Fig. 1). Once *B. subtilis* cells reach a certain cell density, a transcriptional response that leads to genetic competence is initiated (103, and references therein). This response is modulated by at least four peptide signaling molecules (14, 103, 142). Three of these peptides are intercellular Phr signaling peptides that are imported into the cell through the oligopeptide permease to interact with their cognate receptor proteins, which are members of the Rap family of regulatory proteins (14, 92, 142). These peptides are discussed in detail in Chapter 2. The fourth peptide is the extracellular signaling peptide ComX.

B. subtilis encodes eleven Rap proteins (88), six of which have been characterized in molecular detail. Three Rap proteins bind to response regulator proteins and interfere with DNA

binding (14, 32, 118). The remaining three Rap proteins interact with a response regulator protein to stimulate auto-dephosphorylation (125, 160). Rap protein activity is thought to be mediated by tetratricopeptide repeat (TPR) domains, protein-protein interaction domains that compose approximately two-thirds of each Rap protein's sequence (32, 81, 124). Phr peptides are thought to competitively inhibit binding of Rap proteins to response regulators (32, 81).

The ComX signaling peptide also activates the initiation of competence development. The active form of ComX is an isoprenylated oligopeptide that can vary in size from 5-10 amino acids depending upon the strain of *B. subtilis* (3, 4, 103, 156, 157). ComX is produced as a precursor polypeptide that is thought to be modified by the ComQ protein; *comQ* is required for production of mature ComX (103), expression of *comX* and *comQ* is sufficient for *E. coli* to produce mature ComX pheromone (4, 156), and mutations in the isoprenoid binding domain of ComQ inhibit production of mature ComX pheromone (8). Although modifications of the mature ComX peptide vary among different strains of *B. subtilis* in the number of isoprenyl groups added (4), isoprenylation of ComX is required for functionality (4, 103). Isoprenylation is also important for the activity of the *Saccharomyces cerevisiae* **a**-factor mating peptide; isoprenylation is thought to promote membrane association (23, 179).

ComX interacts extracellularly with its receptor, the histidine kinase, ComP (4, 103, 129, 143, 157). This likely stimulates ComP autophosphorylation, followed by donation of phosphate to the response regulator protein, ComA, which results in its activation for DNA binding (129, 137, 170, 171). ComA activates the expression of several genes including the surfactin synthetase operon, *srfA*, which also encodes a small gene, *comS*, that is required to activate genetic competence (31, 35, 36, 110, 111, 137). ComS inhibits degradation of the major competence transcription factor, ComK (159), which activates transcription of operons encoding

DNA uptake and processing machinery, as well as several other genes (12, 119, and references therein).

comQ, *comX*, *comP*, and *comA* are encoded together in a single operon (103, 171). This arrangement is similar to the *comCDE* operon of *Streptococcus* species, as well as signaling cassettes in several other gram-positive bacteria (16, 85, 115, 146). The *comQXPA* locus is conserved in several *B. subtilis* strains and closely related *Bacillus* species, although an extensive amount of diversity exists in the sequence of *comQ*, *comX,* and the 5' end of the *comP* gene (signal reception domain) (3, 4, 156, 157). Diversity in *comQ* and *comX* results in production of different forms of the mature ComX peptide; variations in *comP* allows response to different ComX peptides (4, 156, 157).

The different forms of the mature ComX peptide have been classified into pherotypes based on the ability of these peptides to affect ComP-ComA signaling in other strains (Fig. 1D) (4). Some forms of the mature ComX peptide stimulate ComP-ComA signaling of strains that produce different peptides, whereas other forms of mature ComX peptides antagonize ComP-ComA signaling of non-cognate strains (4). Although interference has not been observed for *Streptococcus* mating pheromones, the AgrD pheromones of *Staphylococcus* species do exhibit cross-species inhibition and it is thought that this may serve to isolate populations (reviewed in 115). Similarly, it is thought that specificity in ComX-ComP-ComA signaling may improve fitness of these strains by providing a mechanism for sexual isolation (3).

F. Benefits of regulating competence development through intercellular signaling. Sexual isolation is thought to be one of the major reasons that competence development is regulated by quorum sensing mechanisms in both *B. subtilis* and *Streptococci* (reviewed in 141, 155). DNA uptake in other naturally competent bacteria, such as *Neisseria gonorrhoeae* and

Haemophilus influenzae, requires the presence of specific sequences in incoming DNA (38, 49). These uptake sequences are dispersed throughout the chromosomes of *N. gonorrhoeae* and *H. influenzae* and utilization of these sequences for DNA uptake likely limits acquisition to DNA of closely related species (141). In contrast, DNA uptake occurs non-specifically in *B. subtilis* and *Streptococci*, so additional mechanisms are needed to limit DNA acquisition to closely related species (141, 155). It is thought that acquisition of DNA from closely related species is more likely to reduce the possibility of gene disruption or production of toxic products encoded on foreign DNA (155). Alternatively, it is also thought that incoming DNA may be utilized as a nutrient source (51, 134). Therefore, density-dependent signaling may play a role in activating competence under conditions when cells are crowded and more likely to have limited nutrients available.

G. Transfer of a *B. subtilis* mobile genetic element. Transfer of the *B. subtilis* integrative and conjugative element ICE*Bs1* is regulated by intercellular Phr peptide signaling (Fig. 4). The initial characterization of this element and its regulation by intercellular peptide signaling is described in Chapter 3. Further insights into the molecular mechanisms that govern regulation of ICE*Bs1* are discussed in Chapter 4.

ICE*Bs1* is normally integrated in the *B. subtilis* chromosome. Under certain conditions, ICE*Bs1* can excise from the chromosome, transfer to recipient cells through conjugation, and integrate into the chromosome of the new host. Two mechanisms of peptide signaling control transfer of ICE*Bs1*. One mechanism limits expression of genes involved in excision and transfer of ICE*Bs1* to conditions when recipient cells are likely to be present. This mechanism acts through transcriptional regulation of RapI, a protein that stimulates expression of genes involved in excision and transfer of ICE*Bs1*. Transcription of *rapI* is repressed during exponential growth

by the repressor protein AbrB. When cells sense starvation and are at high cell density, transcription of AbrB is repressed. This results in increased expression of *rapI* under conditions of high cell density and starvation that likely correlate with the presence of recipient cells.

A second mechanism limits transfer of ICE*Bs1* in the presence of other cells that contain the element. This self-recognition is accomplished through PhrI peptide signaling. The ICE*Bs1*-encoded PhrI peptide antagonizes the activity of RapI, thereby inhibiting expression of ICE*Bs1* genes required for excision and transfer when the concentration of ICE*Bs1*-containing, PhrI-producing cells is high. Therefore, dual regulation of the transcription and activity of RapI limits ICE*Bs1* excision and transfer to conditions when it is most likely to disseminate to cells lacking the element.

These mechanisms of regulation likely provide ICE*Bs1* with several benefits. Constitutive expression of genes required for excision and transfer is detrimental to the element, as it results in increased excision of ICE*Bs1* and occasional loss of the element from the population of cells, and also makes the host cells sick (Appendix B and Chapter 4). Therefore, mechanisms that limit expression of genes required for excision and transfer increase the stable association of ICE*Bs1* with a healthy host cell. However, regulatory mechanisms that permit dissemination of ICE*Bs1* to cells lacking the element ensure that copies of ICE*Bs1* are found throughout the population and limit the chance that it will be lost due to inability of the host cell to transmit the element to its progeny cells through vertical gene transmission.

Limiting transfer of ICE*Bs1* into cells that already contain a copy of the element may provide the element with several benefits. Mechanisms that limit transfer into cells that already contain the element may help maintain the genetic stability of ICE*Bs1* by limiting the chance of acquisition of a deleterious mutation or deletion through inter-element recombination in cells

containing multiple copies of the element. Furthermore, limiting the number of copies of ICE*Bs1* in the cell may also limit the burden imposed on host cells due to replication of additional DNA; similar benefits have been proposed for other mobile genetic elements (163). Further insights will likely be obtained from more detailed analysis of those strains that contain multiple copies of ICE*Bs1*.

IV. Interference with intercellular signaling

As many cells in the environment do not exist as pure cultures (33), it is important to consider the roles that other cells play in regulating cellular responses controlled by intercellular signaling molecules. The presence of other cells and secreted products in the environment can directly influence responses regulated by intercellular signaling (reviewed in 40, 140). Enzymes that degrade *N*-acyl HSL's have been identified in gram-negative and gram-positive bacteria (39, 40, 93, 181), mammalian sera (26, 178), and diverse soil samples (166). Several of these enzymes have been shown to degrade *N*-acyl HSLs through hydrolysis of the lactone ring or acyl side chain (reviewed in 40). In addition, signals that mimic *N*-acyl HSLs and interfere with signaling have also been identified (140, 182). AI-2 signaling can also be disrupted by mimicry (140, 182), as well as by the AI-2 uptake machinery of *E. coli* and *S. typhimurium* (175). Molecular mimicry can also inhibit quorum sensing in *S. aureus* and *B. subtilis*. In these cases, peptides produced by different strains of *S. aureus* and *B. subtilis* can interfere with signaling (4, 115). Furthermore, extracellular proteases and the peptide uptake machinery of other cells could also interfere with peptide signaling.

Several roles for quorum sensing interference have been proposed. Signal turnover, which could allow cells to exit from quorum sensing and also serve to insulate spatially separated populations of cells, is one potential benefit proposed for cells that produce both *N*-acyl HSL and

HSL acylases (40, 166, 180, 181). Other cells may degrade intercellular signaling molecules for use in metabolism; peptides can be degraded as a source of amino acids and cells that metabolize *N*-acyl HSLs have been identified (93). Interfering with processes regulated by intercellular signaling in another bacterium, such as the production of degradative enzymes and antibiotics, likely provides a competitive advantage to microbes not reliant upon that type of intercellular signaling molecule in colonization of specific niches (40, 175). Since several species of bacteria use intercellular signaling molecules to regulate production of virulence factors, signal degradation by host cells can provide a defense mechanism against microbial virulence (26, 40, 178). Further work is needed to understand the roles that signal interference plays in modulating intercellular signaling in the environment.

V. Mechanisms of horizontal gene transfer

Three general mechanisms have been recognized for horizontal transfer of DNA: conjugation, transformation of DNA into competent cells, and transduction of DNA by phage (Fig. 7) (116, 133). In addition, other mobile genetic elements such as transposons and mobilizable plasmids can take advantage of these transfer mechanisms to mediate their own intercellular transfer. For example, the first evidence of transfer of vancomycin resistance from *E. faecalis* to *S. aureus in vivo* is thought to have been mediated by transfer of a conjugative plasmid carrying a transposon that encoded vancomycin resistance (52).

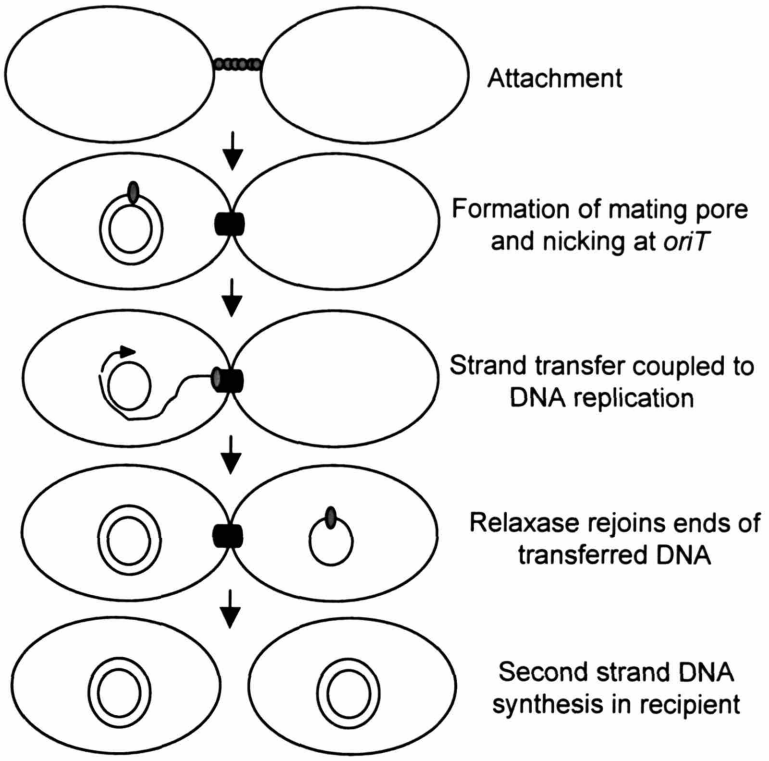
A. Acquisition of DNA through conjugation. Although both conjugative plasmids and integrative and conjugative elements have been characterized, the molecular mechanisms of DNA transfer have only been characterized in a few gram-negative conjugative plasmids (reviewed in 25, 62, 138). With gram-negative conjugative plasmids, donor cells attach to

recipient cell utilizing a sex pilus, which may retract to bring cells into contact (138). For some gram-positive plasmids, such as the pheromone responsive plasmids of *E. faecalis*, a surface protein present on donor cells, known as aggregation substance, mediates attachment (62). For other conjugative systems, it is not known what, if anything, specifically mediates attachment.

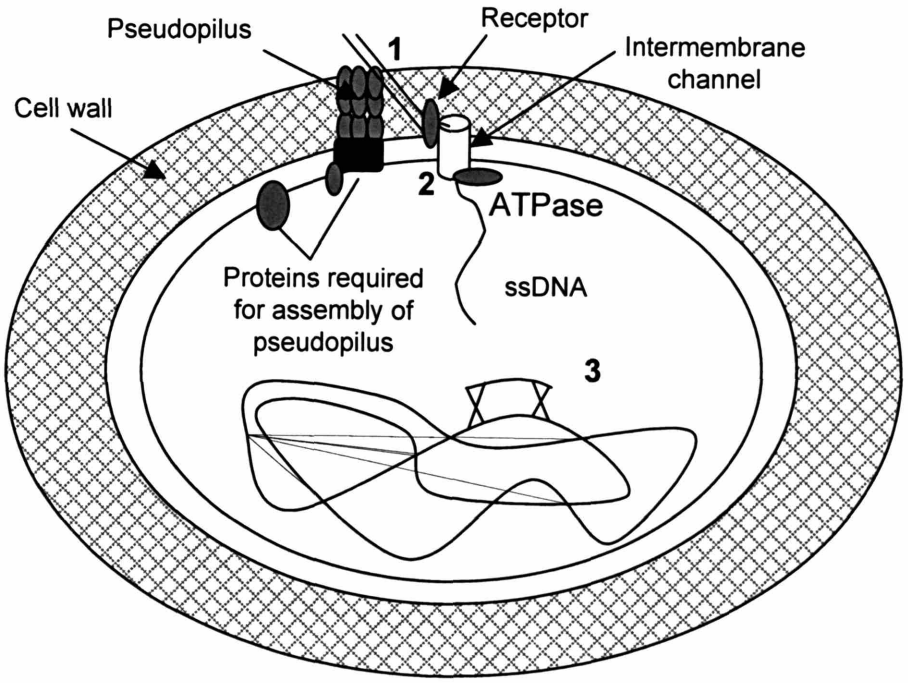
DNA is transferred from one cell to another through a mating pore composed of several protein subunits encoded by the conjugative plasmid (25, 62, 138). The protein subunits that form the mating pore of some gram-negative plasmids have been characterized and are members of the Type IV secretion apparatus family (25, 138). Several proteins contain ATPase domains and it is thought that ATP hydrolysis may be used to pump DNA into the intermembrane channel that spans the inner and outer membranes (138). The pore-forming proteins in gram-positive bacterial plasmids have been defined genetically, but there are currently few insights into the structure that is formed, as only three proteins required for DNA transfer are recognizable homologs of the gram-negative transfer machinery (62).

Prior to DNA transfer, the double stranded plasmid is nicked at the origin of transfer by the action of a relaxase protein; this has been demonstrated for plasmids from both gram-positive and gram-negative bacteria (62, 138). The relaxase is covalently attached to the 5' end of the DNA and is thought to interact with host-encoded proteins to unwind the DNA from its complimentary strand during a round of rolling circle-type replication (62, 138). In gram-negative bacteria, it has been shown that the single-stranded DNA/relaxase complex interacts with the mating pore and both the relaxase and single-stranded DNA are transferred to recipient cells (138). A similar mechanism likely functions in gram-positive conjugation systems. Once in the recipient cell, the two ends of the DNA are rejoined by the relaxase and second strand synthesis occurs (138).

A.


Attachment
Formation of mating pore and nicking at *oriT*
Strand transfer coupled to DNA replication
Relaxase rejoins ends of transferred DNA
Second strand DNA synthesis in recipient

B.


Pseudopilus
Receptor
1
Intermembrane channel
Cell wall
2
ATPase
Proteins required for assembly of pseudopilus
ssDNA
3

C.

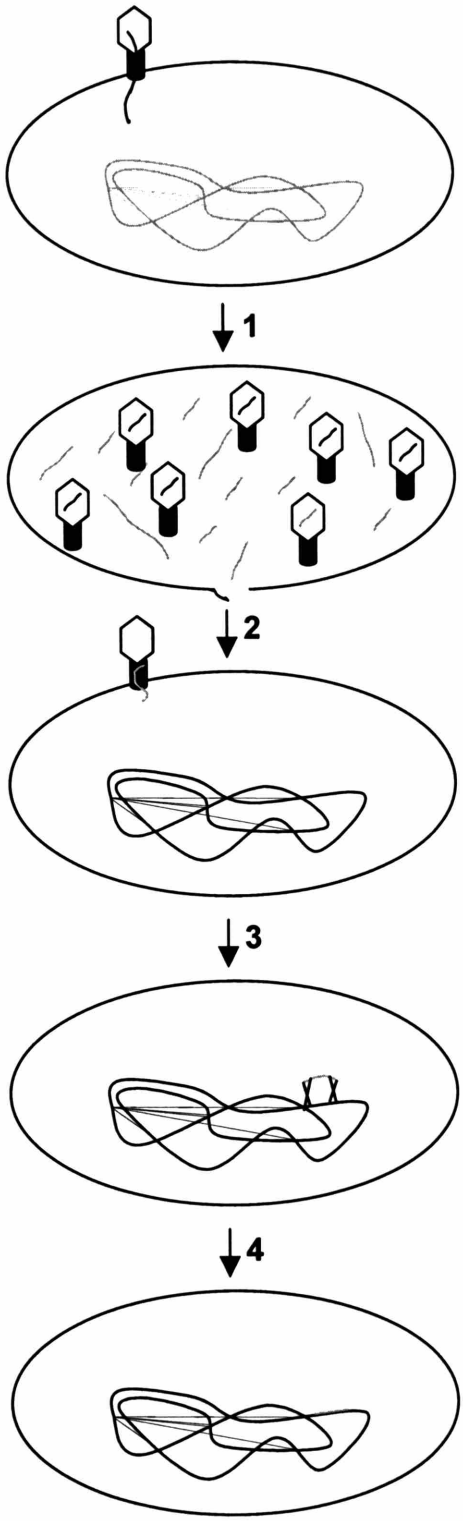


Figure 7. Mechanisms of horizontal gene transfer in bacteria.

A. Acquisition of DNA through conjugation. Steps of conjugal transfer are shown on the left. Additional details are described in the text.

B. Acquisition of DNA through natural transformation.
   1. Double-stranded DNA passes through the cell wall through its association with the pseudopilus and interacts with the DNA binding receptor.
   2. Single-stranded DNA passes through the intermembrane channel. Translocation is powered by the associated ATPase subunit.
   3. Once inside the cell, single-stranded DNA (ssDNA) can recombine into the chromosome.

C. Acquisition of DNA through generalized transduction.
   1. Phage DNA enters a host cell and undergoes a normal lytic cycle. However, upon packaging of DNA into phage heads, some phage heads accidentally package host cell DNA instead of phage DNA.
   2. Phage particles are released from the host cell. The phage particle containing DNA from the old host can initiate infection of a new cell.
   3. DNA is released into the new cell. However, as the DNA is not of phage origin, it does not promote lytic development.
   4. The DNA can be incorporated into the chromosome of the new cell through recombination.

Some conjugative elements that have integrated into the chromosome will also undergo nicking and transfer (77, and references therein). However if circularization of the element does not occur prior to DNA transfer, DNA transfer continues past the element and into the flanking chromosomal DNA sequence. If this DNA can be integrated into the chromosome of the recipient cell, it will be maintained.

B. Acquisition of DNA through natural transformation. The competence machinery of naturally competent gram-positive and gram-negative bacteria has been characterized. Many of the proteins involved in forming the DNA uptake machinery in these bacteria are homologous to each other and to components of the Type IV secretion machinery (reviewed in 6, 22, 43). In gram-positive bacteria, DNA uptake is initiated by binding of DNA to a membrane-bound receptor (reviewed in 22, 43). This requires the presence of a pilus-like structure, composed of several pseudopilin subunits, that transverses the cell wall (22). Pseudopilin assembly requires the action of several dedicated proteins (22). Double-stranded breaks are introduced into the DNA through the action of endonucleases (22, 43). DNA is transferred from the membrane-bound receptor to a specific intermembrane channel, which is coupled to an ATPase that drives transport across the membrane (22, 43). Only a single strand of DNA is transferred into the cell; the second strand is degraded (22, 43). Once inside the cell, the incoming DNA can recombine with homologous DNA (22, 43).

DNA taken up by gram-negative bacteria must also transverse the outer membrane. In *N. gonorrhoeae*, DNA binds to an outer-membrane associated protein that recognizes the signal sequence required for uptake and DNA is brought through the membrane through a structure formed of proteins known as secretins (6, 22). DNA passes through the periplasmic space either

in association with a pseudopilus (22) or pilus (6). DNA then interacts with the periplasmic DNA binding protein, a homolog of the gram-positive membrane-associated DNA binding protein (6, 22). The remaining steps in DNA import into the cytoplasm are thought to be similar to gram-positive bacteria, although a specific ATPase that drives transport into the cell has not been identified (6, 22).

C. Acquisition of DNA through transduction. Several bacteriophages have been shown to occasionally package chromosomal DNA into a phage particle instead of, or in addition to, phage DNA (reviewed in 53, 116, 133). These transducing phage are capable of mediating transfer of DNA from the host cell to a recipient cell. If the incoming DNA can be incorporated into the chromosome of the host, new genes may be added (53, 116, 133). In cases where host DNA has been packaged into the phage head in addition to phage DNA, integration of DNA into the chromosome of the new host cell can be mediated by phage-encoded proteins (116).

VI. Significance of horizontal gene transfer in bacterial evolution

Horizontal gene transfer is thought to have played an important role in bacterial evolution (41, 116, 117, 133). The large amount of DNA present in bacterial chromosomes that appears to have been acquired by horizontal transfer has even led some researchers to question the idea of evolution of distinct bacterial species through divergence and descent, the "tree of life" model of bacterial evolution, and to argue for a "web of life", reflecting evolution through transfer of DNA from one species to another (10, 41, 61). These hypotheses are based primarily on incongruences observed when different loci are used to construct phylogenetic relationships among organisms (61). Other researchers insist that although there is abundant evidence of horizontal gene transfer, insights into phylogenetic relationships among bacterial species can still

be determined by looking at sequence divergence in multiple conserved loci (58) or across whole genomes (117).

Estimates on the amount of horizontally acquired DNA in bacterial genomes range from 0% to >25% of total DNA content depending upon the species of bacteria (116, 122). DNA putatively acquired through horizontal gene transfer is usually identified through discordant nucleotide composition compared to the overall nucleotide composition of the organism's genome, incongruent phylogenetic relationships of genes encoded in the region with a gene or genes used to classify the species, and/or atypical codon usages in open reading frames encoded in the region (61, 116, 117). Although authors have alternatively argued that using these measures may overestimate the occurrence of horizontal gene transfer due to uncharacterized mechanisms that generate unusual DNA composition (61), or underestimate the amount of horizontal gene transfer that occurs due to transfer of DNA from closely related species that have similar nucleotide composition and codon usage (116) or absence of evidence of transfer of genes that are detrimental to cells (133), it appears that utilizing a combination of these approaches likely gives a rough approximation of the amount of successful horizontal gene transfer that occurs.

The likelihood that horizontal transfer of genes may result in acquisition of genes that are detrimental to the cell (133) is one mechanism that selects for regulatory processes that limit horizontal gene transfer. As discussed previously, a similar argument has been applied to the regulation of natural genetic competence by quorum sensing; transformation is primarily limited to conditions when cells are surrounded by cells of the same species and are less likely to take up DNA that would encode functions detrimental to the cell or that would spuriously integrate into and disrupt the sequence of chromosomal genes required for survival. In addition, in *B. subtilis*,

regulation ensures that cells enter the differentiated, growth-arrested competence state only under conditions when this is likely to be more beneficial to the cell (12).

Selective forces that act on the evolution of regulatory mechanisms for mobile genetic elements must be thought of in terms of the ability of the element to survive and propagate. In the case of integrative and conjugative elements, survival and propagation depends upon maintaining genetic identity of the element and avoiding disruption of essential host cell functions. These forces may have led to the evolution of mechanisms that prevent constitutive expression of transfer functions of ICE*Bs1*, which is detrimental to the cell (Appendix B and Chapter 4), and limit transfer into cells that already contain a copy of the element, thereby limiting the possibility of inter-element recombination which could result in loss of function of the element. In addition, the ability of ICE*Bs1* to sense when host cells are undergoing DNA damage and to initiate transfer from these cells, may have evolved as a mechanism for a copy of the element to escape the distressed host.

VII. Conclusion

Intercellular signaling regulates many processes in bacteria. This thesis focuses on describing how intercellular peptide signaling regulates two mechanisms of horizontal gene transfer in *B. subtilis*. As described in Chapter 2, at least 4 signaling peptides promote natural genetic competence for DNA transformation. Chapters 3 and 4 describe how peptide signaling regulates transfer of the mobile genetic element ICE*Bs1*. Both mechanisms of horizontal gene transfer, DNA transformation and mobile genetic element transfer, are regulated in ways that ensure successful DNA transfer and have likely shaped the evolution of *B. subtilis*.

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Chapter 2: Multiple Rap proteins and Phr peptides regulate the ComA response of *Bacillus subtilis*

- Manuscript in preparation to be submitted to the Journal of Bacteriology -

Abstract

In *Bacillus subtilis*, extracellular peptide signaling regulates several biological processes. Secreted Phr signaling peptides are imported into the cell and act intracellularly to antagonize the activity of regulators known as Rap proteins. *B. subtilis* encodes several Rap proteins and Phr peptides, and the processes regulated by some of these Rap proteins and Phr peptides are known. We used DNA microarrays to characterize the roles that several *rap-phr* signaling modules play in regulating gene expression. We found that *rapK-phrK* regulates the expression of several genes activated by the response regulator ComA. ComA activates expression of genes involved in competence development and the production of several secreted products. Two Phr peptides, PhrC and PhrF, were previously known to stimulate the activity of ComA. We assayed the roles that PhrC, PhrF, and PhrK play in regulating gene expression and found that these three peptides stimulate ComA-dependent gene expression to different levels and are all required for full expression of genes activated by ComA. The involvement of multiple Rap proteins and Phr peptides in a regulatory network allows several physiological cues to modulate the timing and levels of the ComA response.

Introduction

Many bacteria utilize extracellular signaling molecules to coordinate biological processes (44, 84). Extracellular signaling molecules can be used to provide information about population density, often referred to as quorum sensing (44, 84) or diffusion sensing (66). In the Gram-positive bacterium, *Bacillus subtilis*, several processes are known to be regulated by extracellular peptide signaling (reviewed in 32), including the initiation of genetic competence (the ability to incorporate exogenous DNA from the environment) (42, 73), sporulation (62, 73), production of degradative enzymes (11, 29, 54, 79) and exopolysaccharides (11, 81), and antibiotic synthesis (11, 42, 73).

Two types of secreted peptide signaling molecules have been identified in *B. subtilis*: a modified five to ten amino-acid peptide, ComX, that interacts extracellularly with its receptor, the histidine kinase ComP (42, 65, 80, 81), and unmodified pentapeptides, known as Phr peptides, that are internalized to inhibit the activity of their target proteins, known as Rap proteins (reviewed in 32, 59). Whereas some (perhaps all) Phr peptides act as signals of high cell density (3, 35, 73), other Phr peptides are hypothesized to act in a cell-autonomous fashion and serve as either molecular timing devices or as signals of a functioning secretion machinery (58, 59). However, no evidence in direct support of these alternative roles for Phr peptide signaling has been presented, and it has been shown that one of the peptides proposed to act as a molecular timing device can act non-autonomously in mixed cultures (62).

B. subtilis encodes a family of seven Phr peptides and eleven Rap proteins (31). Each of the seven Phr peptides is encoded in an operon with a gene encoding a Rap regulatory protein (31), and each characterized Phr peptide inhibits the activity of its co-transcribed Rap protein (7, 26, 54, 62, 73). The PhrC peptide (also known as CSF for competence and sporulation stimulating

factor, 73) inhibits the activity of an unpaired Rap protein, RapB (58), in addition to its cognate RapC protein (12, 73). It is possible that the other unpaired Rap proteins are also inhibited by non-cognate Phr peptides.

*phr*s are transcribed and translated as pre-Phr peptides that are 35-45 amino acids in length. Each *phr* is expressed from the upstream *rap* promoter (33, 62), and for six of the seven *phr*s, a promoter upstream of the *phr* that is transcribed by RNA polymerase containing the alternative sigma factor, σ^H (33, 43, 48). This regulation by σ^H causes the levels of *phr* transcription to increase as cells transition from exponential growth to stationary phase (33, 43).

After transcription and translation, the pre-Phrs are exported and processed (reviewed in 32, 59). Mature Phr peptides are imported through the oligopeptide permease (Opp, also known as Spo0K) (35, 62, 73), an ATP-binding cassette (ABC) transporter that imports small peptides into the cell (61, 68). Once inside the cell, Phr pentapeptides can inhibit the activities of Rap proteins (7, 26, 35, 54, 58).

Several Rap proteins antagonize the activities of response regulator proteins, either by stimulating dephosphorylation (26, 60, 82), or by binding to the response regulator and interfering with DNA binding (7, 12, 54) (Table 1). RapA, RapB, and RapE antagonize the activity of Spo0F (26, 60). Spo0F is involved in activating a second response regulator, Spo0A (9), that regulates the transcription of genes involved in several post-exponential phase processes, including the initiation of sporulation (17, 47, and references therein). The activities of RapA, RapB, and RapE are inhibited by the PhrA, PhrC, and PhrE peptides (26, 58, 62). RapG antagonizes the activity of DegU (54), a response regulator that activates transcription of genes involved in competence development, inhibits transcription of flagellar genes, and stimulates transcription of genes encoding degradative enzymes (1, 16, 22, 30, 49, 55). RapG's

Table 1: Processes regulated by Rap proteins and Phr peptides in *B. subtilis*.

Rap	Phr	Target Protein of Rap	Mechanism of Rap	Responses regulated by Target Protein
RapA	PhrA	Spo0F~P	Stimulates autodephosphorylation of Spo0F (inactivates)	Activates post-exponential phase gene expression and sporulation indirectly through Spo0A
RapB	PhrC	Spo0F~P	Stimulates autodephosphorylation of Spo0F	Activates post-exponential phase gene expression and sporulation indirectly through Spo0A
RapC	PhrC	ComA	Inhibits binding of ComA to DNA (inhibits activation)	Activates expression of genes involved in production of degradative enzymes, antibiotics, and competence
RapE	PhrE	Spo0F~P	Stimulates autodephosphorylation of Spo0F	Activates post-exponential phase gene expression and sporulation indirectly through Spo0A
RapF	PhrF	ComA	Inhibits binding of ComA to DNA	Activates expression of genes involved in production of degradative enzymes, antibiotics, and competence
RapG	PhrG	DegU	Inhibits binding of DegU to DNA	Activates expression of genes involved in competence and degradative enzyme production; inhibits expression of flagellar genes
RapI	PhrI	Unknown	Unknown	[RapI stimulates gene expression, excision, and transfer of ICE*Bs1* (3, Chapter 3)]

activity is antagonized by the PhrG peptide (54). RapC and RapF inhibit the activity of ComA (7, 12, 73), which activates expression of genes involved in antibiotic synthesis (11, 52, 53, 86), degradative enzyme production (11, 50), exopolysaccharide production (11), fatty acid metabolism (11), and the initiation of genetic competence (14, 15, 53). The PhrC and PhrF peptides inhibit the activities of RapC and RapF (7, 12, 73). RapI stimulates gene expression, excision, and transfer of the mobile genetic element ICE*Bs1* by regulating the activity of an unknown protein; PhrI antagonizes the activity of RapI (3, Chapter 3). The regulatory targets of the remaining Rap proteins have not been identified.

The activity of the ComA protein is also regulated by signaling through the ComX peptide. (42). ComX is an extracellular signaling peptide that promotes the kinase activity of its membrane-bound receptor, the histidine kinase ComP (2, 42, 74, 80, 81). ComP then donates phosphate to ComA (85), which results in activation of ComA, as it is the phosphorylated form of ComA that activates transcription (52, 67). *comX*, *comP*, and *comA*, are encoded together in an operon along with *comQ*, which encodes a protein required for the production of active ComX peptide (2, 4, 80).

In the course of characterizing the roles of several Raps and Phrs in regulating gene expression, we found that RapK, in addition to RapC and RapF, inhibits the expression of genes activated by ComA. RapK's activity is inhibited by PhrK, which stimulates expression of ComA-dependent genes. PhrC, PhrF, and PhrK stimulate ComA-dependent gene expression to different levels and all three peptides are required for full expression of ComA-dependent genes. The involvement of these three Phr signaling peptides, in addition to the ComX peptide, allows the cell to modulate the levels and timing of ComA-dependent gene expression in response to multiple physiological cues.

Materials and Methods

Media. Cells were grown at 37° C in Schaeffer's nutrient broth sporulation medium (DSM) (23) or S7 minimal salts medium (83) (containing 50 mM MOPS instead of 100 mM) supplemented with 1% glucose, 0.1% glutamate, tryptophan (40 μg/ml), phenylalanine (40 μg/ml), and threonine (120 μg/ml, when necessary) as indicated. LB (69) was used for routine growth of *B. subtilis* and *Escherichia coli*. Antibiotics, when appropriate, were used at the following concentrations: ampicillin (100 μg/ml), chloramphenicol (5 μg/ml), neomycin (2.5

μg/ml), spectinomycin (100 μg/ml); erythromycin (0.5 μg/ml) and lincomycin (12.5 μg/ml) together to select for macrolide-lincosamide-streptogramin B (MLS) resistance, and tetracycline (12.5 μg/ml). Isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma) was used at a final concentration of 1 mM.

Strains and alleles. Strains used in this study are listed in Table 2. All *B. subtilis* strains were derived from the parental strain JH642 (63). The *E. coli* strain used for cloning is an MC1061 derivative with F'(*lacI*^{q}) *lacZM15* Tn*10* (*tet*). Standard techniques were used for cloning and strain construction (23, 69). P*spank*(*hy*) (3), Δ*rapC*::*cat* (73), Δ*phrC*::*erm* (73), and *amyE*::*srfA-lacZ*Ω682 (4) were previously described.

For overexpression in *B. subtilis, rapC*, *rapF*, *rapH, rapJ,* and *rapK* were cloned downstream of the IPTG-inducible promoter P*spank*(*hy*) (8), a generous gift from D. Rudner (Harvard Medical School), and integrated into the *amyE* locus by homologous recombination.

rapF-phrF was deleted by replacing +542 of *rapF* to +149 of *phrF* with a tetracycline resistance gene derived from pDG1513 (20). *rapK* was deleted by replacing +36 to +980 of *rapK* with the chloramphenicol resistance gene from pGEM-*cat* (87); *cat* was replaced with *erm* by integration of the plasmid pCm::Er (77). *phrF* was deleted by replacing +38 to +103 of *phrF* with the chloramphenicol resistance gene from pGEM-*cat*. *phrK* was deleted by replacing +64 to +100 of *phrK* with spectinomycin resistance derived from pDL55 (5).

Δ*oppBCDE*::*spc* (Δ*spo0KBCDE*::*spc*) was created by replacing the *Rsr*II/*Cla*I fragment in plasmid pDR9 (68, contains *oppBCDE*) with the *Bgl*I/*Nde*I fragment containing *spc* from pUS19 (5). Both plasmid and insert DNA was recessed/filled-in by treatment with the Klenow fragment of *E. coli* DNA polymerase I prior to ligation. The recombinant plasmid was transformed into *B. subtilis*, selecting for double crossover recombination into the chromosome.

Table 2: Strains used in this study.

Strain name	Genotype*
JH642	*trpC2 pheA1*
CAL7	Δ*phrK7*::*spc*
CAL8	*amyE*::{*srfA-lacZ*Ω682 *neo*} Δ*phrK7*::*spc*
CAL9	*amyE*::{*srfA-lacZ*Ω682 *neo*} Δ*phrC*751::*erm* Δ*phrK7*::*spc*
CAL10	*amyE*::{*srfA-lacZ*Ω682 *neo*} Δ*phrF*163::*cat* Δ*phrK7*::*spc*
CAL11	*amyE*::{*srfA-lacZ*Ω682 *neo*} Δ*phrC*751::*erm* Δ*phrF*163::*cat* Δ*phrK7*::*spc*
JMA26	*amyE*::{P*spank(hy)-rapF spc*}
JMA27	*amyE*::{P*spank(hy)-rapH spc*}
JMA29	*amyE*::{P*spank(hy)-rapJ spc*}
JMA30	*amyE*::{P*spank(hy)-rapK spc*}
JMA47	*amyE*::{*srfA-lacZ*Ω682 *neo*} Δ*rapC*::pJS79 *cat*
JMA48	*amyE*::{*srfA-lacZ*Ω682 *neo*} Δ*rapK*41::*cat*::*erm*
JMA51	*amyE*::{*srfA-lacZ*Ω682 *neo*} Δ*sigH*380::*cat*::*spc*
JMA52	*amyE*::{*srfA-lacZ*Ω682 *neo*} Δ*oppBCDE*585::*spc*
JMA54	*amyE*::{*srfA-lacZ*Ω682 *neo*} Δ*oppBCDE*585::*spc* Δ*rapC*::pJS79 *cat*
JMA56	*amyE*::{*srfA-lacZ*Ω682 *neo*} Δ*oppBCDE*585::*spc* Δ*rapK*41::*cat*::*erm*
JMA57	*amyE*::{*srfA-lacZ*Ω682 *neo*} Δ*sigH*380::*cat*::*spc* Δ*rapC*::pJS79 *cat* Δ*rapK*41::*cat*::*erm*
JMA58	*amyE*::{*srfA-lacZ*Ω682 *neo*} Δ*oppBCDE*585::*spc* Δ*rapC*::pJS79 *cat* Δ*rapK*41::*cat*::*erm*
JMA76	*amyE*::{P*spank(hy)-rapF spc*} *thrC*::{*pel-lacZ erm*}
JMA77	*amyE*::{P*spank(hy)-rapK spc*} *thrC*::{*pel-lacZ erm*}
JMA78	*amyE*::{P*spank(hy)-rapC spc*} *thrC*::{*pel-lacZ erm*}
JMA79	*amyE*::{P*spank(hy) spc*} *thrC*::{*pel-lacZ erm*}
JMA117	*amyE*::{*srfA-lacZ*Ω682 *neo*} Δ*rapFphrF*312::*tet*
JMA122	*amyE*::{*srfA-lacZ*Ω682 *neo*} Δ*oppBCDE*585::*spc* Δ*rapFphrF*312::*tet*
JMA129	*amyE*::{*srfA-lacZ*Ω682 *neo*} Δ*rapFphrF*312::*tet* Δ*rapK*41::*cat*::*erm*
JMA134	*amyE*::{*srfA-lacZ*Ω682 *neo*} Δ*oppBCDE*585::*spc* Δ*rapC*::pJS79 *cat* Δ*rapFphrF*312::*tet*
JMA135	*amyE*::{*srfA-lacZ*Ω682 *neo*} Δ*sigH*380::*cat*::*spc* Δ*rapC*::pJS79 *cat* Δ*rapFphrF*312::*tet*
JMA138	*amyE*::{*srfA-lacZ*Ω682 *neo*} Δ*oppBCDE*585::*spc* Δ*rapFphrF*312::*tet* Δ*rapK*41::*cat*::*erm*
JMA139	*amyE*::{*srfA-lacZ*Ω682 *neo*} Δ*rapC*::pJS79 *cat* Δ*rapK*41::*cat*::*erm*
JMA142	*amyE*::{*srfA-lacZ*Ω682 *neo*} Δ*rapC*::pJS79 *cat* Δ*rapFphrF*312::*tet* Δ*rapK*41::*cat*::*erm*
JMA144	*amyE*::{*srfA-lacZ*Ω682 *neo*} Δ*oppBCDE*585::*spc* Δ*rapC*::pJS79 *cat* Δ*rapFphrF*312::*tet* Δ*rapK*41::*cat*::*erm*
JMA149	*amyE*::{*srfA-lacZ*Ω682 *neo*} Δ*sigH*380::*cat*::*spc* Δ*rapC*::pJS79 *cat* Δ*rapFphrF*312::*tet* Δ*rapK*41::*cat*::*erm*
JMA163	Δ*phrF*163::*cat*
JMA165	*amyE*::{*srfA-lacZ*Ω682 *neo*} Δ*phrC*751::*erm*
JMA166	*amyE*::{*srfA-lacZ*Ω682 *neo*} Δ*phrF163*::*cat*
JMA169	*amyE*::{*srfA-lacZ*Ω682 *neo*} Δ*phrC*751::*erm* Δ*phrF163*::*cat*
JMA752	*amyE*::{*srfA-lacZ*Ω682 *neo*} Δ*phrK7*::*spc* Δ*rapK*38::*cat*
JMS682	*amyE*::{*srfA-lacZ*Ω682 *neo*}
RSM121	Δ*phrC*751::*erm*

* All strains are derived from JH642 and contain *trpC2* and *pheA1* alleles.

Δ*sigH*::*cat*::*spc* (Δ*spo0H*::*cat*::*spc*) was created by integrating plasmid pJL62 (36) into a strain containing the Δ*sigH*::*cat* (25) mutation.

The *pel-lacZ* promoter fusion was generated by cloning the DNA from –371 to +39 of *pel* upstream of the promoter-less *lacZ* in the vector pDG793 (19), followed by integration into the *thrC* locus by homologous recombination. A similar fusion at *amyE* was previously described (11).

DNA Microarrays. P*spank*(*hy*)-*rapF* (JMA26) and P*spank*(*hy*)-*rapK* (JMA30) cells were grown in defined minimal medium for at least four generations to O.D. 600 ~ 0.5. IPTG was added to half the cultures, and samples were collected from induced and uninduced cultures 30 min. later. Wild-type (JH642), Δ*phrC* (RSM121), Δ*phrF* (JMA163), and Δ*phrK* (CAL7) cells were grown in defined minimal medium for at least four generation to an optical density at 600 nm (O.D. 600) ~ 1, when samples were collected.

Cells were harvested and total RNA was prepared as described (8). RNA from each sample was reverse transcribed and labeled as described (3). In the experiments monitoring gene expression in cells overexpressing the indicated *rap* gene, labeled cDNA from induced (+IPTG) and uninduced samples (no IPTG) were co-hybridized to cDNA microarrays as described (3). In the experiments monitoring gene expression in wild-type, Δ*phrC*, Δ*phrF*, and Δ*phrK* cells, labeled cDNA from each experimental sample was hybridized with a labeled reference cDNA sample to 65-mer oligonucleotide arrays as described (3).

Arrays were scanned and analyzed as described (3). Iterative outlier analysis (8, 38) was used as described (3) to identify genes whose expression changed significantly with 95% or greater confidence. The mean ratio for a set of triplicate experiments is reported. Lists of significant genes were arranged into known or putative operons based on the prediction of co-orientation of

transcription and the absence of predicted rho-independent terminators. If a gene or genes that were part of a known or predicted operon changed significantly, the average fold changes in gene expression for the other genes in the operon were also assessed. If the expression of those genes changed similarly, but these changes were below the significance threshold of the analysis, the values of these fold changes were included in Fig. 1 and Table 3 (Appendix A).

β-galactosidase assays. β-galactosidase specific activity of the indicated fusions was assayed as described (25). Specific activity was calculated relative to the O.D. 600 of the samples. β-galactosidase activity is plotted relative to time or the optical density of the sample as indicated. In each graph, the results from a single experiment are presented and are representative of the results observed in at least two independent experiments.

Results

Identification of Rap proteins that inhibit the expression of genes activated by ComA. We used DNA microarrays as an initial approach to characterize genes whose mRNA levels were affected by several Rap proteins. We examined the effects of *rapF, rapH, rapJ,* and *rapK* overexpression on global mRNA levels. Although changes in global mRNA levels may result from changes in the level of gene transcription as well as changes in the levels of RNA stability, for simplicity we assumed that changes in mRNA levels reflect changes in gene expression. This same microarray-based strategy successfully elucidated the role that RapI plays in activating expression of genes in the ICE*Bs1* mobile element (3, Chapter 3).

Each *rap* gene was overexpressed from the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter P*spank(hy)* during exponential growth in defined minimal medium and RNA transcript levels were compared between induced and uninduced cells 30 min. after induction.

We analyzed the results of three independent experiments to identify those genes whose expression changed significantly in response to overproduction of each Rap. We compared the results of these experiments to the published genome-wide analyses of several response regulator regulons (11, 17, 28, 41, 47, 57) in order to identify response regulators whose activities were potentially regulated by these Rap proteins.

Overproduction of each Rap resulted in changes in the expression of several genes. Overexpression of *rapH* caused small changes in the expression of 14 genes; there was no significant overlap between those genes effected by *rapH* overexpression and characterized response regulator regulons (Table 3 in Appendix A). *rapJ* overexpression affected the expression of several operons known to be regulated by Spo0A (20/38; Table 3). It is not known whether this inhibition by RapJ is through direct inhibition of Spo0A, or occurs indirectly. Overexpression of either *rapF* or *rapK* inhibited the expression of genes known to be activated by ComA (Fig. 1), with *rapK* overexpression inhibiting the expression of a larger number of ComA-regulated genes and to a greater magnitude than overexpression of *rapF*. Effects of *rapF* overexpression on ComA-dependent gene expression are consistent with previously published data (7). We decided to focus on those Rap proteins that primarily affected the expression of ComA-regulated genes to further understand how multiple Raps and Phrs affect the activity of this regulator.

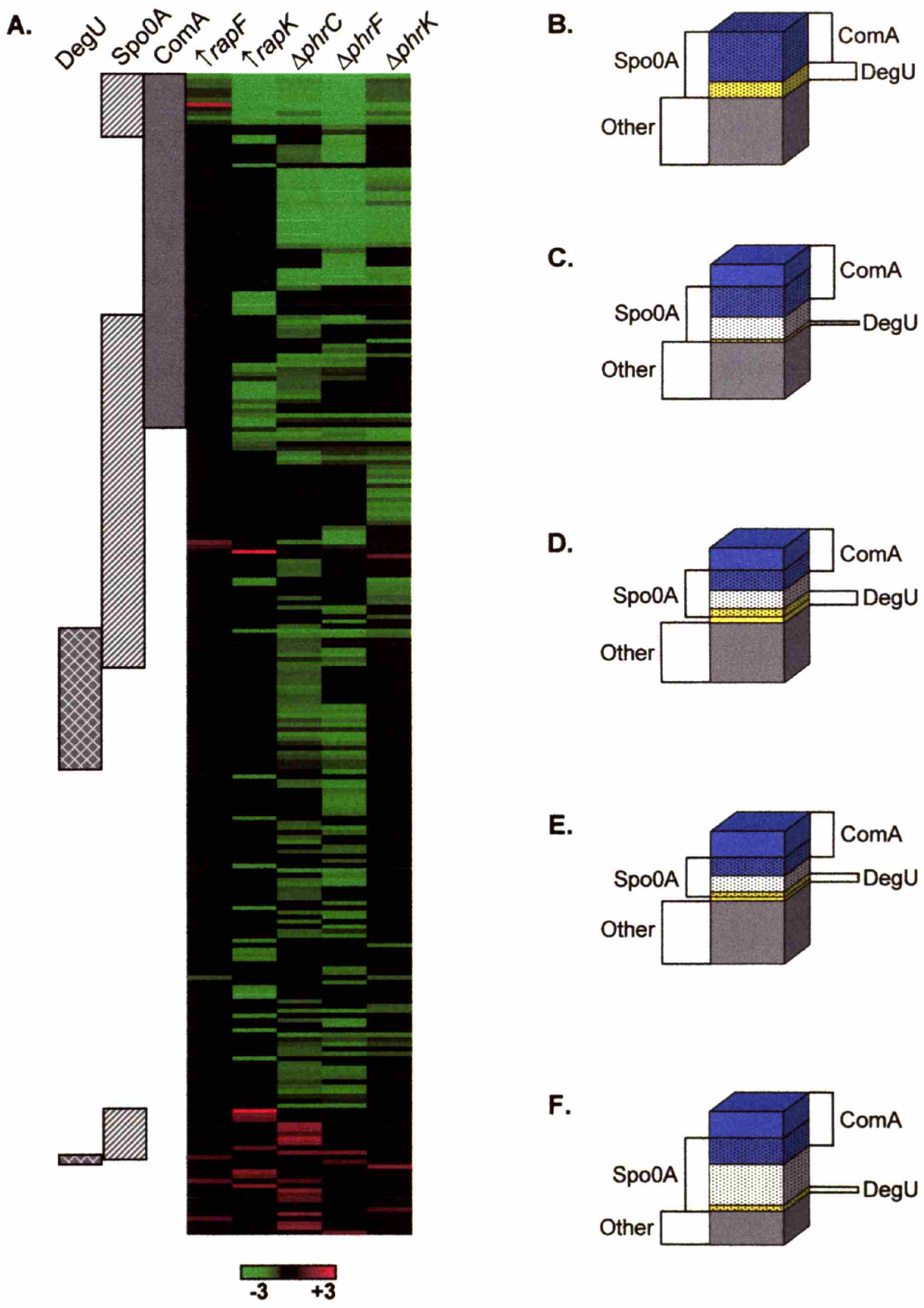
Effects of overexpression of *rapF* on gene expression. Overproduction of RapF had small effects on gene expression, with significant changes in only 8 operons (Fig. 1, Table 3). The expression of 4 operons decreased in response to *rapF* overexpression: 3 are directly activated by ComA and indirectly activated by Spo0A, while 1 is not known to be regulated by a response regulator. Overexpression of *rapF* also resulted in increased expression of 4 operons: the *rapI-*

phrI operon, which is known to be activated indirectly by Spo0A (3), 1 operon whose transcription is repressed by DegU and Spo0A, and 2 operons that are not known to be regulated by response regulators.

Although *rapF* overexpression inhibited only a small subset of the ComA regulon, the genes that were inhibited show some of the largest changes in gene expression in response to perturbations in the levels of *comA* (11). Therefore, it is likely that overexpression of *rapF* modestly inhibited the expression of genes in the ComA regulon and that any additional changes in expression of other genes in the ComA regulon were below the significance threshold of the microarrays.

Effects of *rapK* overexpression on gene expression. We detected significant changes in the expression of 36 operons in response to RapK overproduction (Fig. 1, Table 3), with expression of 29 operons decreased and 7 operons increased. Of the 29 operons whose expression decreased, previous studies have shown that 14 are activated by ComA, 11 by Spo0A, 1 by DegU, 1 by YclG and YkoG, and the remaining 11 are not known to be regulated by response regulators. Several operons that decrease in expression are regulated by more than one response regulator. DegU and Spo0A activate the expression of 1 operon, and ComA and Spo0A activate the expression of 8 operons. Of the 7 operons whose expression increased in *rapK* overexpressing cells, 4 are known to be repressed by Spo0A and the remaining 3 are not known to be regulated by a response regulator.

Based on these results, we infer that RapK inhibits the activity of ComA, either directly or indirectly, when overexpressed. However, several genes whose expression changed in response to overexpression of *rapK* are not part of the ComA regulon. Spo0A may be another direct or


A.
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ComA
↑rapF
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ΔphrC
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-3
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DegU
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Other
F.
ComA
Spo0A
DegU
Other

Figure 1: Overexpression of *rapF* or *rapK* or deletion of *phrC*, *phrF*, or *phrK* inhibits expression of genes activated by ComA.

We used DNA microarrays to examine changes in mRNA levels in response to overexpression of *rapF* or *rapK* and deletion of *phrC*, *phrF*, or *phrK*.

A. Genes whose expression changed significantly in response to overexpression of *rapF* (↑*rapF*) or *rapK* (↑*rapK*), or to deletion of *phrC* (Δ*phrC*), *phrF* (Δ*phrF*), or *phrK* (Δ*phrK*) were identified as described in Materials and Methods and are represented in the figure by a box shaded to represent the magnitude of the mean fold change in gene expression. A 3-fold or greater decrease in gene expression is shaded bright green and a 3-fold or greater increase in gene expression is shaded bright red. Those genes whose expression did not change significantly are shaded black. Additional microarray results, including the gene names and numerical values of the fold-changes in gene expression are in Table 3 (Appendix A).

The boxes to the left of the visualization indicate those genes whose expression were previously shown to be regulated by the response regulators ComA (6, 11, 56, 57)(gray box), Spo0A (17, 47) (hatched boxes), and DegU (41, 57) (stippled boxes).

B-F. These illustrations show the number of operons whose expression changed significantly in response to overexpression of *rapF* or *rapK* or deletion of *phrC, phrF,* or *phrK* and are known to be regulated by the response regulators ComA (blue segments), Spo0A (stippled segments), DegU (yellow segments) or other regulators (gray segments).

B. Gene expression changes in cells overexpressing *rapF*.

C. Gene expression changes in cells overexpressing *rapK*.

D. Gene expression changes in Δ*phrC* cells.

E. Gene expression changes in Δ*phrF* cells.

F. Gene expression changes in Δ*phrK* cells.

indirect target of RapK, as several of the operons affected by *rapK* overexpression are known to be regulated by Spo0A.

Overexpression of RapF and RapK inhibits expression of *pel-lacZ*. In order to further investigate the roles that RapF and RapK play in regulating ComA-dependent gene expression, we monitored the effects of RapF and RapK overproduction on the expression of the ComA-activated gene *pel* using a fusion of the *pel* promoter to the reporter gene, *lacZ*. We compared expression of *pel-lacZ* in cells overexpressing *rapF* (Pspank(*hy*)-*rapF*) or *rapK* (P*spank*(*hy*)-*rapK*) to that in control cells with an empty overexpression vector (Fig. 2). Consistent with previous observations (11), *pel* expression was initially low and increased with increasing cell density in control cells (Fig. 2A); other ComA-regulated genes also exhibit similar patterns of expression (33, 42). Overexpression of *rapF* or *rapK* prevented the density-dependent increase in *pel* expression (Fig. 2). A similar effect was observed when *rapC*, the other known ComA inhibitor, was overexpressed (Fig. 2). These data further demonstrate that RapF and RapK inhibit ComA-dependent gene expression when overexpressed and indicate that RapF and RapK likely have roles in regulating ComA-dependent gene expression under certain conditions.

PhrC, PhrF, and PhrK stimulate ComA-dependent gene expression. All characterized Rap proteins are inhibited by their cognate Phr peptides. Therefore, we examined the roles that PhrF and PhrK play in regulating the expression of ComA-regulated genes. We used DNA microarrays to compare mRNA levels in Δ*phrF* and Δ*phrK* mutants to mRNA levels in wild-type cells. We expected that loss of *phrF* and *phrK* should result in decreased ComA-dependent gene expression, as the activities of RapF and RapK should increase due to the absence of their inhibitory peptides. We also tested the effects of deletion of *phrC* on global gene expression. The expression of several ComA-regulated genes is known to decrease in Δ*phrC* mutants (12,

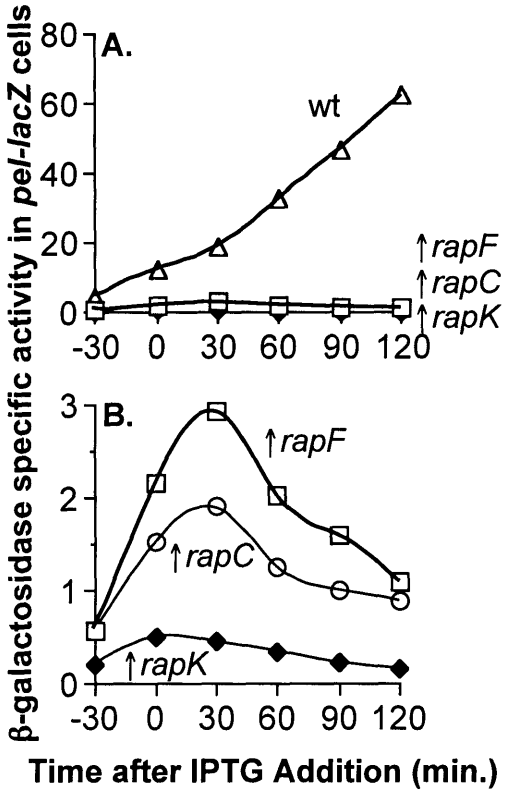

Figure 2: Overexpression of *rapC, rapF*, or *rapK* inhibits expression of the ComA-activated gene *pel.*

Cells containing *pel-lacZ* and P*spank(hy)-rapC* (JMA77), P*spank(hy)-rapF* (JMA76), P*spank(hy)-rapK* (JMA78), or P*spank(hy)* (JMA79) were grown in defined minimal medium. IPTG was added to cells at O.D. 600 ~ 0.4-0.6. Samples were collected from cells 30 min. prior to IPTG addition, at the time of IPTG addition, and 30, 60, 90 and 120 min. after IPTG addition. β-galactosidase activity was assayed as described in Methods and is plotted relative to the time of IPTG addition. *pel-lacZ* expression in P*spank(hy)* (△, wt), P*spank(hy)-rapC* (○, ↑*rapC*), P*spank(hy)-rapF* (□,↑*rapF*), and P*spank(hy)-rapK* (◇,↑*rapK*) cells.

A. Data plotted with y-axis from 0-80.

B. Data from (A) re-plotted with y-axis from 0-3.

33, 73); this is due to increased RapC activity. We found that deletion of all three *phr*s resulted in decreased expression of genes activated by ComA, with Δ*phrF* and Δ*phrK* mutants having the largest and smallest decreases in expression of ComA-dependent genes, respectively (Fig. 1, Table 3).

Δ*phrC*. Deletion of *phrC* significantly changed the expression of 66 operons (Fig. 1, Table 3); expression of 54 operons decreased and 12 operons increased. The transcription of 21 of the 54 operons that decreased are activated by ComA. 21 of the 54 are activated by Spo0A, including 10 that are also activated by ComA and 4 that are also activated by DegU. DegU activates the expression of 3 additional operons and YbdG is thought to repress 1 operon that decreased in expression in Δ*phrC* cells. The remaining 23 operons that decreased in expression are not known to be regulated by response regulators. Of the 8 operons that increased in expression in Δ*phrC* cells, 2 are repressed by Spo0A and the remaining 6 operons are not known to be regulated by response regulators.

Δ*phrF*. Deletion of *phrF* resulted in significant changes in the expression of 72 operons (Fig. 1, Table 3); expression of 69 operons decreased and 3 operons increased. Of the 3 operons with increased expression, 1 is known to be repressed by DegU, 1 by Spo0A, and the third operon is not known to be regulated by a response regulator. Of the 69 operons that decreased in Δ*phrF* cells, 24 are known to be activated by ComA, including the majority of the operons demonstrating the largest fold changes in gene expression, 21 are activated by Spo0A, including 10 that are activated by ComA, 4 are activated by DegU, including 3 that are activated by Spo0A, 1 is thought to be repressed by the response regulator YdbG, and 32 are not known to be regulated by response regulators.

ΔphrK. Deletion of *phrK* resulted in significant changes in the expression of 40 operons (Fig. 1), 38 of which decreased. The expression of 2 operons not known to be regulated by response regulators increased in Δ*phrK* mutants. Of the 38 operons whose expression decreased in Δ*phrK* cells, 16 are activated by ComA, 22 by Spo0A, including 8 that are also activated by ComA and 2 that are also activated by DegU, 2 by DegU, and 8 are not known to be regulated by response regulators.

Targets for Phr peptides and Rap proteins. Consistent with previous studies and our results from overexpression of RapC, RapF, and RapK, our analysis of Δ*phrC*, Δ*phrF*, and Δ*phrK* mutants demonstrate that all three Phr peptides play a role in activating expression of genes regulated by ComA. PhrK likely stimulates ComA-dependent gene expression by inhibiting the activity of RapK; PhrC and PhrF are known to stimulate the expression of ComA-dependent genes by inhibiting RapC and RapF, respectively (7, 73). RapC and RapF have been previously shown to directly interact with ComA and to inhibit its activity to bind to DNA (7, 12). RapK may also inhibit the activity of ComA directly, or by affecting the activity of another regulator that is known to regulate the transcription of these genes (17, 21, 34, 47, 70). RapK may also play a role in directly or indirectly regulating the expression of genes activated by Spo0A because a number of Spo0A-controlled genes were also affected by overproduction of RapK and deletion of *phrK*.

Although one may expect that overexpression of each *rap* and deletion of each cognate *phr* would have similar effects on gene expression, this is not what we observed. Overexpression of *rapK* and deletion of *phrK* both resulted in decreased expression of ComA-regualted genes. However, overexpression of *rapK* had much more pronounced effects on ComA-dependent gene expression than did deletion of *phrK*. These results may be partially explained by the fact that

transcription of *rapK* was likely low at the time at which changes in gene expression were measured in Δ*phrK* mutants. Transcription of *rapK* has been proposed to be activated indirectly by the transcription factor Spo0A, which activates gene expression at the end of exponential growth, a few generations later than the time of assay in Δ*phrK* mutant cells.

In contrast, overexpression of *rapF* had modest effects on ComA-depenent gene expression while deletion of *phrF* had much larger effects. These observations may also be explained by the transcriptional regulation of *rapF*. Transcription of *rapF* is activated by ComA, and its expression is activated early in response to increased ComA activity (11). Therefore, *rapF* was expressed at high levels under the conditions used to assay the effects of a Δ*phrF* mutation on gene expression. Overexpression of *rapF* may have had modest effects on gene expression because it failed to raise the levels of RapF significantly above the levels of PhrF present in the cell. This is likely due to the presence of the native copy of *rapF*. Transcription from this locus, which is activated by ComA, likely decreased in response to increased *rapF* expression from the P*spank*(*hy*) promoter due to the autoregulatory loop controlling *rapF* transcription from its native promoter. This regulation likely explains most of the differences in the magnitude of effects observed in response to overexpression of *rapF* or *rapK* and deletion of *phrF* or *phrK*.

In addition to the genes regulated by ComA, deletion of each *phr* also resulted in changes in the expression of several other genes. Many of these genes were not detected in the *rap* overexpression experiments. These differentially regulated genes may be regulated by both the *rap* and *phr* and reflect differences in the way the experiments were performed - transient overexpression of each *rap* compared to absence of each *phr* throughout growth. Alternatively, each Phr may affect the activity of a protein or proteins in addition to its cognate Rap protein. The PhrC and PhrG peptides are known to affect the activities of proteins in addition to their

cognate Rap proteins (54, 58, 73). PhrC interacts with two additional proteins (73), one of which is the indirect Spo0A antagonist RapB (58), so the effects of the Δ*phrC* mutation on Spo0A-regulated gene expression may reflect increased activity of RapB. The other target of PhrC has not been identified, so it is possible that some of the gene expression changes observed in the Δ*phrC* mutant could reflect changes in the activity of this protein.

PhrC, PhrF, and PhrK play different roles in stimulating ComA-dependent gene expression. The results of our microarray analysis of the Δ*phrC*, Δ*phrF*, and Δ*phrK* mutants indicated that the Δ*phrF* mutation had the largest effect on the expression of genes activated by ComA under the condition tested, mid-exponential growth in defined minimal medium (O.D. 600 ~ 1). However, these experiments did not give a dynamic picture of the effects of the Δ*phrC,* Δ*phrF,* and Δ*phrK* mutations on ComA-dependent gene expression throughout growth. We used a fusion of *lacZ* to the ComA-dependent promotor *srfA* (*srfA-lacZ*) to monitor the effects of these mutations throughout growth (Fig. 3A).

As described previously (73), we found that *srfA* expression was ~2-fold lower in Δ*phrC* mutants relative to wild-type and that the increase in *srfA* expression was delayed by about one half of a generation. In Δ*phrF* mutant cells, *srfA* expression was reduced ~10-20-fold and the increase in *srfA* expression was delayed several generations relative to wild-type cells. In contrast, in the Δ*phrK* mutant, *srfA* expression was ~75% of that in wild-type cells and was not detectably delayed.

These results provide further evidence that all 3 Phrs are required for full levels of ComA-dependent gene expression and that PhrC, PhrF, and PhrK stimulate ComA-dependent gene expression to different levels. Furthermore, the effects of the *phrC*, *phrF*, and *phrK* mutations on *srfA* expression were dependent upon the presence of their cognate *rap*s, as *srfA* expression was

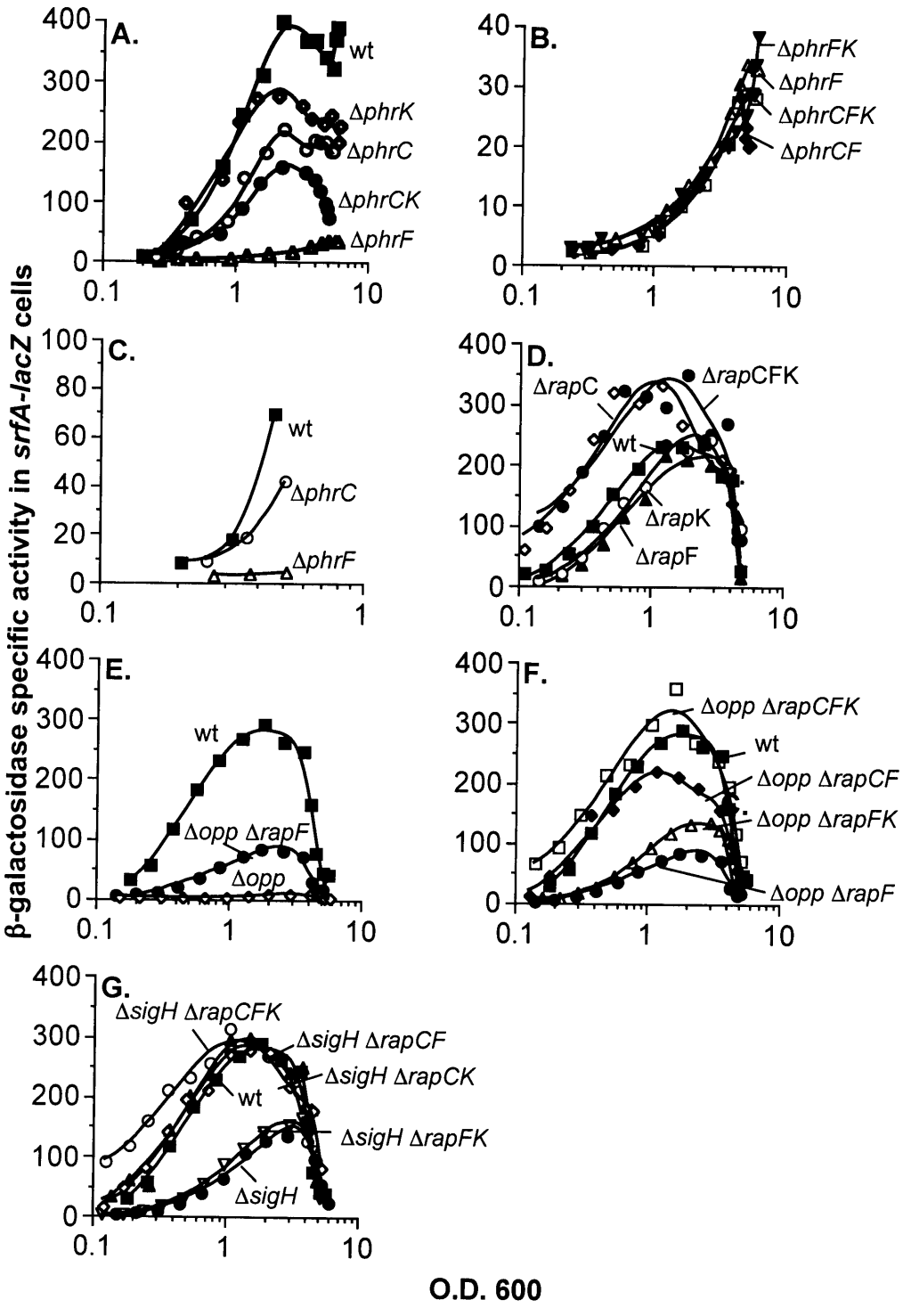
β-galactosidase specific activity in srfA-lacZ cells
A.
wt
ΔphrK
ΔphrC
ΔphrCK
ΔphrF
B.
ΔphrFK
ΔphrF
ΔphrCFK
ΔphrCF
C.
wt
ΔphrC
ΔphrF
D.
ΔrapC
ΔrapCFK
wt
ΔrapK
ΔrapF
E.
wt
Δopp ΔrapF
Δopp
F.
Δopp ΔrapCFK
wt
Δopp ΔrapCF
Δopp ΔrapFK
Δopp ΔrapF
G.
ΔsigH ΔrapCFK
ΔsigH ΔrapCF
ΔsigH ΔrapCK
wt
ΔsigH ΔrapFK
ΔsigH
O.D. 600

Figure 3. Effects of *rap* and *phr* deletions on expression of *srfA-lacZ*

srfA-lacZ containing cells were grown in defined minimal medium and samples were removed for β-galactosidase activity assays throughout growth. β-galactosidase specific activity was determined as described in Materials and Methods and is plotted relative to the O.D. 600 values of the samples.

A. wild-type (wt, JMS682, ■), Δ*phrK* (CAL8,◇), Δ*phrC* (JMA165, ○), Δ*phrC* Δ*phrK* (Δ*phrCK*, CAL9, ●), and Δ*phrF* (JMA 166, △) cells.

B. Δ*phrF* (△, replotted from part A), Δ*phrF* Δ*phrK* (Δ*phrFK*, CAL10, ▼), Δ*phrC* Δ*phrF* Δ*phrK* (Δ*phrCFK*, CAL11, □), and Δ*phrC* Δ*phrF* (Δ*phrCF*, JMA169, ◆) cells.

C. Expression at the early time points in wild-type (■), Δ*phrC* (○), and Δ*phrF* (△) cells. The data from part A are replotted to allow better visualization of the differences.

D. wild-type (JMS682, ■), Δ*rapC* (JMA47, ◇), Δ*rapF* (JMA117, ▲), Δ*rapK* (JMA48, ○), and Δ*rapC* Δ*rapF* Δ*rapK* (Δ*rapCFK*, JMA142, ●) cells.

E. wild-type (JMA682, ■), Δ*opp* (Δ*opp*, JMA52, ◇), and Δ*opp* Δ*rapF* (Δ*opp* Δ*rapF*, JMA122, ●) cells.

F. wild-type (■, replotted from part E), Δ*opp* Δ*rapF* (Δ*opp* Δ*rapF*, ●, replotted from part A), Δ*opp* Δ*rapF* Δ*rapK* (Δ*opp* Δ*rapFK*, JMA138, △), Δ*opp* Δ*rapF* Δ*rapC* (Δ*opp* Δ*rapCF*, JMA134, ◆) and Δ*opp* Δ*rapC* Δ*rapF* Δ*rapK* (Δ*opp* Δ*rapCFK*, JMA144, □) cells.

G. wild-type (JMS682, ■, data replotted from part E), Δ*sigH* (Δ*sigH*, JMA51, ●), Δ*sigH* Δ*rapF* Δ*rapK* (Δ*sigH* Δ*rapFK*, JMA139, ▽), Δ*sigH* Δ*rapC* Δ*rapF* (Δ*sigH* Δ*rapCF*, JMA135, ▲), Δ*sigH* Δ*rapC* Δ*rapK* (Δ*sigH* Δ*rapCK*, JMA57, ◇), and Δ*sigH* Δ*rapC* Δ*rapF* Δ*rapK* (Δ*sigH* Δ*rapCFK*, JMA149, ○) cells.

not inhibited when both *rap* and *phr* were inactivated (Fig. 3D, data not shown). These observations indicate that ComA-dependent gene expression decreases in Δ*phrC*, Δ*phrF*, and Δ*phrK* mutants due to the increased activities of RapC, RapF, and RapK. Based on the delay in *srfA* expression observed in Δ*phrC* and Δ*phrF* cells, it also appears likely that RapC and RapF are active at lower cell densities, while RapK activity is limited to higher cell density. This would be consistent with the known transcriptional activation of *rapC* and *rapF* by ComA (11, 33, 57) and the proposed (indirect) transcriptional activation of *rapK* by Spo0A (17, 47), which is activated by signals of starvation and high cell density (reviewed in 10, 18, 75).

Multiple Phrs act independently to inhibit *srfA* expression. We also examined the effects of multiple *phr* deletions on *srfA* expression. We found that the Δ*phrC* Δ*phrK* double mutant had levels of *srfA* expression below those observed in a Δ*phrC* mutant (Fig. 3A). The effects of these *phr* mutations on *srfA* expression indicate that RapC and RapK act independently to inhibit ComA-dependent gene expression. Δ*phrC* Δ*phrF* double mutant cells exhibited a very small decrease in *srfA* expression relative to Δ*phrF* mutant cells (Fig. 3B). Although deletion of *phrK* in Δ*phrF* mutant cells had no effect on *srfA* expression, deletion of *phrK* in Δ*phrC* Δ*phrF* mutant cells resulted in a small increase in *srfA* expression to the level of that seen in Δ*phrF* mutants at high cell densities (Fig. 3B). Although the explanation for this small increase in expression is not readily apparent, it may be due to the combined activities of additional regulator proteins affected by PhrC, PhrF, and PhrK.

Deletion of *rapF* and *rapK* has no effect on ComA-dependent gene expression. Deletion of *rapC* in otherwise wild-type cells results in increased expression of *srfA* relative to wild-type (73). However, deletion of *rapF* or *rapK* has no detectable effect on *srfA* expression in otherwise wild-type cells when assayed in minimal medium (Fig. 3D), nutrient broth sporulation

medium (DSM), or DSM supplemented with glucose and glutamate (both buffered and unbuffered, described in (13) (data not shown)). Furthermore, in preliminary microarray experiments there were no detectable effects on the expression of any other ComA-regulated genes in Δ*rapF* and Δ*rapK* mutants (data not shown). Similarly, Bongiorni *et al.* (7) did not observe changes in expression of *rapA* in Δ*rapF* mutant cells.

We were also unable to detect synergistic effects on *srfA* expression when combining the Δ*rapC* mutation with either the Δ*rapF*, Δ*rapK*, or both the Δ*rapF* and Δ*rapK* mutations (Fig. 3D). This is in contrast with the results reported by Bongiorni *et al.* (7), who observed that combination of Δ*rapF* and Δ*rapC* mutations had synergistic effects on *rapA* expression. This conflicting observation could be due to the differences in the promoters assayed (*rapA* compared to *srfA*) and the growth conditions (DSM, where ComA-dependent gene expression increases at the end of exponential growth, versus minimal glucose medium, where ComA-dependent gene expression increases during exponential growth (42, 53)).

Rap proteins inhibit ComA-dependent gene expression in strains defective for synthesis and uptake of Phrs. The inability to detect effects of Δ*rapF* and Δ*rapK* mutations on *srfA* expression in cells grown in minimal media was surprising as our previous results indicated that under these growth conditions, RapC, RapF, and RapK all actively repress *srfA* expression in the absence of their inhibitory peptides. Therefore, we looked for additional insights into the roles that RapC, RapF, and RapK play in regulating ComA-dependent gene expression by examining the effects of *rapC*, *rapF* and *rapK* mutations in the presence of mutations, Δ*opp* and Δ*sigH*, that also affect ComA-dependent gene expression, likely by affecting import and synthesis of Phr peptides.

Δopp. Previous work has shown that the oligopeptide permease (Opp) is required for competence development, sporulation, and expression of *srfA* (21, 61, 68). It was previously demonstrated that the only role for Opp in sporulation is to inhibit the activities of the RapA and RapB proteins; deletion of *rapA* and *rapB* in an *opp* mutant restores sporulation efficiency to wild-type levels (62). However, the role for Opp in competence development and *srfA* expression has not been elucidated, although it has been proposed to inhibit the activities of RapC and other Rap proteins, likely through its role in importing Phr peptides (35, 73).

Based on our observations that RapC, RapF, and RapK all inhibit expression of *srfA* and other ComA regulated genes, we thought it was likely that the role that Opp played in regulating *srfA* expression was to antagonize the activities of RapC, RapF, and RapK. Therefore, we tested the ability of *rapC*, *rapF*, and *rapK* deletions to suppress the defects in *srfA* expression that occurred in an *opp* mutant. We found that deletion of *rapC*, *rapK,* or deletion of both *rapC* and *rapK* was not able to suppress the defect in *srfA* expression observed in Δ*opp* cells, as *srfA* was expressed at the same low level in Δ*opp*, Δ*opp* Δ*rapC*, Δ*opp* Δ*rapK*, and Δ*opp* Δ*rapC* Δ*rapK* cells (data not shown). However, deletion of *rapF* in Δ*opp* mutant cells resulted in a significant restoration of *srfA-lacZ* expression (Fig. 3E). *srfA* expression was further enhanced in Δ*opp* Δ*rapF* cells by deletion of *rapC*, *rapK*, or both *rapC* and *rapK* (Fig. 3F). *srfA* expression levels in Δ*opp* Δ*rapC* Δ*rapF* Δ*rapK* cells were slightly higher than wild type and were similar to the levels of *srfA* expression in *opp*$^{+}$ Δ*rapC* cells (73, Fig. 3D).

These results indicate that the primary reason that *opp* mutant cells exhibit low levels of *srfA* expression is due to increased activity of RapF. In addition, the similarly low levels of *srfA* expression observed in Δ*phrF* and Δ*opp* mutant cells indicate that the increase in RapF activity in *opp* mutant cells is primarily due to the inability to import the PhrF peptide. The changes in

the timing and level of *srfA* expression in the Δ*opp* Δ*rapF* Δ*rapC* and Δ*opp* Δ*rapF* Δ*rapK* mutants also correlate well with the changes in the timing and level of *srfA* expression in the Δ*phrC* and Δ*phrK* mutants. Taken together, these results indicate that in the absence of Opp and the import of the PhrC, PhrF, and PhrK peptides, RapC, RapF and RapK inhibit *srfA* expression.

Δ*sigH*. σ^H is required for transcription of all *phrs* except *phrA* (33, 43, 48), for full activation of *srfA* expression (25), and seems to play a role in the post-translational production of mature PhrC peptide (33). We tested whether the decrease in *srfA* expression that occurs in *sigH* mutants was due to the increased activity of RapC, RapF, or RapK. We found that deletion of *rapF, rapK,* or both *rapF* and *rapK* in Δ*sigH* mutants had no effect on *srfA* expression (Fig. 3G). However, deletion of *rapC* in Δ*sigH* mutants restored *srfA* expression to near wild-type levels (data not shown), and deletion of *rapC* in addition to *rapF* or *rapK* restored *srfA* expression to wild-type levels in Δ*sigH* mutants (Fig. 3G). Deletion of *rapC*, *rapF*, and *rapK* in Δ*sigH* mutants resulted in levels of *srfA* expression slightly higher than wild-type (Fig. 3G).

These data indicate that the defect in *srfA* expression in *sigH* mutant results from the increased RapC, RapF, and RapK activity that occurs when production of PhrC, PhrF, and PhrK peptides is reduced. Furthermore, the RapC protein plays a more significant role in inhibiting *srfA* expression under these conditions than does RapF or RapK, as deletion of *rapC* is required for deletion of *rapF* or *rapK* to restore *srfA* expression (Fig. 3G).

These results, when compared to the results observed with deletion of *rap*s in *opp* mutant cells, also indicate that there may be different requirements for σ^H activity among the Phr peptides, with some Phr peptides being more dependent upon σ^H activity, either at the level of *phr* transcription or post-translational production. *phrA* does not possess a σ^H-dependent promoter and instead appears to be transcribed only from the upstream *rapA* promoter (43, 62).

phrE and *phrI* are transcribed from at least one promoter in addition to the σ^H-dependent promoter (43). As the role that σ^H plays in the post-translational processing of PhrC and potentially other peptides has not been elucidated, it is currently not clear whether different Phr peptides may be more dependent upon σ^H activity for post-translational processing.

Discussion

PhrK stimulates ComA-dependent gene expression by antagonizing RapK. In this work, we identified an additional *rap-phr* pair, *rapK-phrK*, that regulates the expression of several genes activated by the response regulator ComA (11). We observed that PhrK stimulates expression of genes activated by ComA by antagonizing the activity of RapK. Since other regulatory proteins are also known to regulate transcription of some of the genes that are activated by ComA, (17, 21, 34, 47, 70), the effect mediated by RapK and PhrK may occur through direct inhibition of ComA activity by RapK or through inhibition of the activity of another regulatory protein. *rapK* transcription is thought to be activated indirectly by Spo0A (17, 47). Therefore, regulation of ComA activity directly or indirectly by RapK provides an opportunity for additional signals, such as those that regulate Spo0A activity, to be incorporated into the decision to activate expression of genes regulated by ComA.

RapC, RapF, and RapK play different roles in regulating ComA activity. RapC, RapF, and RapK appear to have different roles in regulating ComA-dependent gene expression. We found that RapK has a modest role in inhibiting the expression of genes in the ComA regulon, and that under the conditions tested, this is only evident in the absence of its inhibitory peptide or when *rapK* is overexpressed. RapF is a potent antagonist of ComA-dependent gene expression in the absence of its inhibitory peptide, but there is little evidence for its role in regulating

ComA-dependent gene expression when this peptide is produced and able to enter the cell. This is in contrast to RapC, whose modest inhibition of ComA-dependent gene expression is apparent in the presence and absence of the PhrC peptide.

The reason that RapF has such profound effects on ComA-dependent gene expression in the absence of PhrF, but not in its presence, is not immediately obvious. Both *rapF* and *rapC* are transcribed from ComA-dependent promoters and are clearly active under the conditions tested. Therefore, it seems unlikely that differences in transcriptional regulation of the *rapC* and *rapF* would explain the differences in the levels of activity of the two Rap proteins.

RapC and RapF could have different levels of activity under the conditions tested due to differences in their respective Phr peptides. One hypothesis is that PhrF peptides accumulate in the cell at a lower cell population density than PhrC peptides, thereby inhibiting RapF activity at lower densities than those observed for RapC. Accumulation of PhrF peptides at lower cell density could be due to higher levels of PhrF transcription, processing, or import into the cell. If this were true, one would expect to observe differences in the amount of RapF and RapC activity at very low cell densities. Consistent with this hypothesis, we observed that even at the earliest time point assayed, *srfA* expression was ~3-5 fold lower in Δ*phrF* mutants than in Δ*phrC* mutants or wild-type cells (Fig. 3C). At these very early time points, there was little difference in *srfA* expression between Δ*phrC* and wild-type cells (Fig. 3C). Furthermore, differences in the import of Phr peptides may explain the observation that mutations in the oligopeptide permease can be obtained that do not respond to the PhrC peptide but still allow more significant levels of ComA-dependent gene expression to occur than is observed in Δ*opp* cells (72).

Integration of multiple signals for complex regulation of gene expression. The involvement of multiple peptides in the activation of ComA provides the opportunity for a

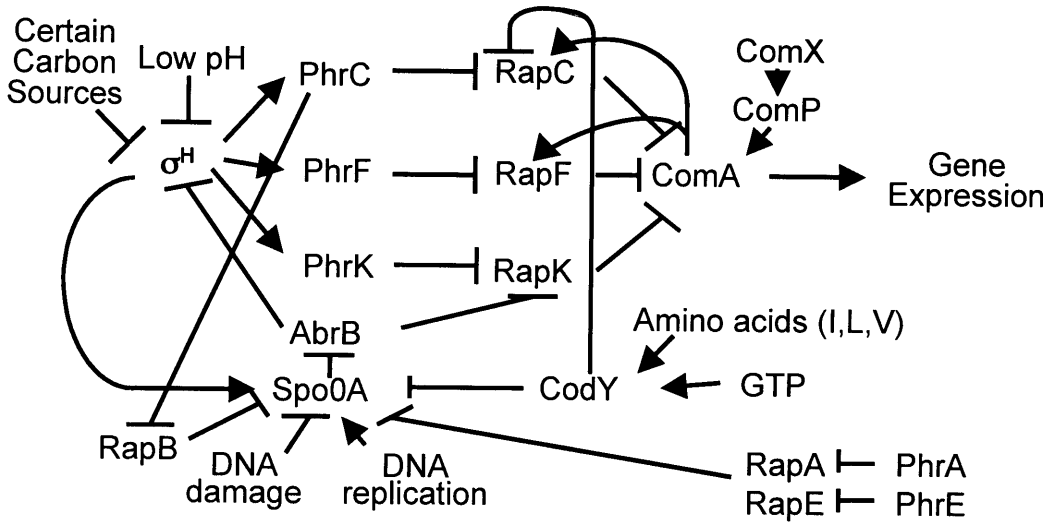

Figure 4: Integration of multiple signals for complex regulation of ComA-dependent gene expression. This network diagram illustrates several of the physiological cues that are known to influence ComA-dependent gene expression. Further information is provided in the text.

variety of physiological signals to modulate the levels of ComA-dependent gene expression (Figure 4). Although ComX production occurs at a consistent rate throughout growth (4), the levels of Rap proteins and Phr peptides are regulated at the level of transcription by proteins that respond to different cellular signals: transcription of *rapC* and *rapF* is activated by ComA (11, 33, 57), which establishes a negative autoregulatory loop for transcription of *rapC* and *rapF*. *rapC* transcription is also repressed by CodY, a protein that is active when cellular pools of branched chain amion acids and GTP are high (reviewed in 73). *rapK* is thought to be activated indirectly by Spo0A (17, 47). Several factors are known to regulate the transcription and activity of Spo0A: CodY represses transcription of Spo0A, RNA polymerase containing σ^H provides additional transcription of Spo0A, high population density signals and ongoing replication promote the activity of Spo0A, and DNA damage inhibits the activity of Spo0A (reviewed in 10, 18). Transcription from σ^H-dependent promoters affect the levels of *phrC*, *phrF*, and *phrK* transcripts (32, 43, 48), in addition to the factors that regulate read-through expression of their upstream *rap*s. σ^H is also regulated at the level of transcription and activity by a variety of physiological signals, including indirect activation of *sigH* transcription by Spo0A, and inhibition of the activity of σ^H by certain carbon sources and low pH (reviewed in 10).

Differential regulation could also occur during production of the active PhrC, PhrF, and PhrK peptides, which is thought to rely upon the secretion machinery and at least one extracellular protease (32, 59, 78), or import of Phr peptides into the cell through the oligopeptide permease. Therefore, it is likely that this regulatory network serves to modulate the levels and timing of the ComA response under a variety of different conditions.

Similarly, the activity of the Spo0A protein, which activates expression of genes involved in sporulation and other post-exponential phase processes, is regulated by multiple Phr peptides

[acting indirectly through Spo0F, (26, 58, 60, 62)]. It is thought that the involvement of multiple Raps and Phrs also allows additional signals to regulate this response, as *rapA* and *rapE* expression is controlled by ComA, while *rapB* is expressed during exponential phase (26, 51, 60).

The involvement of multiple quorum sensing signals in coordinating biological responses is not unique to *B. subtilis*. In the γ-proteobacterium *Pseudomonas aeruginosa*, a complex network involving at least 3 quorum sensing signals modulates expression of several virulence genes (reviewed in 27, 71, 84). In several of the γ-proteobacterial *Vibrio* species, 2-3 quorum sensing signals control specific biological responses, including bioluminescence in *V. harveyi* and *V. fischeri* (24, 39, and references therein) and virulence in *V. harveyi* and *V. cholerae* (24, 45). In both cases, it is thought that the involvement of multiple signals plays a role in fine-tuning the level of responses to specific conditions (37, 46, 71, 84). Furthermore, sequential mechanisms of quorum-sensing activation have also been described in both *V. fischeri* and *P. aeruginosa* (40, 64). In all these systems, the utilization of quorum sensing systems that integrate multiple signals provides the cells the ability to modulate specific biological responses under a variety of conditions.

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Appendix A: Supplementary Table for Chapter 2

Table 3: Effects of *rapF*, *rapH*, *rapJ*, or *rapK* overexpression or Δ*phrC*, Δ*phrF*, or Δ*phrK* mutations on global gene expression[1]

Name[2]	RapF	RapH	RapJ	RapK	PhrC	PhrF	PhrK
Activated directly by ComA[3]							
fapR*plsXfabDfabG*					(-1.4)	-2.0	
*fapR****plsX****fabDfabG*					-1.5	-2.4	
*fapRplsX****fabD****fabG*					-1.5	-2.5	
*fapRplsXfamD****fabG***					(-1.4)	-2.4	
rapA*phrA*				-2.2		-3.0	
*rapA****phrA***				-2.7		-2.0	
Activated directly by ComA and indirectly by Spo0A							
srfAA*srfABcomSsrfACsrfAD*	-1.5			-38.4	-1.7	-13.2	(-1.3)
*srfAA****srfAB****comSsrfACsrfAD*	-1.5			-20.1	-2.2	-21.0	-1.4
*srfAAsrfAB****comS****srfACsrfAD*	(-1.2)			-9.4	-2.3	-20.5	(-1.4)
*srfAAsrfABcomS****srfAC****srfAD*	(-1.2)			-9.1	-2.1	-24.2	(-1.4)
*srfAAsrfABcomSsrfAC****srfAD***	(-1.4)			-15.9	-2.3	-27.7	(-1.5)
pel	-1.8			-33.5	-4.3	-27.4	-2.0
rapE*phrE*						-1.6	
*rapE****phrE***						(-1.2)	
rapF*phrFywhH*	2.8[4]			-10.8	-3.1	-5.7	-2.2
*rapF****phrF****ywhH*	(-1.1)			-2.1	-2.3	-8.9	-1.8
*rapFphrF****ywhH***	(-1.3)			-3.3	-1.5	-2.3	-1.4
rapC*phrC*	-1.7			-5.5	-2.5	-7.1	-1.9
*rapC****phrC***	(-1.2)			(-1.9)	-1.8	-2.7	(-1.6)
Activated indirectly by ComA							
abfA				-3.5			
comC					-3.2	-5.5	-2.1
comEA*comEBcomECcomER*					-2.6	-3.4	-1.8
*comEA****comEB****comECcomER*					-2.3	-2.5	(-1.7)
*comEAcomEB****comEC****comER*					-2.5	-5.7	(-1.6)
*comEAcomEBcomEC****comER***					-1.9	-3.8	(-1.4)
comFA*comFBcomFC*					-3.8	-5.9	-2.1
*comFA****comFB****comFC*					-2.7	-2.9	-1.8
*comFAcomFB****comFC***					-1.9	-2.0	(-1.4)
comGA*comGBcomGCcomGDcomGEcomGFcomGGyqzE*					-8.8	-10.9	-2.4
*comGA****comGB****comGCcomGDcomGEcomGFcomGGyqzE*					-7.5	-9.3	-2.3
*comGAcomGB****comGC****comGDcomGEcomGFcomGGyqzE*					-7.5	-12.4	-2.5
*comGAcomGBcomGC****comGD****comGEcomGFcomGGyqzE*					-7.4	-10.4	-2.4
*comGAcomGBcomGCcomGD****comGE****comGFcomGGyqzE*					-6.1	-9.0	-2.2
*comGAcomGBcomGCcomGDcomGE****comGF****comGGyqzE*					-5.7	-5.9	-2.0
*comGAcomGBcomGCcomGDcomGEcomGF****comGG****yqzE*					-5.5	-8.0	-2.1
*comGAcomGBcomGCcomGDcomGEcomGFcomGG****yqzE***					-5.5	-9.4	-2.1
comK					-1.6	-1.8	-1.6
fabHA*fabF*						(-1.4)	
*fabHA****fabF***						-1.9	

Name	RapF	RapH	RapJ	RapK	PhrC	PhrF	PhrK
Activated indirectly by ComA (cont.)							
fabHB						-2.4	
fabI						-2.5	
nin*nucA*					-4.3	-5.5	-2.2
*nin****nucA***					-2.9	-4.8	-1.8
smf					-2.3	-2.7	-1.8
ybdN					-1.5		
yczC			-2.0				
ydaK*ydaL*				-3.0			
*ydaK****ydaL***				-2.2			
yesO				-4.2			
yhaN				-2.2			
ywpH					-5.6	-11.8	-2.2
yvfH				-2.1			
Activated directly by Spo0A and indirectly by ComA							
yxbC*yxbD*			-3.3	-2.0			
*yxbC****yxbD***			-2.6	(-1.4)			
skfA*skfBskfCskfDskfEskfFskfGskfH*			(-1.6)	(-1.7)	-2.0	-2.1	-2.2
*skfA****skfB****skfCskfDskfEskfFskfGskfH*			-2.6	-2.2	(-1.2)	(-1.1)	(-1.2)
*skfAskfB****skfC****skfDskfEskfFskfGskfH*			-2.4	-2.9	ND	ND	ND
*skfAskfBskfC****skfD****skfEskfFskfGskfH*			(-1.2)	(-1.2)	-1.6	(-1.8)	-2.0
*skfAskfBskfCskfD****skfE****skfFskfGskfH*			-2.2	(-1.8)	-1.9	(-1.6)	-2.0
*skfAskfBskfCskfDskfE****skfF****skfGskfH*			(-1.7)	(-1.6)	(-1.6)	(-1.7)	-1.9
*skfAskfBskfCskfDskfEskfF****skfG****skfH*			-2.1	(-1.4)	ND	ND	ND
*skfAskfBskfCskfDskfEskfFskfG****skfH***			(-1.4)	(-1.3)	(-1.1)	(-1.1)	(-1.2)
Activated indirectly by ComA and Spo0A							
yxbB*yxbAyxnBasnHyxaM*			-4.1	-2.6	(-1.5)		
*yxbB****yxbA****yxnBasnHyxaM*			-3.2	-2.4	(-1.4)		
*yxbByxbA****yxnB****asnHyxaM*			-3.7	-2.8	(-1.6)		
*yxbByxbAyxnB****asnH****yxaM*			-3.5	-2.4	-1.7		
*yxbByxbAyxnBasnH****yxaM***			-2.1	(-1.4)	(-1.2)		
sunA*sunTbdbAyolJbdbB*			-2.5				-2.4
*sunA****sunT****bdbAyolJbdbB*			-1.8				
*sunAsunT****bdbA****yolJbdbB*			-1.7				
*sunAsunTbdbA****yolJ****bdbB*			(-1.6)				
*sunAsunTbdbAyolJ****bdbB***			(-1.4)				
lip			-3.3		-1.9	-1.9	-1.5
yqxM*sipWtasA*					-1.6		
*yqxM****sipW****tasA*					(-1.4)		
*yqxMsipW****tasA***					-1.5		
yfmH*yfmG*			(-1.5)			-1.7	
*yfmH****yfmG***			-1.9			(-1.4)	
yuaB			-1.7		-2.1	-2.2	-1.6
yweA			-2.2				

Name	RapF	RapH	RapJ	RapK	PhrC	PhrF	PhrK
Activated indirectly by ComA and Spo0A (cont.)							
yydF *yydGyydHyydIyydJ*			(-1.6)	(-1)	(-1.7)	(-1.6)	
yydF ***yydG*** *yydHyydIyydJ*			-3.9	-2.2	-1.9	-1.8	
yydFyydG ***yydH*** *yydIyydJ*			-1.8	(-1.8)	(-1.3)	(-1.2)	
yydFyydGyydH ***yydI*** *yydJ*			-1.6	(-1.4)	-1.4	(-1.1)	
yydFyydGyydHyydI ***yydJ***			(-1.5)	(-1.1)	(-1.4)	(-1.3)	
ycxA					-1.5	-2.3	
Activated directly by Spo0A							
spoIIAA *spoIIABsigF*			-1.8				-1.4
spoIIAA ***spoIIAB*** *sigF*			(-1.6)				-1.7
spoIIAAspoIIAB ***sigF***			(-1.5)				-1.5
spoIIGA *sigE*			-1.8				-2.3
spoIIGA ***sigE***			(-1.3)				(-1.2)
yppD							-1.4
Activated indirectly by Spo0A							
sdpA *sdpBsdpC*			-2.2		(-1.6)	(-1.2)	(-1.3)
sdpA ***sdpB*** *sdpC*			-1.8		-2.1	-1.6	-1.7
sdpAsdpB ***sdpC***			(-1.3)		(-1.6)	(-1.2)	(-1.2)
glgB *glgCglgDglgAglgP*							(-1.8)
glgB ***glgC*** *glgDglgAglgP*							-1.7
glgBglgC ***glgD*** *glgAglgP*							(-1.9)
glgBglgCglgD ***glgA*** *glgP*							(-1.6)
glgBglgCglgDglgA ***glgP***							(-1.3)
maa						-1.5	
pksD *pksEacpKpksF*					-1.6		
pksD ***pksE*** *acpKpksF*					-1.5		
pksDpksE ***acpK*** *pksF*					-1.5		
pksDpksEacpK ***pksF***					-1.6		
rapG *phrG*						-2.2	
rapG ***phrG***						-2.1	
rapI *phrI*	1.8				-1.7	-2.6	
rapI ***phrI***	(1.3)				(-1)	(-1.1)	
rapK *phrK*				9.1[4]			(1.3)
rapK ***phrK***				(-1.3)			1.8
rok				-3.6			
ywcI *sacT*		-1.5	-3.4	-2.4			(-2)
ywcI ***sacT***		(-1.2)	-2.5	-1.9			-1.7
sigG							-1.7
spoIIQ							-1.5
spoIIIAA							-1.6
ydgC *ydgD*			-2.2				
ydgC ***ydgD***			(-1.4)				

Name	RapF	RapH	RapJ	RapK	PhrC	PhrF	PhrK
Activated indirectly by Spo0A (cont.)							
ygaB							-1.6
yjbX					-1.5		-1.4
yknWyknYyknY			-1.7				
yknW***yknY***yknY			(-1.4)				
yknWyknY***yknY***			(-1.4)				
ykuP		-1.3					
yneE							-1.6
yobB					-1.6	-1.8	
yobW						-1.7	
yqzG						-2.2	
yybN					-1.5		
Activated indirectly by Spo0A and activated by DegU							
dhbAdhbCdhbEdhbBdhbF		(-1.1)					
dhbA***dhbC***dhbEdhbBdhbF		-1.3					
dhbAdhbC***dhbE***dhbBdhbF		(-1.3)					
dhbAdhbCdhbE***dhbB***dhbF		(-1.1)					
dhbAdhbCdhbEdhbB***dhbF***		-1.7					
ycdA				-2.4	-2.3	-3.8	-1.6
yitM					-1.7	-1.9	-1.5
yqxIyqxJ					-1.4		
yqxI***yqxJ***					(-1.4)		
ywfBywfCywfDywfEywfG			(-1.7)				
ywfB***ywfC***ywfDywfEywfG			-1.8				
ywfBywfC***ywfD***ywfEywfG			-1.7				
ywfBywfCywfD***ywfE***ywfG			-1.6				
ywfBywfCywfDywfE***ywfG***			-1.7				
ywqIywqJywqKywqL					(-1.4)	-1.8	
ywqI***ywqJ***ywqKywqL					-1.7	-2.5	
ywqIywqJ***ywqK***ywqL					(-1.2)	(-1.4)	
ywqIywqJywqK***ywqL***					-1.6	-1.7	
pksGpksHpksIpksJpksLpksMpksNpksR					-1.5		
pksG***pksH***pksIpksJpksLpksMpksNpksR					-1.4		
pksGpksH***pksI***pksJpksLpksMpksNpksR					-1.5		
pksGpksHpksI***pksJ***pksLpksMpksNpksR					-1.5		
pksGpksHpksIpksJ***pksL***pksMpksNpksR					-1.7		
pksGpksHpksIpksJpksL***pksM***pksNpksR					-1.6		
pksGpksHpksIpksJpksLpksM***pksN***pksR					-1.4		
pksGpksHpksIpksJpksLpksMpksN***pksR***					(-1.5)		
yfjAyfjByfjCyfjDyfjEyfjF					(-1.6)	(-1.8)	
yfjA***yfjB***yfjCyfjDyfjEyfjF					(-1.7)	(-1.7)	
yfjAyfjB***yfjC***yfjDyfjEyfjF					-1.9	-2.0	
yfjAyfjByfjC***yfjD***yfjEyfjF					(-1.3)	(-1.6)	
yfjAyfjByfjCyfjD***yfjE***yfjF					(-1.6)	-1.9	
yfjAyfjByfjCyfjDyfjE***yfjF***					(-1.1)	(-1.2)	

Name	RapF	RapH	RapJ	RapK	PhrC	PhrF	PhrK
Activated indirectly by Spo0A and activated by DegU							
yukE*yukDyukCyukByukAyueByueCyueD*					-1.9	-2.0	
*yukE****yukD****yukCyukByukAyueByueCyueD*					-1.6	-2.0	
*yukEyukD****yukC****yukByukAyueByueCyueD*					(-1.2)	-1.7	
*yukEyukDyukC****yukB****yukAyueByueCyueD*					(1.1)	(-1.1)	
*yukEyukDyukCyukB****yukA****yueByueCyueD*					(-1.4)	-1.8	
*yukEyukDyukCyukByukA****yueB****yueCyueD*					(-1.3)	(-1.6)	
*yukEyukDyukCyukByukAyueB****yueC****yueD*					(-1.1)	(-1.3)	
*yukEyukDyukCyukByukAyueByueC****yueD***					(-1.1)	(-1.3)	
Activated by YclJ and YkoG							
yngA				-2.0			
Repressed by YbdG							
ybdK*ybdL*					-1.5	(-1.2)	
*ybdK****ybdL***					-1.5	-1.8	
Not regulated by response regulator/decreased expression in *rap* overexpressing or *Δphr* cells							
acoB						-2	
albB				-3			
aroE					-1.6	-1.8	
comX					-1.6	-3.0	
cotF						-1.6	
cotG						-1.8	
cotV*cotWcotX*						-2.2	
*cotV****cotW****cotX*						-1.9	
*cotVcotW****cotX***						-1.7	
cydB						-1.6	
ebrA*ebrB*			-1.7				
*ebrA****ebrB***			(-1.5)				
feuC		-1.3					
fnr				-2.0			
glcR					-1.4		
hipO			-1.9				
hxlR					-1.6	-1.9	
mleA						-1.5	
mntB					-1.4		
nasD					-1.9		
nhaC						-1.6	
nucB					-1.4	-1.6	
spo0B*obg*							
*spo0B****obg***		-1.3				-1.5	

Name	RapF	RapH	RapJ	RapK	PhrC	PhrF	PhrK
Not regulated by response regulator/decreased expression in *rap* overexpressing or Δ*phr* cells (cont.)							
pckA				-3.8			
pelB					-2.8	-10.7	
pgcM						-1.7	
phy			-1.8		-1.7	-2.8	
qcrA*qcrBqcrC*			(-1.6)		-1.4		
*qcrA****qcrB****qcrC*			-1.7		(-1.4)		
*qcrAqcrB****qcrC***			-2.4		(-1.4)		
radC						-1.8	
rapD					-1.6	-2.3	
rapH		16*			-2.4	-3.1	-1.4
rplC		-1.3					
sacC					-1.6	-1.8	
sacX						-2.1	
sboX					-1.7		
xkdP						-2.2	
xtrA			-1.6				
ybeF				-2.0			
ycbL							-1.7
yclD			-1.8				
ydaP				-1.8			
ydgB*ydgAcotP*				(-1.7)			
*ydgB****ydgA****cotP*				-1.8			
*ydgBydgA****cotP***				ND			
yeeF						-1.6	
yesE						-1.7	
yflD*yflCyflB*			-1.5				
*yflD****yflC****yflB*			-1.6				
*yflDyflC****yflB***			(-1.6)				
yfmC	-1.5	-1.4					-1.5
yfmD						-1.7	
yfnC				-1.8			
yhcD				-2			
yhcQ							
yhdC				-2.9			

Name	RapF	RapH	RapJ	RapK	PhrC	PhrF	PhrK
Not regulated by response regulator/decreased expression in *rap* overexpressing or Δ*phr* cells (cont.)							
yjbF					-1.5		
yjfA							-1.4
ykpC			-1.7				
yndE					-1.7	-2.1	-1.5
yoqB			-1.5				
yorD			-2.0				
yorM			-1.6				
yqaJ					-1.4	-1.8	
yqiX						-2.9	
yrzE				-2.3			
yrzH			-1.5				
yvaW			-2.2				
yvaX			-1.8		-2.1	-1.6	-1.7
yvcA*yvcByvzAyvcS*					(-1.1)	(-1.3)	(-1.1)
*yvcA****yvcB****yvzAyvcS*					(-1.3)	-1.9	(-1.3)
*yvcAyvcB****yvzA****yvcS*					-1.5	-1.7	-1.3
*yvcAyvcByvzA****yvcS***				-2.8			
yvgL					-1.5		
yvqJ					-1.5		
yyaF						-2.0	-2.3
yvrP*yvrOyvrN*			(-1.6)		-1.6	-1.7	
*yvrP****yvrO****yvrN*			-1.6		-1.5	-1.6	
*yvrPyvrO****yvrN***			-1.7		-1.4	(-1.5)	
yxiB*yxiCyxiDyxxDyxxE*					(-1.3)	(1)	
*yxiB****yxiC****yxiDyxxDyxxE*					-1.5	-1.7	
*yxiByxiC****yxiD****yxxDyxxE*					-1.7	-2.0	
*yxiByxiCyxiD****yxxD****yxxE*					(-1.2)	(-1.2)	
*yxiByxiCyxiDyxxD****yxxE***					(1)	(-1.1)	
Repressed directly by Spo0A							
abrB			3.9	3.1			
med			2.0	2.0			
yuxH			1.9	1.8			

Name	RapF	RapH	RapJ	RapK	PhrC	PhrF	PhrK
Repressed indirectly by Spo0A							
pstS*pstCpstApstBApstBB*					(2.3)		
*pstS****pstC****pstApstBApstBB*					2.1		
*pstSpstC****pstA****pstBApstBB*					(1.5)		
*pstSpstCpstA****pstBA****pstBB*					2.6		
*pstSpstCpstApstBA****pstBB***					2.1		
rbsR				1.8			
ywsB					1.9	2.0	
Repressed indirectly by Spo0A and repressed by DegU							
fliJ	1.5						
yvzB			1.6				
Repressed by DegU							
ywtD						1.5	
Not regulated by response regulator/increased expression in *rap* overexpressing or Δ*phr* cells							
argB							2.1
argF				2.2			
coxA			1.5				
fbaB				1.7			
feuA						1.6	
gsiB	1.6				1.5		
hisB			1.6				
immR*ydcMint*		(1.4)					
*immR****ydcM****int*		1.7					
*immRydcM****int***		(1.4)					
prkA							
pyrAA				2.2			
rapJ			14*				
ybyB					2.2		
yddM		1.7					
yeaA*ydjPydjO*					1.6		
*yeaA****ydjP****ydjO*					1.6		
*yeaAydjP****ydjO***					ND		
yfiB		1.5					
yjmF							1.5

Name	RapF	RapH	RapJ	RapK	PhrC	PhrF	PhrK
Not regulated by response regulator/increased expression in *rap* overexpressing or Δ*phr* cells							
yndN					2.2		
yoaF		1.7					
yopO		1.5					
yoqL	1.5						
ypbG		1.5					
yqaM		1.4					
yqjD		1.5					
yrdB			1.5				
yuaG					1.9		
yvqH					2.1		

[1] The average fold changes in mRNA levels for genes whose expression changed significantly are reported. The average fold changes in expression for those genes whose expression did not change significantly, but were in operons with genes whose expression changed significantly are reported in parentheses.

[2] Genes are shown in the context of other genes in known or putative operons. The gene whose expression change is reported is in bold-face type and is underlined.

[3] Categories were determined as described in the text and Figure 1.

[4] Indicates changes in mRNA levels of specific *rap* that is altered in the experiment. For *rap* overexpression, *rap* transcripts expressed from P*spank(hy)* promoter cannot be distinguished from transcripts expressed from the native copy of the gene.

Chapter 3: Regulation of a *Bacillus subtilis* mobile genetic element by intercellular signaling and the global DNA damage response

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Abstract

Horizontal gene transfer contributes to the evolution of bacterial species. Mobile genetic elements play an important role in horizontal gene transfer, and characterization of the regulation of these elements should provide insight into conditions that influence bacterial evolution. We characterized a mobile genetic element, ICE*Bs1*, in the gram-positive bacterium *Bacillus subtilis*, and found that it is a functional integrative and conjugative element (ICE) capable of transferring to *Bacillus* and *Listeria* species. We identified two conditions that promote ICE*Bs1* transfer: conditions that induce the global DNA damage response and crowding by potential recipients that lack ICE*Bs1*. Transfer of ICE*Bs1* into cells that already contain the element is inhibited by an intercellular signaling peptide encoded by ICE*Bs1*. The dual regulation of ICE*Bs1* allows for passive propagation in the host cell until either potential mating partners lacking ICE*Bs1* are present or the host cell is in distress.

Introduction

Horizontal gene transfer and mobile genetic elements play a significant role in bacterial evolution (14, 16, 32, 50). Conjugative transposons (CTns) (11, 51), also known as integrative and conjugative elements (ICEs) (5, 50), are mobile genetic elements that are normally integrated into the chromosome. They can excise and transfer to recipients through conjugation (mating) and integrate into the chromosome of the recipient (11, 51). ICEs encode proteins required for conjugal transfer, and can also encode proteins involved in resistance to antibiotics (11, 51), metabolism of alternative carbon sources (31, 50), symbiosis (49), and other processes (7). ICEs and putative ICEs have been found in many bacteria (7) and are important agents of horizontal gene transfer because they are capable of moving themselves and other DNA to recipients (2, 41, 46, 51).

Mechanisms that regulate transfer have been determined for several ICEs. In some cases, an antibiotic induces transfer of an element that encodes resistance to that antibiotic (3, 11, 51). Transfer of the *Streptomyces* ICE pSAM2 is inhibited by the presence of a pSAM2-encoded protein in the recipient (40). Recently, it was shown that the DNA damage response stimulates transfer of SXT, an ICE from *Vibrio cholerae* (3).

We characterized a 20-kb ICE, ICE*Bs1* (6), found in *Bacillus subtilis* and found that ICE*Bs1* excision and transfer is regulated by a secreted peptide encoded by ICE*Bs1*.

Many Gram-positive bacteria use secreted signaling peptides to coordinate physiological processes with population density, often called quorum sensing (48). In *B. subtilis*, several secreted peptides contribute to quorum sensing, including Phr peptides encoded by *phr* genes (reviewed in 23). It has been suggested that Phr peptides act as autocrine signals and not in cell-

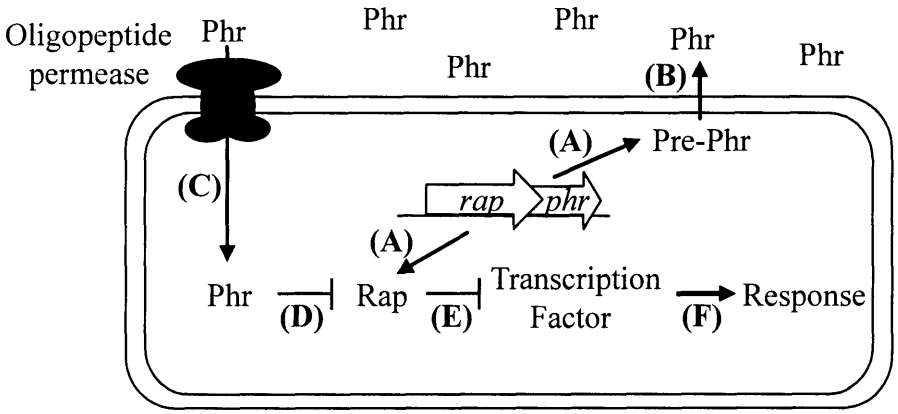

Fig. 1. Phr peptide signaling in *B. subtilis*. *rap* and *phr* genes are transcribed and translated (**A**); pre-Phr peptides are secreted and processed (**B**); mature Phr peptides are transported into the cell by the oligopeptide permease (**C**); once inside the cell, Phr peptides inhibit the activities of regulators known as Rap proteins (**D**); each characterized Rap protein inhibits the activity of a transcription factor, either directly or indirectly (**E**); and inhibition of transcription factors lead to cellular responses (**F**).

cell signaling (reviewed in 35), although this is clearly not true for all Phr peptides (24, 47). Nonetheless, all characterized Phr peptides have a common mechanism of action. Following secretion and extracellular accumulation, Phr pentapeptides are imported through the oligopeptide permease (Opp); once inside the cell, Phr peptides directly inhibit the activities of intracellular regulators, known as Rap proteins (20, 24, 33, 37, 47) (Fig. 1). The characterized Rap proteins directly (13, 33) or indirectly (20, 36) inhibit the activities of transcription factors that regulate sporulation, competence development, and production of degradative enzymes and antibiotics (20, 33, 37, 47).

RapI and PhrI are encoded by ICE*Bs1*. We found that RapI activates ICE*Bs1* gene expression, excision, and transfer, and that the PhrI peptide antagonizes the activity of RapI. Furthermore, expression of *rapI* and *phrI* is stimulated by conditions of low nutrient availability and high cell density. This combined regulation activates ICE*Bs1* excision and transfer when host cells are crowded by potential recipients that lack ICE*Bs1* and do not produce the PhrI peptide.

In addition, we observed that the global DNA damage (SOS) response activates ICE*Bs1* excision and transfer, independently of *rapI* and *phrI*. Therefore, at least two conditions promote ICE*Bs1* excision and transfer: the presence of a high concentration of cells lacking ICE*Bs1* and host cell distress. In the absence of these conditions, ICE*Bs1* is propagated by the host through vertical gene transfer to progeny cells.

Materials and Methods

Media. Cells were grown at 37° C with agitation in LB medium (45), defined minimal medium (43) (supplemented with required amino acids when necessary), Schaeffer's nutrient

broth sporulation medium (18), or Brain Heart Infusion medium (18) as indicated. Antibiotics and other chemicals were used at the following concentrations: ampicillin (100 μg/ml), chloramphenicol (5 μg/ml), kanamycin (5 μg/ml), spectinomycin (100 μg/ml), streptomycin (100 μg/ml), erythromycin (0.5 μg/ml) and lincomycin (12.5 μg/ml) together to select for macrolide-lincosamide-streptogramin B (MLS) resistance, Isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma) (1 mM), and mitomycin C (MMC, Sigma) (1 μg/ml).

Strains and alleles. Strains used in this study are listed in Table 3 (Appendix B). The *Escherichia coli* strain used for cloning is an MC1061 derivative carrying F'(*lacI*^{q}) *lacZM15* Tn*10* (*tet*). Standard techniques were used for cloning and strain construction (18, 45).

For overexpression in *B. subtilis, rapI, phrI*, and *rapI phrI* were cloned downstream of the IPTG-inducible promoters P*spank*(*hy*) (4) or P*spank* (43), both generous gifts from D. Rudner (Harvard Medical School, Boston), and integrated into the *amyE* locus by homologous recombination. P*spank* and P*spank*(*hy*) (with no inserts) were also integrated into *amyE.*

The *rapI-lacZ* promoter fusion was generated by cloning the DNA from 329 to 12 bp upstream of the *rapI* ORF upstream of the promoter-less *lacZ* in the vector pDG793 (17), followed by integration into the *thrC* locus by homologous recombination.

Isolation of spontaneous streptomycin-resistant mutants and construction of the following alleles is described in *Supporting Methods* (Appendix B): ICE*Bs1*::*kan*, an insertion of a kanamycin resistance gene between the 3' end of *yddM* and attachment (*att*) site *attR*; ICE*Bs1*^{0}, a complete loss of ICE*Bs1* that leaves the chromosomal *att* site intact; and Δ(ICE*Bs1*)*206*::*cat*, a deletion of the entire ICE, including *attR*, and insertion of a chloramphenicol resistance gene. Null mutations included Δ(*rapI phrI*)*342*::*kan*, Δ*int205*::*cat*, and Δ*immR208*::*cat*. S. Branda and R. Kolter generously provided Δ*phrI173*::*erm* and Δ(*rapI phrI*)*260*::*erm*.

comK::*spc* and *comK*::*cat* (29), Δ*abrB*::*cat* (38), *recA260* (10, 25), and *opp*::*cat* [*opp*::Tn*917lac*::pTV21Δ2*cat* (*opp* = *spo0K*)] (44), were described previously.

DNA Microarrays. Cells were harvested, and total RNA was prepared as described (4). RNA from each sample was reverse transcribed and labeled with Cy3 or Cy5. Labeled samples were combined and purified with Qiagen PCR purification columns and hybridized to microarrays containing PCR products of virtually all the *B. subtilis* ORFs (4). Similar hybridization experiments were performed using microarrays containing a unique DNA oligonucleotide for each *B. subtilis* open reading frame. Additional details are described in *Supporting Methods*.

Arrays were scanned and analyzed with the program GENEPIX 3.0 (Axon Instruments, Union City, CA). Cy3 and Cy5 signals for each spot were normalized to the total Cy3 and Cy5 signals of the array and were obtained for each spot that had a signal above background for 50% of pixels. Iterative outlier analysis (4, 28) was used to identify spots (genes) whose experimental mean ratio was >2.5 SDs away from the mean ratio of the population of genes in the third iteration of the calculation (outlier cutoff). The probability that the mean ratios of these outliers were greater than the outlier cutoff was calculated using the normal distribution function for each spot; those genes with ≥95% probability were considered significantly changed. The mean ratio for a set of triplicate experiments is reported.

Excision assays. DNA was extracted using Qiagen's DNEasy tissue Kit (protocol for Gram-positive bacteria with RNase A treatment). PCR with the primer pair oJMA93 and oJMA100 detected the chromosomal junction formed after ICE*Bs1* excision. PCR with the primer pair oJMA95 and oJMA97 detected the excised ICE*Bs1* circle. Primer sequences, PCR conditions, and cycling parameters are described in *Supporting Methods*. Products were visualized on 2%

agarose gels stained with ethidium bromide. PCR was performed on at least two independent, biological replicates. Representative results are shown.

For linear-range (quantitative) PCR, known concentrations of DNA were diluted serially, and regions were amplified using the indicated primer pairs. Products were visualized on 2% agarose gels stained with ethidium bromide and quantified using the ChemiImager gel documentation system (Alpha Innotech, San Leandro, CA). Reactions were deemed in the linear range when three 2-fold serial dilutions of input DNA produced linearly decreasing amounts of PCR product.

The relative increase in excision is reported for circular intermediate PCR products. Fold-increase was determined by calculating the amount of PCR product of the ICE*Bs1* circle in each experimental sample, compared to the amount of ICE*Bs1* circle PCR product from the control sample for each experiment. These fold-increases were normalized to the amount of PCR product from *cotF* for each sample. *cotF*, a chromosomal site unaffected by ICE*Bs1* excision, was amplified with primers oLIN93 and oLIN94 (27). The fold-increase is reported as the mean (± SEM) from at least two independent experiments.

In experiments with mixed cultures, an additional normalization was done to take into account only the cells capable of excision of ICE*Bs1*. PCR was also done with the primer pair oJMA177 and oJMA178 that amplifies DNA [*amyE*::P*spank*(*hy*)] unique to the population of cells capable of excision. The amount of this product in the experimental sample was compared to the amount of this product in the control to determine the number of cells in the experimental sample capable of excision. All cells in the control were capable of excision and contained *amyE*::P*spank*(*hy*).

Mating experiments. Donors and recipients were grown in LB (for matings with *Bacillus*) or brain heart infusion (BHI) medium (for matings with *Listeria*) when assaying transfer from

cells overexpressing *rapI* or in defined minimal medium when assaying transfer from cells treated with MMC. ICE*Bs1* excision in donor cells was induced either by overexpression of *rapI* [P*spank*(*hy*)-*rapI* Δ(*rapI phrI*), strain JMA168] or by addition of MMC [Δ(*rapI phrI*), strain IRN342]. At 1 hr after induction, equal volumes of donor and recipient cultures were mixed and filtered onto a sterile nitrocellulose filter (0.2 μM pore size, Nalgene), placed on LB or BHI agar plates, and incubated at 37° for ~3 hrs. Cells were removed from filters by washing with 5 ml of Spizizen minimal salts (18). Transconjugants were isolated by selecting for antibiotic resistance unique to the recipient and the kanamycin resistance in ICE*Bs1*. Donor and recipient numbers were also determined by selective plating. Concentrated, unmixed donor and recipient cultures spread on the double antibiotic agar did not give rise to spontaneous antibiotic-resistant mutants. Transfer of DNA to the donor through transformation was not observed.

Mating frequencies were calculated by dividing the number of transconjugants by the number of donor cells, except in the case of donor cells treated with MMC, where mating frequencies were calculated relative to recipients. The reported transfer frequencies are the mean (± SEM) of at least two independent biological replicates.

β-galactosidase assays. β-galactosidase specific activity of a *rapI-lacZ* fusion was assayed throughout growth of wild-type and Δ*abrB* cultures in sporulation media as described (19).

Results and Discussion

Identification of a Mobile Genetic Element Regulated by Peptide Signaling. *B. subtilis* encodes seven *phr* genes (22), each located in an operon with a *rap* gene. To identify biological

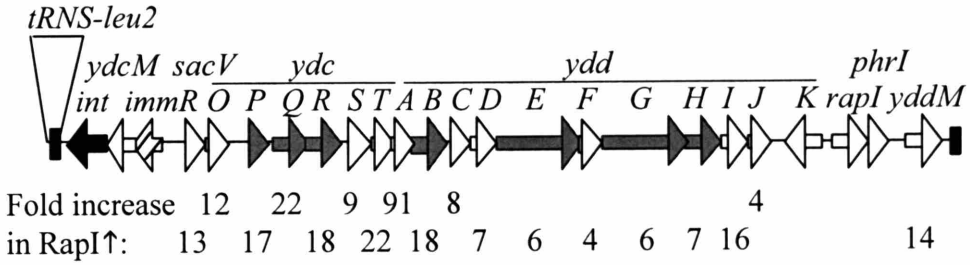

Fig. 2. Overexpression of *rapI* activates expression of genes in ICE*Bs1*.

The diagram shows the organization of ICE*Bs1*, which contains at least 24 ORFs. The name of each gene is indicated above its respective arrow. Black boxes at the left and right ends indicate the *att* sites, *attL* and *attR*. *attL* of ICE*Bs1* is in the 3' end of a leucyl-tRNA gene (*trnS-leu2*). The black arrow indicates *int*, encoding the putative integrase. The hatched arrow indicates *immR*, encoding the putative immunity repressor. Gray arrows indicate genes similar to genes found in other ICE's (6). The numbers below the cartoon of ICE*Bs1* indicate the mean fold-increase in mRNA levels in cells overexpressing *rapI*. P*spank*(*hy*)-*rapI* (JMA28) cells were grown for at least 4 generations to mid-exponential phase in minimal medium. IPTG was added to half of the cultures to induce *rapI* expression. Samples were collected 30 min. later from induced and uninduced cultures. RNA was isolated, labeled, and hybridized, and genes that changed significantly upon overproduction of RapI were identified as described in Materials and Methods. Expression of the three genes at the left end did not change significantly, nor did the expression of almost all chromosomal genes. Experimental details and additional microarray results are in Table 4 and *Supporting Text* (Appendix B).

processes regulated by the uncharacterized *rapI-phrI* operon, we used whole-genome DNA microarrays to monitor changes in mRNA levels caused by overexpression of *rapI* from an IPTG-inducible promoter [P*spank*(*hy*)-*rapI*].

In two types of microarray experiments, overproduction of RapI caused mRNA levels of 18 genes to increase ≥4-fold (Fig. 2; Table 4, Appendix B). All 18 genes cluster around *rapI* and *phrI*, and are in the 20-kb ICE*Bs1* element (Fig. 2) previously identified by comparative sequence analysis (6). ICE*Bs1* is flanked by 60 bp direct repeats, the likely *att* sites. One of the potential *att* sites is in the 3' end of a tRNA gene, a common integration site for mobile elements (8). ICE*Bs1* contains *int* (previously *ydcL*) (6), encoding a putative λ-like integrase, *immR* (previously *ydcN*), encoding a putative bacteriophage-like immunity repressor with 50% amino acid similarity to the repressor of *B. subtilis* phage ϕ105 (1, 15), and seven genes similar to genes from other ICE's (6). Our results demonstrate that RapI activates ICE*Bs1* gene expression. This activation is most likely by directly or indirectly inhibiting the activity of the putative immunity repressor, ImmR (Chapter 4). Furthermore, activation of ICE*Bs1* gene expression is specific to overexpression of *rapI*, as overproduction of other *B. subtilis* Rap proteins did not stimulate ICE*Bs1* gene expression (Chapter 2 and Appendix A).

ICE*Bs1* excises and transfers. Before conjugal transfer, an ICE excises from the chromosome, forming a circular intermediate and a repaired chromosomal junction (11). We used a PCR-based assay to detect products formed upon ICE*Bs1* excision (Fig. 3A). We detected a low level of circular ICE*Bs1* intermediates and repaired chromosomal junctions in control cells of *B. subtilis* (Fig. 3B), indicating that excision occurs at a low level in this population of cells. Overexpression of *rapI* greatly stimulated ICE*Bs1* excision (Fig. 3B). Because expression of the putative Integrase is not activated by *rapI* overexpression, RapI likely stimulates excision by

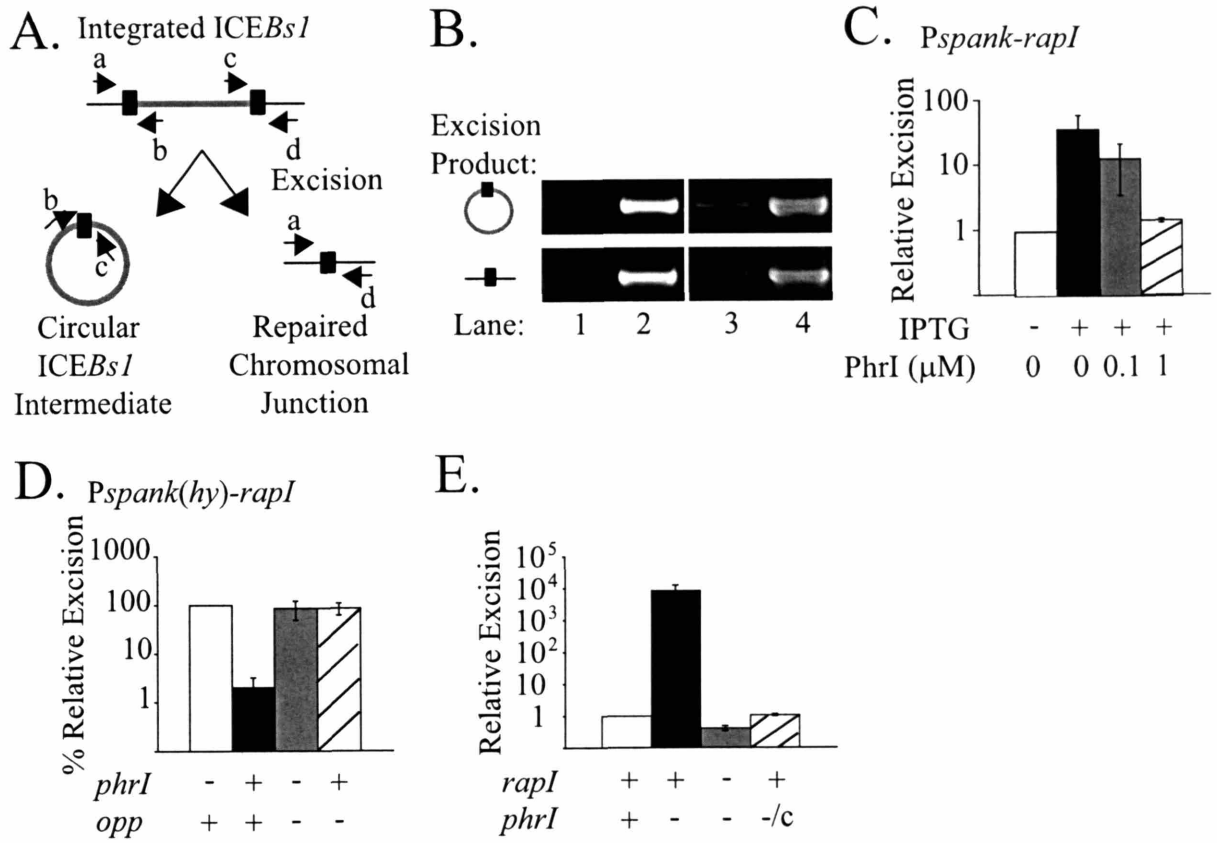

Fig. 3. Excision of ICE*Bs1*.

A. PCR assay for determining excision of ICE*Bs1*. Primers **a** and **d** (oJMA93 and oJMA100) anneal to sequences surrounding ICE*Bs1* and amplify the repaired chromosomal junction formed upon excision. Primers **b** and **c** (oJMA95 and oJMA97) anneal to sequences inside ICE*Bs1* and amplify the circular intermediate generated upon excision.

B. Overproduction of RapI and treatment with MMC induce ICE*Bs1* excision. Cells were grown to mid-exponential phase in minimal medium. Samples were collected 1 hr after treatment with IPTG (to induce *rapI* overexpression) or MMC (to cause DNA damage and induce the SOS response). 100 ng of template DNA was used to amplify the indicated products. Shown are: lane 1, control cells [P*spank*(*hy*), JMA35]; lane 2, P*spank*(*hy*)-*rapI* (JMA28); lane 3, wild type cells

(JH642), untreated; and lane 4, wild type cells treated with MMC. Induction of ICE*Bs1* excision by MMC was *recA*-dependent (data not shown).

C. PhrI pentapeptide inhibits ICE*Bs1* excision. Cells [P*spank-rapI* Δ(*rapI phrI*); JMA342] were grown to mid-exponential phase in minimal medium. Where indicated, the synthetic PhrI pentapeptide (DRVGA) in potassium phosphate buffer pH 7 (Genemed Synthesis, South San Francisco, CA) was added to cultures at 100 nM and 1 μM. Buffer was added to the control cultures; all cultures had a final buffer concentration of 1 mM. Ten minutes later, IPTG was added to induce RapI overproduction. Samples were collected 1 hr after IPTG addition, and linear-range PCR was performed as described (Materials and Methods). P*spank-rapI* [rather than P*spank*(*hy*)-*rapI*] was used because transcription from P*spank* is better repressed in the absence of inducer. Open bar, uninduced cells, defined as 1; black bar, overproduction of RapI; shaded bar, overproduction of RapI, in 100 nM PhrI pentapeptide; hatched bar, overproduction of RapI, in 1 μM PhrI pentapeptide.

D. Opp is required for *phrI* to inhibit excision. Cells were grown to mid-exponential phase in minimal medium. Samples were collected 1 hr after addition of IPTG and analyzed by linear-range PCR. Open bar, overexpression of *rapI* alone [P*spank*(*hy*)-*rapI* Δ(*rapI phrI*), JMA168], defined as 100%; black bar, overexpression of *rapI* and *phrI* [P*spank*(*hy*)-(*rapI phrI*) Δ(*rapI phrI*), JMA186]; shaded bar, overexpression of *rapI* in an oligopeptide permease null mutant [Pspank(*hy*)-*rapI* Δ(*rapI phrI*) Δ*opp*, CAL51]; hatched bar, overexpression of *rapI* and *phrI* in an oligopeptide permease null mutant [P*spank*(*hy*)-(*rapI phrI*) Δ(*rapI phrI*) Δ*opp,* CAL52].

E. Excision of ICE*Bs1* increases in a *phrI* null mutant. Cells were grown in nutrient broth sporulation medium. Samples were collected from cells ~2 hours after the entry into stationary phase, and relative excision of ICE*Bs1* was determined by linear range PCR. Open bar, wild-type (NCIB3610), defined as 1; black bar, Δ*phrI* (SSB173); shaded bar, Δ(*rapI phrI*) (SSB260); hatched bar, Δ*phrI* P*spank(hy)-phrI* (JMA298). -/c indicates complementation of Δ*phrI* mutation.

activating expression of an accessory protein required for excision. Many integrase proteins require accessory proteins for excision (26).

Table 1. Frequency of ICE*Bs1* mating into recipients.

Recipient	Mating Frequency*
B. subtilis ICE*Bs1*^{0} (CAL89)	$1 \times 10^{-2} \pm 3 \times 10^{-3}$
B. subtilis ICE*Bs1*$^+$ (CAL88)	$2 \times 10^{-4} \pm 1 \times 10^{-4}$
B. anthracis (UM44-1C9)	$6 \times 10^{-3} \pm 5 \times 10^{-3}$
B. licheniformis (REM42)	$2 \times 10^{-4} \pm 5 \times 10^{-6}$
L. monocytogenes (10403S)	$8 \times 10^{-6} \pm 6 \times 10^{-6}$

*Mating was assayed 1 hr after induction of *rapI* overexpression from donor cells (P*spank(hy)*-*rapI* Δ(*rapI phrI*)::*kan*, JMA168). Mating frequency is the number of transconjugants per donor (± SEM).

rapI overexpression also stimulated ICE*Bs1* transfer to recipients. To assay transfer from donor cells, we replaced *rapI* and *phrI* with an antibiotic-resistance marker. Deletion of *rapI* and *phrI* had minimal effects on excision of ICE*Bs1* in wild-type cells (Fig. 3E) and in cells overexpressing *rapI* (data not shown). We assayed transfer of ICE*Bs1* on a solid surface (filter mating) by mixing donor cells [P*spank(hy)*-*rapI* Δ(*rapI phrI*)::*kan*], in which *rapI* overexpression had been induced for 1 hr, with an equal number of recipient *B. subtilis* cells that lacked ICE*Bs1* (ICE*Bs1*^{0}).

ICE*Bs1* transferred at an average frequency of ~1×10^{-2} transconjugants (recipients that received ICE*Bs1*) per donor (Table 1). Transfer into recipients that contained ICE*Bs1* occurred with ~50-fold lower frequency (Table 1), indicating that ICE*Bs1* encodes at least one mechanism that inhibits acquisition of a second element. Acquisition of ICE*Bs1* by recipients was not due to natural transformation, as the recipients were *comK* mutants incapable of transformation (29). Transfer of ICE*Bs1* from non-activated donor cells [Δ(*rapI phrI*)::*kan*, IRN342] was not detected under these conditions ($<2 \times 10^{-8}$ transconjugants per donor).

Transfer of ICE*Bs1* into *Bacillus* and *Listeria* recipients. The putative bacterial chromosomal *att* site of ICE*Bs1* is conserved (≥52/60 base pairs identical) in *Bacillus*, *Listeria*, and *Staphylococcus* species (Fig. 5, Appendix B). We assayed transfer of ICE*Bs1* from *B. subtilis* donor cells overexpressing *rapI* into *B. anthracis*, *B. licheniformis*, and *L. monocytogenes* and found that ICE*Bs1* mated into all three species (Table 1). The efficient transfer of ICE*Bs1* into *Bacillus* and *Listeria* species, and potentially *Staphylococcus* species (not tested), indicates that ICE*Bs1* may be a useful tool to facilitate genetic manipulation of these organisms.

Inhibition of ICE*Bs1* excision by the PhrI peptide. As the activities of the characterized Rap proteins are inhibited by their cognate Phr peptides and *rapI* overexpression activates ICE*Bs1* excision and transfer, we investigated whether PhrI peptide signaling inhibits ICE*Bs1* excision and transfer. Excision of ICE*Bs1* in cells overexpressing *rapI* was inhibited by addition of synthetic PhrI peptide (Fig. 3C). The active PhrI peptide, the five C-terminal amino acids of the 38-aa precursor protein, was predicted based on its similarity to characterized Phr peptides (23, 35). The addition of 1 μM synthetic PhrI peptide inhibited RapI-dependent excision of ICE*Bs1* ~20-fold, and addition of 100 nM PhrI peptide inhibited excision ~3-fold (Fig. 3C). These concentrations of peptide are similar to the biologically active concentrations of other Phr peptides (20, 24, 34). These results demonstrate that the PhrI pentapeptide inhibits RapI-dependent activation of ICE*Bs1* excision.

Excision in ICE*Bs1* cells overexpressing *rapI* was inhibited ~50-fold by co-overexpression of *phrI* (Fig. 3D). This depended on the presence of Opp, a transporter required for uptake of Phr peptides (20, 37, 47). Excision occurred at similar levels in *opp*$^-$ cells co-overexpressing *rapI* and *phrI* and in *opp*$^+$ cells overexpressing *rapI* alone (Fig. 3D). These data provide further evidence

that the secreted PhrI peptide is imported through Opp and inhibits RapI-dependent activation of ICE*Bs1* excision.

PhrI also inhibits ICE*Bs1* excision when *rapI* is expressed from its native promoter. Deletion of the gene encoding PhrI (Δ*phrI*), in otherwise wild-type cells, activated ICE*Bs1* excision >5,000-fold, relative to wild-type cells (Fig. 3E). This required RapI; excision in Δ(*rapI phrI*) cells was similar to wild-type (Fig. 3E). Ectopic expression of *phrI* complemented the Δ*phrI* phenotype, reducing ICE*Bs1* excision back to a low level (Fig. 3E), indicating that increased excision in the Δ*phrI* mutant was due to loss of *phrI* and not due to effects on neighboring genes.

Regulation of ICE*Bs1* excision and transfer by intercellular signaling. The preceding results indicated that PhrI peptide signaling inhibited ICE*Bs1* excision but did not indicate whether the PhrI peptide acts as an intercellular signaling peptide. If the PhrI peptide acts as an intercellular signaling peptide, then RapI-dependent activation of ICE*Bs1* excision and transfer should be inhibited when the concentration of PhrI peptide produced by the population of cells is high, as when the majority of cells in the population contain ICE*Bs1* and produce PhrI. However, when the concentration of PhrI peptide is low, as when the majority of cells in the population lack ICE*Bs1* and do not produce the PhrI peptide, then RapI-dependent activation of ICE*Bs1* excision and transfer should occur. ICE*Bs1* could use this mechanism to inhibit excision and transfer when surrounded by cells that already contain ICE*Bs1*.

To test this model, we monitored excision in a minority population of ICE*Bs1*$^+$ cells when they were grown together with a majority of ICE*Bs1*-containing cells that either produced PhrI (*phrI*$^+$) or that did not produce PhrI (Δ*phrI*) (Fig. 4A). In these mixed cultures, only the minority ICE*Bs1*$^+$ cells were capable of excision, as cells in the majority lacked integrase (Δ*int*), which is required for ICE*Bs1* excision (C.A.L. and A.D.G., unpublished results).

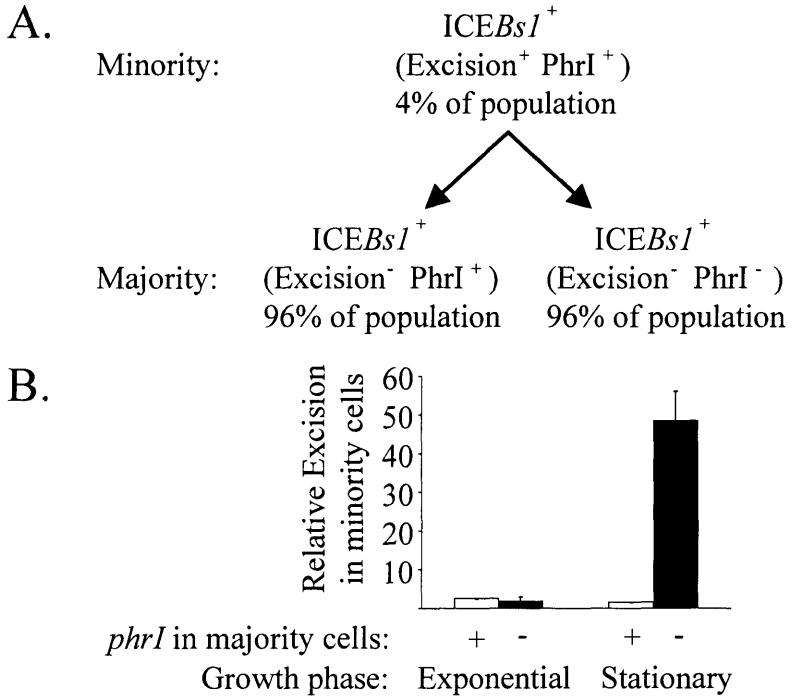

Fig. 4. Excision is inhibited in the presence of PhrI$^+$ cells.

A. Outline of mixing experiments. A minority population (~4% of total) of cells capable of ICE*Bs1* excision and transfer (Excision$^+$ PhrI$^+$) was mixed with a majority population (~96% of total) of cells incapable of ICE*Bs1* excision and transfer that either did (Excision$^-$ PhrI$^+$) or did not (Excision$^-$ PhrI$^-$) encode PhrI.

B. Excision of ICE*Bs1* in cells grown in mixed culture with a majority of ICE*Bs1* Excision$^-$ PhrI$^+$ (JMA205, open bars) or ICE*Bs1* Excision$^-$ PhrI$^-$ (JMA304, black bars) cells was measured during exponential growth and ~2 hours after the entry into stationary phase. Cells were grown separately in nutrient broth sporulation medium to mid-exponential phase. Cells were diluted into fresh medium at a ratio of ~1 minority cell [JMA35, P*spank*(*hy*)] to 24 majority cells [JMA205 (Δ*int*) or JMA304 (Δ*int* Δ*phrI*)] to a total OD_{600} ~ 0.015-0.03 and were co-cultured throughout growth. Samples were collected during mid-exponential growth (OD_{600} ~ 0.2) and ~2 hr after cells entered stationary phase and were used for linear-range PCR assays. In addition to

the circular intermediate and chromosomal control (*cotF*) primer pairs (Materials and Methods), the primer pair, oJMA177 and oJMA178, was used in linear-range PCR assays to amplify a sequence specific to P*spank*(*hy*), which is present only in the minority JMA35 cells. The amount of circular intermediate product from each experimental sample was normalized to the amount of P*spank*(*hy*) and *cotF* products in that sample. This was normalized to the amount of circular intermediate product in an unmixed P*spank*(*hy*) culture (JMA35), also normalized to the amount of P*spank*(*hy*) and *cotF* products, at each time point (defined as 1, not shown) to give the relative increase in excision.

During mid-exponential growth, ICE*Bs1* excision was low whether minority ICE*Bs1*$^+$ cells were grown with excess *phrI*$^+$ or Δ*phrI* cells (Fig. 4B). However, ~2 hours after the cells entered stationary phase, ICE*Bs1* excision was stimulated >40-fold in the ICE*Bs1*$^+$ cells mixed with Δ*phrI* cells, relative to ICE*Bs1*$^+$ cells mixed with *phrI*$^+$ cells (Fig. 4B). We observed a similar increase in excision when ICE*Bs1*$^+$ cells were mixed with cells lacking ICE*Bs1* (data not shown).

These results indicate that the PhrI peptide acts as an intercellular signaling peptide that inhibits ICE*Bs1* excision when cells are crowded by cells that contain ICE*Bs1* and produce the PhrI peptide. Furthermore, ICE*Bs1* excision is inhibited in exponential growth, irrespective of whether cells in the majority population contain *phrI*, indicating that an additional mechanism inhibits ICE*Bs1* excision and transfer. AbrB is a transition-state regulator that represses transcription of several *B. subtilis* genes during exponential phase, and is inactive under conditions of nutrient limitation and high cell density (reviewed in 39). We found that transcription of *rapI*, measured with a *rapI-lacZ* promoter fusion, increased ~5-fold in an *abrB* mutant (CAL26) relative to wild-type cells (CAL15), indicating that AbrB represses *rapI* transcription, either directly or indirectly. Consistent with this model, we also found that ICE*Bs1* excision increased in Δ*abrB* cells relative to wild-type cells; this effect was much larger in exponential phase than in stationary phase (Fig. 6, Appendix B).

Taken together, these observations indicate that at least two mechanisms regulate RapI-dependent activation of ICE*Bs1* excision. When nutrients are abundant and cell density is low, AbrB represses *rapI* transcription, preventing RapI-dependent activation of ICE*Bs1* excision. As cells enter stationary phase, *rapI* transcription is de-repressed and RapI can activate excision, but only when the concentration of PhrI peptide is too low to inhibit RapI.

Table 2. Transfer of ICE*Bs1* is inhibited if the surrounding cells are *phrI*$^+$.

	Recipient	
Donor	ICE*Bs1*$^+$ Excision$^-$ PhrI$^+$	ICE*Bs1*$^+$ Excision$^-$ PhrI$^-$
rapI$^+$ *phrI*$^+$	$1.0 \times 10^{-5} \pm 4.0 \times 10^{-6}$	$3.0 \times 10^{-3} \pm 1.0 \times 10^{-3}$
Δ(*rapIphrI*)	$1.0 \times 10^{-5} \pm 4.0 \times 10^{-6}$	$5.0 \times 10^{-6} \pm 2.0 \times 10^{-6}$

A minority population of ICE*Bs1*-containing cells (potential donors) containing an antibiotic resistance gene in ICE*Bs1* (ICE*Bs1*::*kan rapI*$^+$ *phrI*$^+$, JMA384) was grown in mixed culture with a majority population of ICE*Bs1*-containing cells (potential recipients) that were incapable of excision, defective in competence development, and were either *phrI*$^+$ (*phrI*$^+$ Δ*int comK*, JMA381) or *phrI*$^-$ (Δ*phrI* Δ*int comK*, JMA306), as described in Fig. 4. To show dependence on *rapI* in the donor, a similar experiment was done with potential donors lacking *rapI* and *phrI* [Δ(*rapI phrI*)::*kan*, IRN342] Strains were first grown separately in nutrient broth sporulation medium to mid-exponential phase. Cells were then diluted into fresh medium at a calculated ratio of ~1 potential donor to 24 potential recipients (total OD_{600} ~0.015-0.03) and were grown in co-culture until ~2 hr after entry into stationary phase. A 5-ml aliquot of each co-culture was removed, mixed with 7.5 ml of fresh medium, filtered, and incubated on sporulation medium agar for ~3 hrs. Filters were washed and samples were plated selectively as described (Materials and Methods). The mean number of transconjugants per donor cell (± SEM) for at least 2 independent experiments is reported. ICE*Bs1* transfer occurred much more efficiently under these mating conditions than under the conditions described in Table 1. (See Table 5 and *Supporting Text* in Appendix B).

As expected, transfer of ICE*Bs1* was also inhibited when potential donors were surrounded by cells that produced the PhrI peptide. We introduced an antibiotic resistance cassette into ICE*Bs1* between the last gene of the element (*yddM*) and the attachment site, *attR*. This insertion did not have a significant effect on mating frequency; donor cells overexpressing *rapI* that contained this insertion (JMA448) or an antibiotic insertion in *rapI* and *phrI* (JMA168) mated at similar frequencies (data not shown). We tested transfer of ICE*Bs1* from a minority population (ICE*Bs1*::*kan*) into cells in the majority population that either did (*phrI*$^+$ Δ*int comK*) or did not (Δ*phrI* Δ*int comK*) produce PhrI. ICE*Bs1* transfer in the mixed cultures, measured 2 hr after cells entered stationary phase, was >100-fold higher into recipients that lacked *phrI* than into cells that

contained *phrI* (Table 2). This stimulation was dependent on RapI; it did not occur when the donor cells lacked *rapI* and *phrI* (Table 2).

Taken together, the results of the excision and mating experiments indicate that ICE*Bs1* excision and transfer is more active when cells are crowded by potential mating partners that do not produce the PhrI peptide. Excision and transfer is limited to conditions that are likely to correlate with cell crowding, starvation and high cell density, through the growth phase-dependent regulation of *rapI* transcription. In this way, ICE*Bs1* uses intercellular peptide signaling to coordinate excision and mating with conditions that favor its productive dissemination to recipients lacking ICE*Bs1*.

Activation of ICE*Bs1* excision and transfer by the SOS response. Previous analysis of mRNA levels using DNA microarrays indicated that genes in ICE*Bs1* are activated by a variety of conditions that induce the SOS response (A. Goranov, E. Kuester-Schoeck, R. Britton and A.D.G., unpublished results). Treatment of wild-type cells with MMC, a DNA damaging agent that induces the SOS response in *B. subtilis* (30), stimulated ICE*Bs1* excision (Fig. 3B). Increased gene expression and excision in response to MMC was dependent on *recA*, which is required for the global DNA damage response (30), and was independent of *rapI* and *phrI* (data not shown).

Mating frequency also increased when potential donor cells [Δ(*rapI phrI*)::*kan*, IRN342] were treated with MMC. The mean mating frequency was $2 \times 10^{-4} \pm 8 \times 10^{-5}$ transconjugants per ICE*Bs1*^{0} recipient (CAL89). Mating was undetectable from untreated cells under these conditions ($<2 \times 10^{-8}$ transconjugants per recipient). Mating frequency was determined relative to recipients because MMC treatment reduced the viability of donors. Induction of ICE*Bs1*

excision and transfer by the SOS response may be an attempt by the element to escape the distressed cell for a viable host.

Conserved signals regulate dissemination of mobile genetic elements. We determined that ICE*Bs1* gene expression, excision, and transfer are inhibited by a self-encoded peptide and activated by the global DNA damage response. Intercellular signaling also regulates transfer of some conjugative plasmids. Two well-studied examples are transfer of the Ti plasmid in *Agrobacterium tumafaciens* (reviewed in 52) and transfer of pheromone-inducible plasmids in *Enterococcus faecalis* (reviewed in 9, 12).

Ti plasmid transfer is stimulated by the presence of cells that contain the plasmid; this stimulation depends on the plasmid-encoded signal synthetase, TraI, which synthesizes 3-oxo-8 homoserine lactone, and the plasmid-encoded regulatory protein, TraR (52). In contrast, transfer of ICE*Bs1* is inhibited by the presence of cells that contain the element.

In *E. faecalis,* several mating pheromones (peptides) are encoded in the chromosome. Each pheromone stimulates transfer of a specific conjugal plasmid, and production of these pheromones by cells lacking specific plasmids stimulates transfer of those plasmids from donors (9, 12). Plasmid-containing cells also produce unique plasmid-encoded peptides that inhibit plasmid transfer to potential recipients that already contain the plasmid (9, 12).

Although peptides produced by *E. faecalis* pheromone-responsive plasmids and ICE*Bs1* both inhibit transfer, the regulatory mechanisms are different. With *E. faecalis* plasmids, specific peptide signals produced by recipients trigger transfer from donor cells. ICE*Bs1* transfer is stimulated by conditions (low nutrient availability and high cell density) likely to correlate with a high number of potential recipients. Furthermore, *E. faecalis* inhibitory peptides are thought to be competitive inhibitors of specific mating pheromones (9). There is no evidence that a specific

peptide stimulates transfer of ICE*Bs1* or competes with the inhibitory PhrI peptide for binding to RapI. Hence, multiple molecular mechanisms evolved to inhibit self-transfer of mobile genetic elements utilizing secreted signaling molecules.

Many lysogenic bacteriophage (42) and the ICE SXT (3) are induced by the SOS response. We suspect that the SOS response inactivates the immunity repressor of ICE*Bs1*, as that is how the SOS response induces some other mobile genetic elements (3, 42). However, further work will be needed to reveal the molecular mechanisms regulating SOS-mediated induction of ICE*Bs1*.

Rap-Phr systems in other *Bacillus* mobile elements. In addition to the chromosomally encoded *rap-phr* cassettes in *Bacillus* species, *rap-phr* cassettes are found on the *B. subtilis* plasmids pTA1060, pTA1040, pPOD2000, pLS20, the *B. licheniformis* plasmids pFL5 and pFL7, the *B. cereus* plasmid pBC10987, the *B. subtilis* phage ϕ105, the defective *B. subtilis* prophage *skin*, and the *B. anthracis* bacteriophage λBa04 (Table 6, Appendix B). *rap60* and *phr60*, from pTA1060, have been characterized. Rap60 inhibits degradative enzyme production; this is antagonized by Phr60 (21). Rap60 and Phr60 were studied in the absence of pTA1060 and their effects on mobility of pTA1060 were not reported. To our knowledge, the remaining *rap-phr* systems contained on mobile elements (other than *rapE* and *rapI*) have not been characterized. We postulate that these *rap*s and *phr*s might regulate the mobility of their respective genetic elements, thereby modulating horizontal gene transfer and bacterial evolution.

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Appendix B: Supporting Material for Chapter 3

- Published as Supporting Information on the PNAS webiste -

Contents of Appendix B:

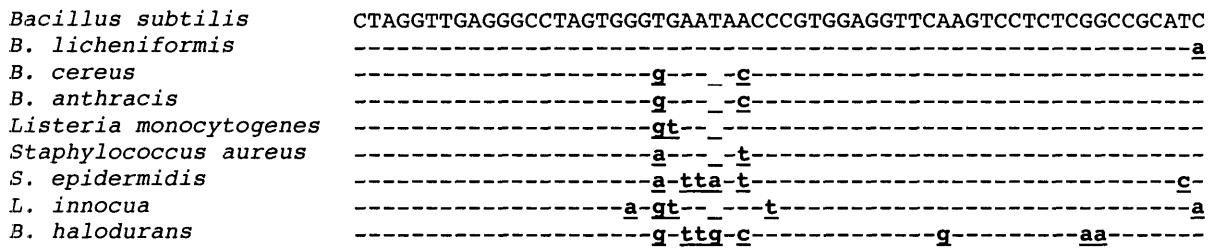

Figure 5. ICE*Bs1 att* site is found in other Gram-positive species. Sequences closely related to the 60-bp direct repeat sequence were identified through BLAST (1) and were aligned with the *B. subtilis* sequence. Consensus nucleotides are identified by a dash. Nucleotides that diverge from the *B. subtilis* sequence, including missing nucleotides, are underlined and in boldface type.

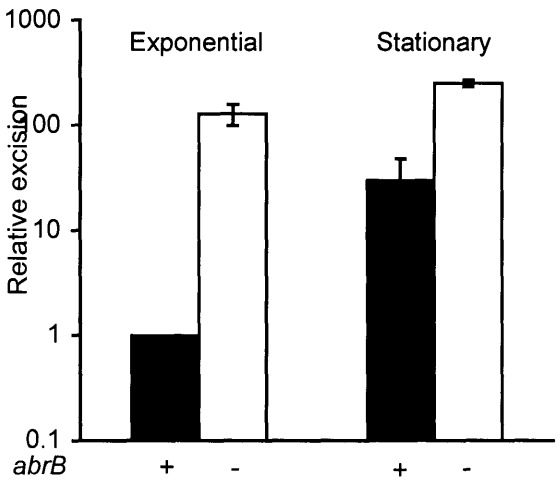

Figure 6. AbrB inhibits ICE*Bs1* excision. Wild-type (JH642, black bar) and Δ*abrB* (AG839, white bar) cells were grown in DSM. Samples were collected from cells during exponential phase (OD600 ~ 0.2) and ~2 hours after the entry into stationary phase. Excision of ICE*Bs1* was determined by linear-range PCR and was normalized to the amount of excision in wild-type cells during exponential phase.

Table 3. Strains used in this study.

Strain	Genotype, comments, reference
JH642[1]	*B. subtilis trpC2 pheA1* (16)
AG839	Δ*abrB*::*cat*
CAL15	*thrC*::(*rapI-lacZ erm*)
CAL26	Δ*abrB*::*cat thrC*::(*rapI-lacZ erm*)
CAL51	*opp*::(Tn*917lac*::pTV21Δ2 *cat*) Δ(*rapI phrI*)*342*::*kan amyE*::{(P*spank*(*hy*)-*rapI*) *spc*} (*rapI* under control of P*spank*(*hy*); *opp* = *spo0K*)
CAL52	*opp*::(Tn*917lac*::pTV21Δ2 *cat*) Δ(*rapI phrI*)*342*::*kan amyE*::{(P*spank*(*hy*)-*rapI phrI*) *spc*} (both *rapI* and *phrI* under control of P*spank*(*hy*))
CAL84	*str* (spontaneous streptomycin-resistant mutant of JH642)
CAL88	*comK*::*spc str*
CAL89	ICE*Bs1*^{0} *comK*::*spc str* (cured of ICE*Bs1*)
CAL419	ICE*Bs1*^{0} *comK*::*cat str*
IRN342	Δ(*rapI phrI*)*342*::*kan* (deletion-insertion of *rapI* and *phrI*)
IRN444	*recA260*:*cm mls* (9)
JMA28	*amyE*::{(P*spank*(*hy*)-*rapI*) *spc*}
JMA35	*amyE*::{(P*spank*(*hy*)) *spc*} (empty vector)
JMA168	*amyE*::{(P*spank*(*hy*)-*rapI*) *spc*} Δ(*rapI phrI*)*342*::*kan*
JMA186	*amyE*::{(P*spank*(*hy*)-*rapI phrI*) *spc*} Δ(*rapI phrI*)*342*::*kan*
JMA205	Δ*int205*::*cat* (integrase null mutation)
JMA206	Δ(ICE*Bs1*)*206*::*cat*
JMA208	Δ*immR*::*cat* (immunity repressor null mutation)
JMA222	ICE*Bs1*^{0} (cured of ICE*Bs1*)
JMA304	Δ*int205*::*cat* Δ*phrI173*::*erm*
JMA306	Δ*int205*::*cat* Δ*phrI173*::*erm comK*::*spc*
JMA342	*amyE*::{(P*spank-rapI*) *spc*} Δ(*rapI phrI*)*342*::*kan*
JMA381	Δ*int205*::*cat comK*::*spc*
JMA384	ICE*Bs1*::*kan*
JMA448	ICE*Bs1*::*kan amyE*::{(Pspank(hy)-*rapI*) *spc*}
NCIB3610	Prototroph (3)
SSB173	NCIB3610 Δ*phrI173*::*erm* (Branda and Kolter)
SSB260	NCIB3610 Δ(*rapI phrI*)*260*::*erm* (Branda and Kolter)
JMA298	NCIB3610 Δ*phrI173*::*erm amyE*::{(P*spank*(*hy*)-*phrI*) *spc*}
Other bacterial species:	
ATCC11946	*B. licheniformis* ATCC11946 (from the Bacillus Genetic Sock Center)
REM42	*B. licheniformis* ATCC11946 *str* (spontaneous streptomycin-resistant mutant of ATCC11946)
UM44-1C9	*B. anthracis* pXO1$^-$ derivative of UM44 *ind str* (7)
10403S	*Listeria monocytogenes str* (2)

[1] All strains through JMA448 are derived from JH642 and contain the *trpC2* and *pheA1* mutations.

Table 4. Changes in mRNA levels caused by overexpression of *rapI*.

	Experiment type[1]:	I	II
Gene	**Description of protein function[2]**	**Fold Change[3]**	
abrB[4]	Transcriptional pleiotropic regulator of transition state genes	2.8	2.4
sacV[5]	Transcriptional regulator of the levansucrase gene	13	57
ydcO[5]	Unknown	12	470
ydcP[5]	Unknown; similar to *orf22* in Tn*916*	17	130
ydcQ[5,6]	Unknown; similar to *orf21* in Tn*916* (putative DNA translocase)	22	280
ydcR[5]	Unknown; similar to *orf20* in Tn*916*	18	69
ydcS[5]	Unknown; similar to unknown proteins from *B. subtilis*	8.6	130
ydcT[5]	Unknown; similar to unknown proteins from *B. subtilis*	22	340
yddA[5]	Unknown	91	260
yddB[5]	Unknown; similar to *orf13* in Tn*916*	18	35
yddC[5]	Unknown	7.6	500
yddD[5]	Unknown	7.4	49
yddE[5]	Unknown; similar to *orf16* in Tn*916*	6.2	26
yddF[5]	Unknown	4.5	6.2
yddG[5]	Unknown; similar to *orf15* in Tn*916*	6.2	32
yddH[5]	Unknown; similar to *orf14* in Tn*916*	6.6	25
yddI[5]	Unknown	16	13
yddJ[5]	Unknown	4.4	7.4
rapI[5]	Response regulator aspartate phosphatase	-[6]	-[6]
phrI[5]	Phosphatase regulator	-[7]	-[7]
yddM[5]	Unknown	14	8.3
yvqH	Unknown; similar to unknown proteins from *B. subtilis*	12	1.7
ggaA	Biosynthesis of galactosamine-containing minor teichoic acid	2.4	1.8
yydB	Unknown	2.7	1.4
yydD	Unknown; similar to unknown proteins	2.8	1.6
glgB	1,4-alpha-glucan branching enzyme	-1.7	-1.6
spoIIGA[4]	Protease (processing of pro-sigma-E to active sigma-E)	-2.9	-2.0
spoIIAA[4]	Anti-anti-sigma factor (sigF)	-2.5	-1.6
spoIIB[4]	Regulator of septal peptidoglycan dissolution during engulfment	-2.2	-2.2
sacT[4]	Transcriptional antiterminator involved in regulating *sacA* and *sacP*	-5.2	-3.1
ywcI[4]	Unknown	-7.2	-2.9

[1] Experiment types are described in Supporting Text.
[2] Description of protein functions are derived from http://genolist.pasteur.fr/SubtiList. Homology with Tn*916* genes was determined through BLAST analysis (1).
[3] The average fold-change in gene expression in the *rapI* overexpressing cells relative to control cells from each set of triplicate experiments is shown. Positive values indicate increased expression in *rapI* overexpressing cells, while negative values indicate decreased expression.

[4] Genes regulated by Spo0A (directly or indirectly) (5, 12, 17). Repression of genes in the Spo0A regulon is consistent with an observed reduction in sporulation caused by *rapI* overexpression (14).
[5] Genes that are part of ICE*Bs1*.
[6] *ydcQ* was identified as encoding a putative DNA translocase due to the presence of a conserved FtsK/SpoIIIE-like domain identified by SMART (10).
[7] We do not report the fold-change in mRNA levels for *rapI* because the arrays do not distinguish between endogenous and ectopically-expressed *rapI* transcripts.
[8] We do not report the fold-change in mRNA levels for *phrI* because the arrays do not distinguish between the endogenous *phrI* and the partial fragment of the *phrI* transcript that is over-expressed from the ectopic *rapI* construct. (The 5' end of *phrI* overlaps the 3' end of *rapI* by 41 nucleotides).

Table 5. Comparison of transfer frequencies using different mating protocols.

Ratio[1]	Agar[2]	Frequency[3]
1:1	DSM	$7 \times 10^{-1} \pm 2 \times 10^{-2}$
1:100	LB	$5 \times 10^{-2} \pm 7 \times 10^{-3}$
1:100	DSM	5 ± 0.9

[1] Ratio of donor (JMA168) cells to recipient (CAL419) cells.
[2] Agar used during 3 hr incubation at 37°C on filter.
[3] Frequency = mean number of transconjugants per donor ± SEM.

Table 6. *rap* and *phr* genes in *Bacillus* mobile genetic elements.

Gene names	Mobile element	Species
rapI phrI	ICE*Bs1*	*B. subtilis*
rapE phrE	*skin* (defective prophage) (21)	*B. subtilis*
rap60 phr60	pTA1060 (11)	*B. subtilis*
rap40 phr40	pTA1040 (11)	*B. subtilis*
rapA rapAB	pPOD2000 (6)	*B. subtilis*
orf50 orf51	phage ϕ105 (4)	*B. subtilis*
orfA orfAB	pLS20 (8)	*B. subtilis*
BA3760 BA3759	phage λBa04 (19)	*B. anthracis* (Ames)
rap5 phr5	pFL5 (15)	*B. licheniformis*
BCEA0148 BCEA0147	pBC10987 (18)	*B. cereus* ATCC1097
rap7 phr7	pFL7 (15)	*B. licheniformis*

Supporting Text

Changes in gene expression caused by overproduction of RapI. We did two types of experiments to evaluate changes in gene-specific mRNA levels caused by overexpression of *rapI* (Table 4). Type I was analyzed on DNA microarrays containing PCR products of virtually all the *B. subtilis* ORFs. Type II was analyzed on DNA microarrays containing a unique oligonucleotide for virtually every ORF.

In type I experiments, RNA was harvested from cells containing the LacI-repressible, IPTG-inducible fusion P*spank(hy)-rapI* (strain JMA28) grown without IPTG (no overexpression) or 30 min after induction with IPTG. Fluorescently labeled cDNA prepared from these samples was co-hybridized to PCR arrays containing DNA amplified from >99% of the *B. subtilis* ORFs.

In type II experiments, RNA was harvested from P*spank(hy)-rapI* cells and from control cells [P*spank(hy)*, JMA35, no insert downstream from P*spank(hy)*]. Fluorescently labeled cDNA was prepared from these samples, mixed with a labeled reference sample, and hybridized to arrays containing 65-mer oligonucleotides complementary to all the annotated *B. subtilis* ORFs.

Both experiments were performed with three independent sets of cultures. Many genes in the integrative and conjugative element ICE*Bs1* appeared to have much greater overexpression in Type II compared to Type I experiments (Table 4). Much of this higher level of expression is likely because of the control sample used for normalization. In the type I experiments, RNA levels in *rapI* overexpressing cells were compared to uninduced P*spank(hy)-rapI* cells, which have a higher level of ICE*Bs1* gene expression and excision than control P*spank(hy)* cells, due to incomplete repression of the P*spank(hy)-rapI* promoter in the absence of inducer (data not shown). However, in the type II experiments, RNA levels in *rapI* overexpressing cells were compared to P*spank(hy)* cells, which do not have increased levels of ICE*Bs1* gene expression and excision.

ICE*Bs1* transfer frequency depends on donor-to-recipient ratio and growth medium. We observed a large range of transfer efficiencies of ICE*Bs1*, depending on the specific mating conditions (compare mating data in Table 1 to Table 2). Under a given set of conditions, mating frequencies were quite consistent. However, when two different sets of mating conditions in two different types of experiments were compared, the differences in transfer frequencies were significant. For example, under one set of conditions, transfer of ICE*Bs1* from Δ(*rapI phrI*) donor cells into ICE*Bs1*$^{+}$ *comK* recipients was not detected (<3 X 10^{-8} transconjugants per donor) but occurred at a frequency of ~1 X 10^{-5} per donor cell under a different set of conditions.

There were many differences between the experiments that gave the various mating frequencies, including different donor and recipient strains, different growth media, differences in the amount of time the donor and recipient were together before filter mating, and differences in the donor-to-recipient ratio. To explore what contributed to the significant differences in mating frequencies, we tested many of these parameters in side-by-side comparisons. We used strain JMA168 {ICE*Bs1* Δ(*rapI phrI*)::*kan amyE*::(P*spank*(*hy*)-*rapI*)} as a donor and strain CAL419 {ICE*Bs1*^{0} *comK*::*cat str*} as a recipient. Excision of ICE*Bs1* in the donor was induced by the addition of IPTG, to overexpress *rapI*, and cells were mixed one hour later. Different mating conditions were tested in parallel, and transconjugants were selected for resistance to kanamycin (from ICE*Bs1*) and streptomycin (from recipient).

We found that the mating frequency was affected both by the donor-to-recipient ratio and by the medium used for the filter mating. Transfer increased ~10-fold in filter matings performed with a ratio of ~1 donor cell to 100 recipient cells, relative to filter matings performed with a ratio of ~1 donor cell to 1 recipient cell (Table 5). Donor-to-recipient ratios of ~1:10, ~1:25, ~1:200 and, ~1:400 gave transfer frequencies similar to the ~1:100 ratio (data not shown). These

results indicate that the availability of recipient cells likely limits the frequency of mating from donor to recipient cells when an equal number of donor and recipient cells are present.

We also observed an ~100-fold increase in transfer efficiency when matings of ~1 JMA168 donor to 100 CAL419 recipients were performed on nutrient broth (Difco) sporulation agar (DSM) as compared to matings performed on LB agar (Table 5). This increase in transfer on DSM could be caused by the presence of divalent cations (Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}) or by the physiological effect of nutritional differences between DSM and LB agar. Taken together, these experiments demonstrate that factors in addition to PhrI peptide signaling affect the efficiency of ICE*Bs1* transfer.

Of note is that matings done DSM at a ratio of ~1 donor to 100 recipients gave rise to multiple transconjugants per donor. It is most likely that a single donor is mating with multiple recipients. It is also possible that transconjugants serve as donors during the course of the experiment.

Supporting Methods

Strain information. Strains used are listed in Table 3 and the construction of specific alleles not described in the main text is described below. Null mutations generated by double crossover recombination of alleles into the chromosome were verified by PCR.

Generation of spontaneous streptomycin-resistant mutants. The *B. subtilis str* strain (CAL84) and the *B. licheniformis str* strain (REM42) were generated by selecting for spontaneous streptomycin resistance of the parental strains JH642 and ATCC11946, respectively, on LB plates containing streptomycin (100 μg/ml). CAL84 and REM42 are resistant to streptomycin and sensitive to spectinomycin. The *str* allele from CAL84 was used to

generate strains CAL88 (*comK*::*spc str*), CAL89 (ICE*Bs1*^{0} *comK*::*spc str*), and CAL419 (ICE*Bs1*^{0} *comK*::*cat str*).

Generation of an ICE*Bs1*-cured (ICE*Bs1*^{0}) strain. A strain cured of ICE*Bs1* (JMA222) was generated by growing Δ*immR208*::*cat* cells (*immR* encodes a repressor of ICE*Bs1* gene expression) in the absence of antibiotic selection for many generations. The *immR* mutant has an increased frequency of ICE*Bs1* excision, and after many generations of growth without selection, 9 of 100 colonies from LB agar plates were sensitive to chloramphenicol, indicating that these cells had lost the *immR208*::*cat* allele. One isolate, JMA222, was chosen for further study.

The absence of ICE*Bs1* at the *att* site was confirmed through PCR using primers (oJMA93 and oJMA100) that amplify across the unoccupied *att* site. Sequencing of this PCR product revealed that it contains a single *att* site surrounded by the chromosomal sequence that normally flanks the integrated ICE*Bs1*. This same unoccupied *att* site structure is observed in sequenced PCR products from cells in which *rapI* overexpression has stimulated excision of ICE*Bs1*. In addition, by using ICE*Bs1*-specific primers, we were unable to detect the element elsewhere in the genome, nor were we able to detect any of the ICE*Bs1* genes using DNA microarrays (data not shown). Based on these data, we believe that ICE*Bs1* excised through the normal excision mechanism in JMA222, failed to reintegrate, and was lost from progeny cells during growth and cell division.

We also found that ICE*Bs1* was missing in some lab strains of *B. subtilis*. We tested for the presence of ICE*Bs1* by using PCR to detect *int*, *immR*, and *rapI phrI*. Sequences of the primers used to amplify these regions are listed below. We also tested for insertion of ICE*Bs1* at *attB* (the chromosomal *att* site in *tRNS-leu2*) by detecting the region spanning *attR* (primers oJMA97 and oJMA100, listed below). In addition, we tested for the unoccupied *attB* site (repaired

chromosomal junction) as described. Presence of the unoccupied *attB* site and absence of the region spanning *attR*, indicates that if ICE*Bs1* is present, it is not integrated at *attB*. We found that in addition to the lab strain JH642, strains 168 (20), CRK6000 (13), and NCIB3610 (3) all contained ICE*Bs1* integrated at *attB*. PCR assays indicated that strains PY79 (23) and YB886 (22) likely do not contain ICE*Bs1*, because the individual regions containing *attR*, *int, immR* and *rapI phrI* were detectable, and the *attB* site was unoccupied (data not shown). It is formally possible that these strains have a form of ICE*Bs1* elsewhere in the genome which has enough sequence divergence that it is not recognized by the primers used for amplification; however, genomic DNA microarrays comparing DNA content between JH642 and YB886 failed to detect any ICE*Bs1* genes (data not shown), indicating that, if present, ICE*Bs1* contains significantly divergent sequences in all of its genes.

Δ(ICE*Bs1*)*206*::*cat*. Δ(ICEBs1)*206*::*cat* is a deletion-insertion of ICE*Bs1*. The entire element, including *attR*, was replaced with the chloramphenicol-resistance gene from pGEM-*cat* (24).

ICE*Bs1*::*kan*. ICE*Bs1*::*kan* is functionally ICE*Bs1*$^+$ and contains the kanamycin- resistance gene from pGK67 (9) inserted between the 3' end of *yddM* and *attR*.

Deletion-insertion of *rapI* and *phrI*. The Δ(*rapI phrI*)*342*::*kan* insertion-deletion was generated by replacing the 3' end of *rapI* and all of *phrI* with the kanamycin- resistance gene in pGK67 (9).

Null mutations in *int* and *immR*. Δ*int205*::*cat* and Δ*immR208*::*cat* were generated by replacing *int* or *immR* with the chloramphenicol-resistance gene from pGEM-*cat* (24).

Preparation of DNA Microarrays

PCR products were resuspended in 50% dimethylsulfoxide (DMSO) and spotted on to Corning GAPS II slides. Oligonucleotides were resuspended in 50% DMSO at a concentration of 25 μM and spotted on to Corning UltraGAPS slides. Slides were stored at room temperature until use. The PCR product arrays stored well for at least 2 years. The oligonucleotide arrays stored well for at least 6 months.

Before hybridization with biological samples, DNA was crosslinked to the glass slides using a UV Stratalinker (Stratagene) at 90 mJ for the PCR product arrays and 600 mJ for the oligonucleotide arrays. After crosslinking, arrays were incubated in pre-hybridization buffer [5X SSC (0.75 M sodium chloride, 75 mM sodium citrate, pH 7), 0.1% sodium dodecyl sulfate (SDS), 1% bovine serum albumin (BSA)] for at least 45 min. at 42° C. Pre-hybridized slides were washed in double-distilled water. Excess water was removed by centrifugation and drying with nitrogen gas.

Reverse transcription and labeling of RNA for microarray experiments

RNA (10 μg) from each sample was reverse transcribed with Superscript II Reverse Transcriptase (RT, Invitrogen) in the presence of aminoallyl-dUTP (Sigma or Ambion). RNA samples were combined with 2.5 μg Random Hexamers (Operon or Qiagen) and incubated at 70° C for 10 min., followed by incubation at 4° C for 5 min. Reverse transcription reactions (30 μl) were started by adding a mix containing additional reaction components to make the final reaction conditions: 1X RT Buffer (Invitrogen), 10 mM dithiothreitol (Invitrogen), 300 units RT, 0.5 mM dATP, dCTP, and dGTP (Invitrogen), 0.1 mM dTTP (Invitrogen), 0.4 mM aminoallyl-dUTP and 20 units of RNase Out (Invitrogen). The reverse transcription reactions were incubated at 25° C for 10 min., 42° C for 70 min., and then shifted to 70° C for 15 min. to stop

reactions. RNA in the reactions was degraded by adding sodium hydroxide (33 mM final concentration) and incubating at 70° C for 10 min. Hydrogen chloride (25 mM final concentration) was added to neutralize the reactions.

Labeled cDNAs were purified with either Qiagen MinElute or QIAquick PCR purification columns according to the manufacturers protocol, with the exception that the columns were washed with 75% ethanol instead of Buffer PE and were eluted with sterile H_2O. Samples purified on Qiagen QIAquick PCR purification columns were dried by centrifugation under vacuum and resuspended in a smaller volume of sterile water. Sodium bicarbonate (pH 9) was added to each sample to adjust the pH prior to coupling. To couple the fluorescent dyes to aminoallyl-modified uracil in the cDNA, the amine reactive Cy5 and Cy3 dyes (Amersham Pharmacia) were added to the cDNA and incubated for 1 hr in the dark, mixing every 15 min. Coupling reactions were quenched by incubation with hydroxylamine (1.125 M final concentration) for 15 min. in the dark.

PCR primer sequences

The following primers (5' to 3') were used to assay excision of ICE*Bs1*.

Chromosomal junction formed after excision of ICE*Bs1*:

oJMA93-GACGAATATGGCAAGCCTATGTTAC

oJMA100-GGGTATACAATCATGGGTGATCGAG;

ICE*Bs1* circular intermediate:

oJMA95-CTGGACTAAGATGTGGTGAAATGCTC

oJMA97-CTGTAAATTATGAATCTCAGATTGTTAATCCTGC;

cotF region as control:

oLIN93-GCAGCGGCGTTCTGCAAGC

oLIN94-CACTTAGTCACCTCGTATCATC;

amyE::P*spank(hy)* region as control for cells in mixed culture:

oJMA177-CTACCGAGATATCCGCACCAACGC

oJMA178-CTCTGACCAGACACCCATCAACAG.

The following primers (5' to 3') were used to detect ICE*Bs1*. Underlined sequences contain added restriction sites and extra nucleotides that are not complementary to ICE*Bs1* sequence.

Primer internal to ICE*Bs1*, upstream of *attR*:

oJMA97-CTGTAAATTATGAATCTCAGATTGTTAATCCTGC;

Primers to amplify *int*:

oJMA127-ATATGCTAGCGCCCACAAACTGCCCACTTACC

oJMA128-ATATGTCGACCAGAATCTATTCACACGAAATAAGCGC;

Primers to amplify *immR*:

oJMA122-ATATAAGCTTCTCTCCATAAAGAAGAAACAAACACTCC

oJMA123-CAGAGCTAGCGTTATCACTCTTTCTTCTTTAATTCGTCAATG;

Primers to amplify *rapIphrI*:

oJMA25-ATAATTGTCGACCGCACAATTTTATGTAAG

oJMA64-ATCTACGCATGCTTCCAATTATCTAAGCTATG.

PCR conditions

Each reaction (50 µl) contained primers at a final concentration of 1 µM, 200 µM dNTPs, 1X Taq Buffer (Roche), and 1.25U Taq DNA polymerase (Roche). For non-linear range PCR, reactions were amplified for 3 min. at 94° C, followed by 30 cycles of 30 sec. at 94° C, 60 sec. at 56° C, and 2 min. at 72° C. These cycles were followed by a 5 min. extension at 72° C. For

linear range (quantitative) PCR, reactions conditions were the same except that the number of cycles was reduced to 26.

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Chapter 4: Identification of a conserved two-protein regulatory system that regulates transfer of the *Bacillus subtilis* mobile genetic element ICE*Bs1*

- Manuscript in preparation -

Abstract

Mobile genetic elements play an important role in shaping bacterial genomes. Characterizing the mechanisms that regulate transfer of mobile genetic elements should provide insights into factors that influence DNA transfer. ICE*Bs1* is a mobile genetic element found in the chromosome of *Bacillus subtilis*. Transfer of ICE*Bs1* is inhibited by an element-encoded intercellular signaling peptide and activated by the global DNA damage response. However, it was not known how these signals regulate ICE*Bs1* gene transfer. We have identified and characterized two ICE*Bs1*-encoded proteins, immunity repressor and immunity repressor antagonist, that directly mediate the regulation of ICE*Bs1* gene expression in response to intercellular peptide signaling and the global DNA damage response. Homologs of immunity repressor and immunity repressor antagonist are found in several other gram-positive mobile genetic elements, including elements that are activated by the global DNA damage response and contain potential signaling peptides, indicating that this two-protein system may be a conserved mechanism for regulating dissemination of mobile genetic elements.

Introduction

Mobile genetic elements play an important role in modulating the evolution of bacterial species (reviewed in 10, 12, 14, 24, 56). Conjugative plasmids, integrative and conjugative elements (ICEs), and bacteriophage have been shown to transfer genes involved in resistance to antibiotics, utilization of alternative carbon sources, and production of virulence factors (10, 12, 14, 15). Characterizing the molecular mechanisms that govern the activity of these mobile genetic elements should provide insights into conditions that promote horizontal gene transfer and bacterial evolution.

We recently characterized ICE*Bs1*, an integrative and conjugative element found in the Gram-positive bacterium *Bacillus subtilis* (5) (Chapter 3). ICE*Bs1* is normally found integrated in the *B. subtilis* chromosome. Under certain conditions, ICE*Bs1* excises from the chromosome and transfers to recipient cells. Excision of ICE*Bs1* requires the site-specific recombinase Int and accessory protein Xis (C.A.L., J.M.A., R. E. Monson, and A.D.G., manuscript in preparation). Transfer of the excised ICE*Bs1* intermediate requires the activities of several element-encoded conjugation proteins, which are likely involved in additional processing of ICE*Bs1* DNA to generate ssDNA and formation of a mating pore through which the element is transferred from donor to recipient (C.A.L. and A.D.G., manuscript in preparation). Expression of *xis* and the ICE*Bs1* conjugation genes is regulated by intercellular peptide signaling and the global DNA damage response (5) (Chapter 3).

Intercellular peptide signaling provides a mechanism for self-recognition by ICE*Bs1*-containing cells and limits transfer of ICE*Bs1* to conditions when it is most likely to result in successful dissemination to cells that lack ICE*Bs1* (5) (Chapter 3). This regulation is mediated by the ICE*Bs1*-encoded signaling cassette, *rapI-phrI*. *rapI* encodes a protein that stimulates

expression of *xis* and the ICE*Bs1* genes required for transfer; *phrI* encodes a secreted signaling peptide that antagonizes the activity of *rapI*. Transcription of *rapI* is regulated by the nutritional status of the cell: *rapI* transcription is repressed during exponential growth and is derepressed when cells are starved and at high population density. When ICE*Bs1*-containing cells are crowded, *rapI* is transcribed, which can lead to increased expression of ICE*Bs1* genes. However, when ICE*Bs1*-containing cells are crowded by other ICE*Bs1*-containing cells, sufficient levels of PhrI peptides accumulate to inhibit RapI-dependent activation of ICE*Bs1* gene expression. This combined regulation of RapI transcription and activity limits activation of ICE*Bs1* gene expression, excision, and transfer to conditions when ICE*Bs1*-containing cells are crowded by cells that lack ICE*Bs1*.

The RecA-dependent global DNA damage response also activates expression of *xis* and the ICE*Bs1* conjugation genes (5) (Chapter 3). This regulation allows ICE*Bs1* to sense host-cell distress and initiate transfer to a new host. Likewise, several bacteriophage and the ICE SXT are also activated by the global DNA damage response (8, 64).

The RecA-dependent global DNA damage response and intercellular peptide signaling act independently to regulate the expression of ICE*Bs1* genes (5) (Chapter 3). However, neither RecA nor RapI are thought to directly regulate transcription. RecA serves several roles in the cell: it mediates homologous recombination and double strand break repair, plays a role in repairing stalled replication forks, and activates a global response to DNA damage (reviewed in 17). In both *Escherichia coli* and *B. subtilis*, RecA is thought to become active to induce the global DNA damage response upon binding to single-stranded DNA (48, and references therein). When bound to single-stranded DNA, RecA stimulates the autoproteolysis of LexA, a protein that represses transcription of several genes, including those encoding proteins involved in DNA

repair and survival after DNA damage (4, 45, and references therein). RecA bound to single-stranded DNA also stimulates autoproteolysis of the λ cI repressor, which results in derepression of late gene expression and lytic development of λ (45). RapI is a member of a family of *B. subtilis* Rap proteins, several of which have been shown to interact with and antagonize the activities of response regulator proteins (9, 16, 41, 57, 60). Therefore, it is likely that both RapI and RecA regulate the expression of ICE*Bs1* genes indirectly, possibly by affecting the activity of an ICE*Bs1*-encoded transcriptional regulatory protein or proteins.

We identified two ICE*Bs1*-encoded regulators, ImmR and ImmA (Immunity Repressor and Immunity Repressor Antagonist), that control transcription of ICE*Bs1* genes. ImmR binds directly to the *xis* promoter and represses transcription of *xis* and the ICE*Bs1* conjugation genes required for transfer. ImmR also regulates its own transcription and confers immunity to cells, inhibiting their acquisition of a second copy of ICE*Bs1*. ImmA is an antagonist of ImmR and is required for derepression of ICE*Bs1* gene expression in response to RapI or RecA activity. We identified homologs of ImmR and ImmA in several other known and putative mobile genetic elements, indicating that this two-protein regulatory mechanism may regulate dissemination of other mobile genetic elements.

Materials and Methods

Media. *B. subtilis* cells were grown at 37° C in LB (67) or S7 minimal salts medium (75) (containing 50 instead of 100 mM MOPS) supplemented with 1% glucose, 0.1% glutamate, tryptophan (40 μg/ml), phenylalanine (40 μg/ml), and threonine (120 μg/ml) as indicated. LB was also used for growth of *E. coli*. *Saccharomyces 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. When appropriate, antibiotics were used at the following concentrations: ampicillin (100 μg/ml, unless otherwise indicated), chloramphenicol (5 μg/ml), kanamycin (5 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. Isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma) was used at a final concentration of 1 mM unless otherwise indicated; mitomycin C (MMC, Sigma) was used at a final concentration of 1 mg/ml, and L-arabinose (Sigma) was used at a final concentration of 0.2%.

Strain construction. Strains used in this study are listed in Table 1. Standard techniques were used for cloning and strain construction (32, 67). The ICE*Bs1*^{0} strain and the Δ*int*205::*cat*, Δ*immR*208::cat, and *amyE*::P*spank*(*hy*)-*rapI* alleles were previously described (5) (Chapter 3).

xis-lacZ was generated by cloning the sequence from –343 to –6 of *xis* upstream of a promoter-less *lacZ* in the vector pDG793 (30). *immR-lacZ* was generated by cloning the same sequence in the opposite orientation upstream of *lacZ* in pDG793. Both vectors were integrated into the *thrC* locus of the chromosome by double crossover homologous recombination, which was verified by conversion to threonine auxotrophy.

Several fusions to the IPTG-inducible P*spank* promoter were generated by cloning into the pDR110 vector (65), a generous gift from David Rudner. P*spank-immR*Ω27 was generated by cloning from –27 to +388 of *immR* into pDR110. P*spank-immR*Ω142 was generated by cloning from –142 to +388 of *immR* into pDR110. P*spank-immA*Ω385 contains the *immA* coding sequence (+4 to +512) along with an engineered ribosome binding site (*rbs*), spacer region, and start codon (AGGAGGAATTACTATG, *rbs* is underlined) (58) upstream of the remaining *immA* coding sequence.

P*immR-immR* and P*immR-immR immA* were generated by cloning the sequence from -268 of *immR* to +388 of *immR* or +512 of *immA* into the integration vector, pMMB124, which was a generous gift from M.B. Berkmen. pMMB124 contains two segments encompassing the entire *cgeD* gene inserted on either side of the kanamycin resistance gene in pGK67 (44). This allows for integration of DNA by double crossover homologous recombination into *cgeD*. Double crossover integrants were distinguished from single crossover integrants by screening for sensitivity to chloramphenicol, as its resistance is encoded on the plasmid backbone outside of the regions of *cgeD* homology.

cgeD::*Pspank(hy)-rapI* was generated by subcloning P*spank*(*hy*)-*rapI* and the *lacI* gene from plasmid pJMA28 (pDR111-P*spank*(*hy*)-*rapI*, (5)) into plasmid pMMB124, followed by integration into the chromosome by double homologous recombination.

Δ*immA* Δ*int*::*cat* was generated through a combination of splicing by overlap extension (SOE) and long-flanking homology PCR (36, 76). This construct creates an in-frame deletion of *immA* linked to a replacement of +53 to +1097 of *int* with the chloramphenicol resistance gene from pGEM-*cat* (79). This *int* deletion removes the same sequence as the previously described Δ*int205*::*cat* deletion (5) (Chapter 3). The in-frame deletion of *immA* joins the first 3 codons at the 5' end of *immA* to the last two codons at the 3' end of *immA*. This construct leaves the 3' end of the *immR,* including its termination codon, intact.

Plasmids encoding ImmR-Gal4 DNA binding domain (Gal4-BD), ImmR-Gal4 activation domain (Gal4-AD), ImmA-Gal4-BD, RapI-Gal4-BD, and RapI-Gal4-AD were generated by cloning the coding sequence of *immR*, *immA*, or *rapI* in the same reading frame as the upstream Gal4-BD coding sequence in plasmid pGAD-c1 (39) or the coding sequences of *immR* and *rapI* in the same reading frame as the upstream Gal4-AD coding sequence in plasmid pGBDu-c3 (39).

Table 1: Strains used in this study

Strain	Genotype
***B. subtilis* strains**[1]:	
JH642[1]	*trpC2 pheA1*
CAL16	Δ*int*205::*cat* *thrC*::{(*xis-lacZ*) *erm*}
JMA201	*thrC*::{(*xis-lacZ*) *erm*}
JMA208	Δ*immR*208::*cat*
JMA214	Δ*immR*208::*cat* *thrC*::{(*xis-lacZ*) *erm*}
JMA226	Δ*immR*208::*cat* *amyE*::{(P*spank-immR*Ω142) *spc*} *thrC*::{(*xis-lacZ*) *erm*}
JMA258	*amyE*::{(P*spank*(*hy*)-*rapI*) *spc*} Δ*int*205::*cat* *thrC*::{(*xis-lacZ*) *erm*}
JMA264	ICE*Bs1*^{0} *thrC*::{(*xis-lacZ*) *erm*}
JMA266	ICE*Bs1*^{0} *amyE*::{(P*spank-immR*Ω142) *spc*} *thrC*::{(*xis-lacZ*) *erm*}
JMA309	*thrC*::{(*immR-lacZ*) *erm*}
JMA310	Δ*immR208*::*cat* *thrC*::{(*immR-lacZ*) *erm*}
JMA362	ICE*Bs1*^{0} *amyE*::{(P*spank-immR*Ω27) *spc*} *thrC*::{(*xis-lacZ*) *erm*}
JMA421	ICE*Bs1*^{0} *cgeD*::{(P*immR-immR*) *kan*} *thrC*::{(*xis-lacZ*) *erm*}
JMA436	ICE*Bs1*^{0} *cgeD*::{(P*immR-immRA*) *kan*} *thrC*::{(*xis-lacZ*) *erm*}
JMA444	ICE*Bs1*^{0} *cgeD*::{(P*immR-immR*) *kan*} *amyE*::{(P*spank*(*hy*)-*rapI*) *spc*} *thrC*::{(*xis-lacZ*) *erm*}
JMA445	ICE*Bs1*^{0} *cgeD*::{(P*immR-immRA*) *kan*} *amyE*::{(P*spank*(*hy*)-*rapI*) *spc*} *thrC*::{(*xis-lacZ*) *erm*}
JMA469	ICE*Bs1*^{0} *cgeD*::{(P*immR-immR*) *kan*} *amyE*::{(P*spank-immA*Ω385) *spc*} *thrC*::{(*xis-lacZ*) *erm*}
JMA541	Δ*immR*208::*cat* *amyE*::{(P*spank-immR*Ω27) *spc*} *thrC*::{(*xis-lacZ*) *erm*}
JMA638	Δ*immR*208::*cat* *amyE*::{(P*spank-immR*Ω27) *spc*} *thrC*::{(*immR-lacZ*) *erm*}
JMA645	ICE*Bs1*^{0} *cgeD*::{(P*immR-immR*:(*immR-his*$_6$ *cat*)) *kan*} *amyE*::{(P*spank-immA*Ω385) *spc*} *thrC*::{(*xis-lacZ*) *erm*}
JMA726	(Δ*immA*720 Δ*int*::*cat*) *thrC*::{(*xis-lacZ*) *erm*}
JMA836	Δ*int*205::*cat* *cgeD*::{(P*spank*(*hy*)-*rapI*) *kan*} *thrC*::{(*xis-lacZ*) *erm*}
JMA838	(Δ*immA*720 Δ*int*::*cat*) *cgeD*::{(P*spank*(*hy*)-*rapI*) *kan*} *thrC*::{(*xis-lacZ*) *erm*}
JMA840	(Δ*immA*720 Δ*int*::*cat*) *amyE*::{(P*spank-immA*Ω385) *spc*} *thrC*::{(*xis-lacZ*) *erm*}
JMA842	(Δ*immA*720 Δ*int*::*cat*) *cgeD*::{(P*spank*(*hy*)-*rapI*) *kan*} *amyE*::{(P*spank-immA*Ω385) *spc*} *thrC*::{(*xis-lacZ*) *erm*}
***E. coli* strains:**	
AG1111[2]	F' (*lacI*^{q}) *lacZM15* Tn*10* (*tet*)
JMA622[3]	F$^-$ *ompT* *hsdS*$_B$(r_B^- m_B^-) *gal* *dcm* *araB*::(T7*RNAP-tetA*) pJMA605
***S. cerevisiae* strain:**	
PJ69-4A[4]	*trp-901* *leu2-3, 112* *ura3-52* *his3-200* *gal4*Δ *gal80*Δ *LYS2*::(*GAL1-HIS3*) *GAL2-ADE2* *met2*::(*GAL7-lacZ*)

[1] All *B. subtilis* strains are derived from *JH642* and contain *trpC2* and *pheA1* (61).

[2] MC1061 derivative strain used for cloning.
[3] BL21-AI strain (Invitrogen) containing pJMA605; used for overexpression and purification of ImmR-His_6.
[4] Strain used for yeast-two hybrid assays (39).

immR-his$_6$ was created by cloning the *immR* coding sequence (+1 to +380) along with an optimized *rbs* and spacer region (AGGAGGAAAAACAT, *rbs* is underlined) downstream of the T7 promoter in the pET21-*cat* vector to create plasmid pJMA605. pET21-*cat* was generated by introducing the chloramphenicol resistance gene from pJH101 (23) into the SphI site of pET21 (Novagen). pJMA605 was introduced into the *B. subtilis* chromosome by single crossover homologous recombination to generate *immR*:*immR-his*$_6$ *cat*.

β-galactosidase assays. β-galactosidase specific activity was assayed as described (38). Specific activity was calculated relative to the optical density at 600 nM (O.D. 600) of the samples and is plotted relative to the time of treatment or optical density of the sample as indicated.

Mating assays. Donor and recipient cells were grown in LB medium prior to mating. IPTG (1 mM) final concentration was present throughout growth of recipient cell cultures that contained P*spank-immR*Ω27. IPTG was added to donor cells to induce expression of *rapI* at O.D. 600 ~ 0.2, and filter matings were performed one hour later as described (5). Transconjugants were identified and mating frequency was calculated as described (5); the reported mating frequency is the mean of two independent experiments ± SEM.

Primer extension assays. The 5' end point of the *xis* transcript was determined through primer extension analysis. RNA was isolated from untreated wild-type cells, wild-type cells treated with MMC, cells overexpressing *rapI*, and control cells using the RNeasy kit from Qiagen according to the manufacturer's protocol. 10 μg of total RNA was reverse transcribed as

described (5), except that ~2 pmol of specific ^{32}P-labeled oligonucleotide was used as a primer. Oligonucleotides oJMA102, which is complementary to –6 to –17 relative to the *xis* translation initiation codon, and oJMA240, which is complementary to +22 to +49 of the *xis* open reading frame, were end-labelled with [γ-^{32}P]ATP (Perkin-Elmer) using T4 polynucleotide kinase (New England Biolabs) as described (73). Labeled olignucleotides were separated from unincorporated ATP prior to use in reverse transcription reactions using Qiagen's Nucleotide Removal Kit. The products of the primer extension reactions were compared to the products of dideoxynucleotide sequencing reactions performed with the fmol DNA Cycle Sequencing System (Promega) using labeled oJMA102 or oJMA240 as primers and PCR products corresponding to –6 to –131 or +22 to –131 of *xis* as template. Primer extension and dideoxynucleotide sequencing reaction products were electrophoresed on 6% polyacrylamide gels containing 7 M urea. Radioactivity was detected through phosphoimaging using the Typhoon imager 9400 (Amersham Biosciences).

Purification of ImmR-his$_6$. ImmR-his$_6$ was purified from *E. coli* cell lysates by nickel-affinity column chromatography. *E. coli* cells containing an arabinose-inducible copy of the T7 RNA polymerase and a plasmid encoding *immR-his$_6$* under the control of a LacI-repressible/IPTG-inducible T7 polymerase-dependent promoter were grown in LB (containing 200 μg/ml ampicillin) at 37° C with shaking. At O.D. 600 ~ 0.4, L-arabinose and IPTG were added to induce expression of the T7 polymerase and derepress expression of *immR-his$_6$*. 15 mls of cells were collected 4 hours after induction, pelleted by centrifugation, decanted, and stored at -20° C.

The cell pellet was thawed on ice and resuspended in 1:10 volume of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). Cells were lysed by a combination of two

cycles of freezing and thawing followed by sonication on ice for 2 X 40 sec. with a Branson 2250 sonicator on tip setting 2 with 25% duty cycle. The supernatant was separated from cell debris by centrifugation at 10,000 X g at 4° C for 20 min.

The total protein concentration of the cell lysate was determined through Bradford assay (69) and was adjusted to 1 mg/ml with fresh lysis buffer prior to purification. ImmR-his_6 was purified from the cell lysate by Ni-NTA column chromatography (Qiagen) according to the manufacturer's protocol for batch purification under native conditions, except that proteins were eluted by a stepwise increase in imidazole concentration (50, 100, 200, and 400 mM imidazole).

Elution fractions were analyzed by SDS-PAGE followed by Coomassie staining (26). The bulk of the ImmR protein was present in the 400 mM elution fraction, which was judged to be ~95% pure. The concentration of the purified ImmR protein was determined through measurement of the absorbance of the protein at 280 nm (A280) and by Bradford assay. Purified protein was stored at -20° C.

Electrophoretic mobility shift assay. Labelled *xis* promoter DNA was generated through PCR using the ^{32}P-labeled oJMA102 primer (described above) and the oJMA109 primer. The product amplified by these primers corresponds to the region cloned into the *xis-lacZ* fusion. Labeled *rapI* promoter DNA was also generated through PCR using the ^{32}P-labeled oKG2 primer and the oKG1 primer. This amplifies the *rapI* promoter region previously described (5) (Chapter 3). Labeled PCR products were purified using Qiagen's PCR purification kit. The concentrations of the labeled PCR products were estimated based on the A260 measurements of unlabeled PCR products that were synthesized and purified under identical conditions.

25 μl reactions containing labeled PCR products (800 pM final concentration) were incubated with increasing concentrations of purified ImmR protein in binding buffer (5 mM Tris,

24 mM HEPES, 50 mM potassium glutamate, 20 mM NaCl, 1.4 mM EDTA, 0.4 mg/ml BSA, 9% glycerol 20 ng/μl poly-(dI-dC) and 5 mM DTT, pH 8 (29)) at 37° C for 30 min. Mobility shift reactions were electrophoresed in a tris-glycine gel as described (11). Radioactivity was detected by phosphoimaging using the Typhoon imager 9400 (Amersham Biosciences).

Results

Characterization of the *xis* promoter. *xis*, which encodes an accessory protein required for excision of ICE*Bs1* (C.A.L., J.M.A., R.E.M. and A.D.G., manuscript in preparation), is the first gene in a putative operon of ICE*Bs1* genes whose expression increases in response to *rapI* overexpression and conditions that induce the DNA damage response (5). We used a combination of primer extension analysis and analysis of the expression of a *xis-lacZ* fusion to characterize the *xis* promoter region.

We used a radiolabelled primer complementary to the 5' end of the *xis* open reading frame to map the 5' end point of the *xis* transcript through primer extension (Fig. 1A). No product was detectable in uninduced wild-type cells (Fig. 1B). In cells overexpressing *rapI* or treated with the DNA damaging agent mitomycin C (MMC), we detected a major and minor primer extension product that terminated 39 and 38 nucleotides upstream of the *xis* start codon, respectively (Fig. 1B). The major product likely represents the primary transcription initiation point. The minor product likely represents an alternative, less utilized transcription initiation point. Both the major and minor products were detected through primer extension with a second radiolabelled primer complementary to sequence internal to the *xis* open reading frame (data not shown).

We examined the sequence upstream of *xis* and identified a sequence 5 base pairs upstream of the start of the major transcript identified through primer extension that has perfect consensus

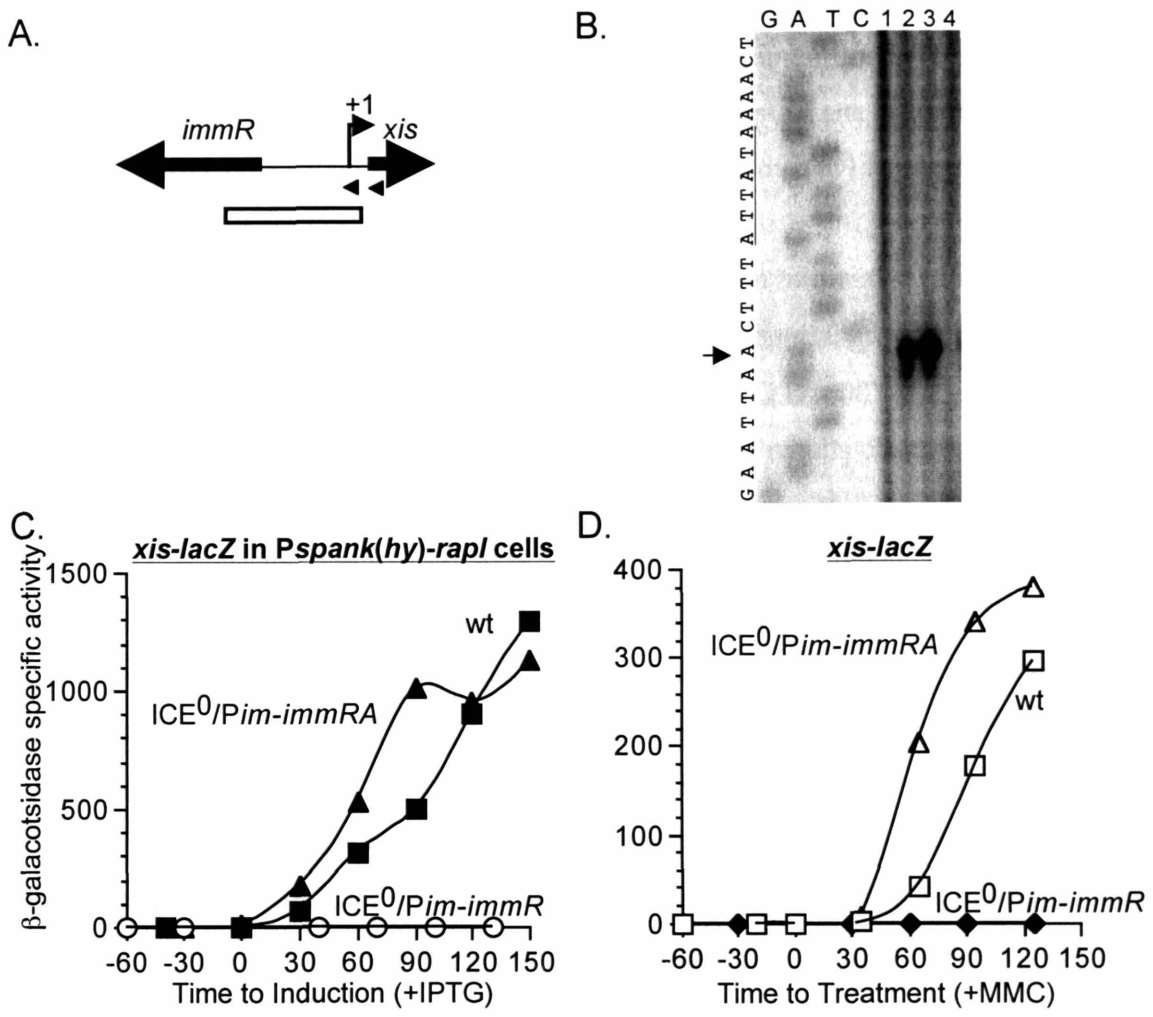

Figure 1. Characterization of the *xis* promoter and its repression by ImmR.

A. Schematic of *xis*, *immR*, and the shared intergenic region. Triangles indicate the positions of primers used for primer extension assays described in part B. The position of the 5' end point of the *xis* transcript identified in part B is indicated by the +1. The white box indicates the region upstream of *xis* cloned in the promoterless *lacZ* vector and used to monitor *xis* expression.

B. The 5' end point of the *xis* transcripts was determined through primer extension assays. RNA was isolated from treated and untreated cells one hour after treatment with MMC or 30 min. after treatment with IPTG. Results of reverse transcription reactions with the primer proximal to +1 are shown; similar results were seen when reverse transcription reactions were carried out with the primer distal to +1 (data not shown). G, A, T, and C indicate the lanes containing dideoxynucleotide sequencing reactions with the indicated nucleotide. The

nucleotides identified in the sequencing reactions are indicated on the left side of the gel image. The sequence complementary to the consensus –10 region is underlined. The arrow indicates the nucleotide complementary to the 5' end of the major transcript. Lane 1, untreated wild-type cells; Lane 2, wild-type cells treated with MMC; Lane 3, P*spank(hy)-rapI* cells treated with IPTG; Lane 4, *Pspank(hy)* cells treated with IPTG.

C and D. Cells containing a *xis-lacZ* fusion were grown in minimal media and samples for β-galactosidase activity assays were collected throughout exponential growth. β-galactosidase specific activity was calculated relative to the cell densities (O.D. 600) of the cultures. Results shown are from a single experiment and are representative of results observed in at least two independent experiments.

C. *xis-lacZ* expression was monitored in P*spank(hy)-rapI* cells. IPTG, at 1 mM final concentration, was added to cells in mid-exponential phase (O.D. 600 = 0.4-0.6). *int* (encoding Integrase) was deleted in Pspank(*hy*)-*rapI* Δ*int xis-lacZ*. Deletion of *int* in P*spank(hy)-rapI* cells prevents excision and loss of ICE*Bs1* which can occur when the P*spank(hy)-rapI* allele is present (data not shown). RapI overexpression induced *xis-lacZ* expression to a similar level in *int*+ cells (data not shown). β-galactosidase specific activities are plotted relative to the time of IPTG addition. P*spank(hy)-rapI* Δ*int xis-lacZ* (JMA258,■, wt); P*spank(hy)-rapI* ICE*Bs1*^{0} P*immR-immR xis-lacZ* (JMA444, ○, ICE^0/P*im-immR*); P*spank(hy)-rapI* ICE*Bs1*^{0} P*immR*-(*immR immA*) *xis-lacZ* (JMA446,▲, ICE^0/P*im-immRA*).

D. *xis-lacZ* expression was assayed in cells treated with MMC, which was added to cells in mid-exponential phase (O.D. 600 = 0.4-0.6). β-galactosidase specific activities are plotted relative to the time of MMC addition. *xis-lacZ* (JMA201, □, wt); ICE*Bs1*^{0} P*immR-immR xis-lacZ* (JMA421,◆, ICE^0/P*im-immR*); ICE*Bs1*^{0} P*immR*-(*immR immA*) *xis-lacZ* (JMA436,△, ICE^0/P*im-immRA*).

to the –10 recognition sequence of the *B. subtilis* housekeeping sigma factor, σ^A, bound to RNA polymerase ($E\sigma^A$) (34, 40). We also identified a near perfect consensus to the –35 recognition sequence of $E\sigma^A$ (TTGACT, differs from consensus sequence at underlined position) 17 base pairs upstream of the –10 recognition sequence. In combination, these data indicate that transcription of *xis* likely initiates from a sigma-A dependent promoter located ~70 bp upstream of *xis* and that transcription from this promoter increases dramatically under inducing conditions.

To facilitate genetic analysis of factors that regulate *xis* transcription, we created a transcriptional fusion of the region upstream of the *xis* open reading frame to *E. coli lacZ* and integrated this fusion at an ectopic chromosomal locus (Fig. 1A). We analyzed the effects of *rapI* overexpression and treatment with MMC on expression of *xis-lacZ*. We found that *xis-lacZ* was normally expressed at very low levels in wild-type cells and that expression increased dramatically in response to overexpression of *rapI* (Fig. 1C) or treatment of cells with MMC (Fig. 1D). These results are in accordance with the results of the transcriptional profiling experiments and primer extension analysis and indicate that this region of DNA contains the sequence necessary for appropriate regulation of *xis* transcription. Furthermore, the *xis-lacZ* fusion is likely also a good indicator of the expression of ICE*Bs1* conjugation genes encoded downstream of *xis*, as transcriptional profiling experiments indicate that the levels of transcripts from the ICE*Bs1* conjugation genes increase concomitant with the level of *xis* transcripts (5) (Chapter 3).

Transcription of *xis* is repressed by the immunity repressor. We previously identified a gene encoding a putative immunity repressor, *immR*, based on the similarity of its predicted protein product to repressors from other Gram-positive bacteriophages (5). We characterized the role that ImmR plays in regulating transcription of *xis* by assaying the effects of deleting *immR*

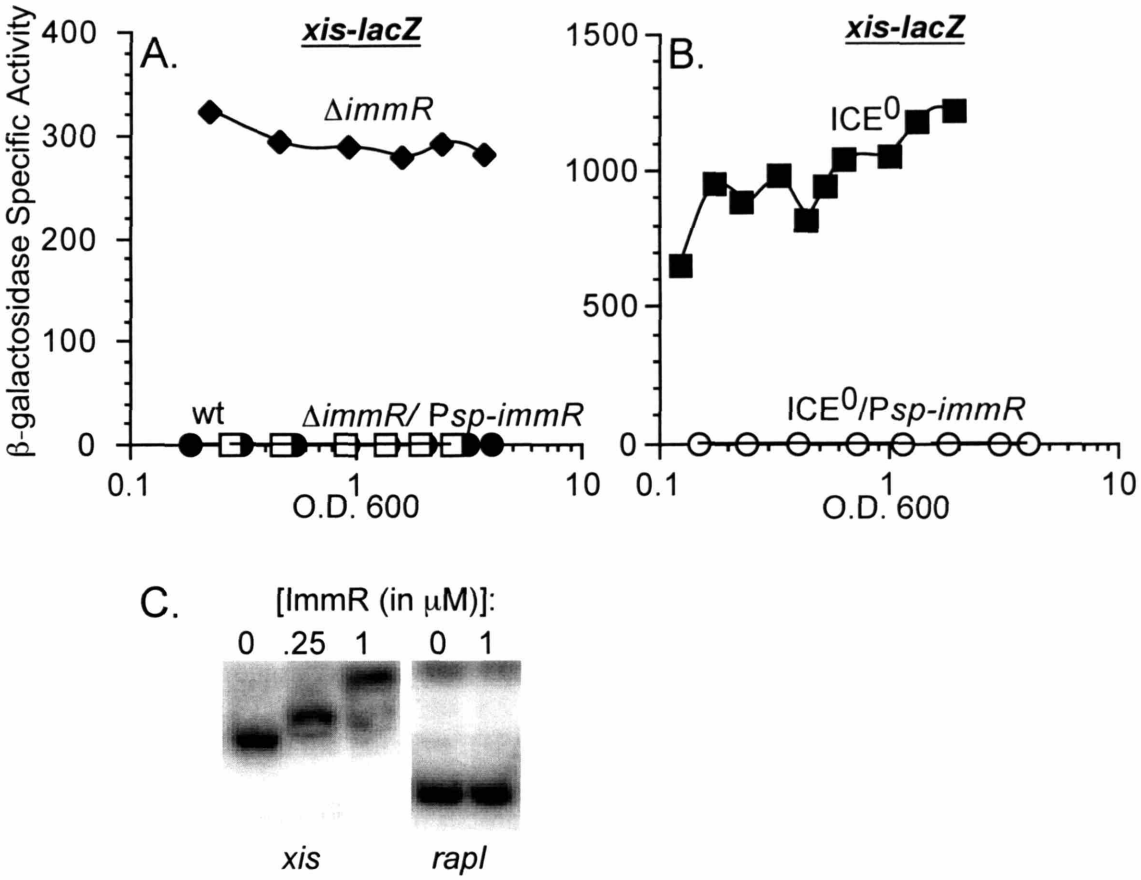

Figure 2. ImmR binds to the *xis* promoter and represses transcription of *xis*.

A and B. *xis-lacZ* expression was monitored throughout exponential growth in minimal medium. IPTG was present throughout growth at 1 mM final concentration when needed to induce expression from P*spank-immR*Ω27. β-galactosidase specific activities are plotted relative to the O.D. 600 measurements of the cultures.

A. *xis-lacZ* expression in wild-type (JMA201, □, wt), Δ*immR* (JMA214, ◆, Δ*immR*), and Δ*immR* P*spank-immR*Ω27 (JMA541, ●, Δ*immR*/P*sp-immR*) cells.

B. *xis-lacZ* expression in ICE*Bs1*^{0} (JMA264, ■, ICE^0) and ICE*Bs1*^{0} P*spank-immR*Ω27 (JMA362, ○, ICE^0/P*sp-immR*) cells.

C. ImmR binding to the *xis* promoter was monitored *in vitro* through electrophoretic mobility shift assays. Purified ImmR was incubated with 800 pM ^{32}P-labeled *xis* or *rapI* promoter region DNA as described in Methods. The concentrations of ImmR in each reaction are indicated.

(Δ*immR*) on *xis-lacZ* expression. We found that *xis-lacZ* expression increased in Δ*immR* cells and that expression of *xis-lacZ* could be restored to wild-type levels in Δ*immR* mutant cells complemented with an ectopic copy of *immR* expressed from the IPTG-dependent promoter, P*spank* (Fig. 2A). These results indicate that ImmR functions as a repressor of *xis* transcription.

In addition to increased expression of *xis-lacZ*, deletion of *immR* resulted in increased excision of ICE*Bs1* and premature lysis of colonies grown on LB plates; these phenotypes could also be suppressed by complementation of the Δ*immR* mutation with P*spank-immR* (data not shown). Increased excision in Δ*immR* cells is likely due to increased expression of *xis,* as increased expression of *xis* alone is sufficient to stimulate excision (C.A.L., J.M.A., R.E.M. and A.D.G., manuscript in preparation). Lysis of colonies lacking *immR* may be due to high levels of expression of genes that form the putative ICE*Bs1* mating pore (5), as cells that completely lack ICE*Bs1* do not exhibit increased lysis in stationary phase (data not shown).

These results demonstrated that ImmR was necessary to repress *xis-lacZ* expression, but did not indicate if additional ICE*Bs1*-encoded proteins were required for repression. In order to test this, we monitored the effects of *immR* expression on *xis-lacZ* expression in cells that lack ICE*Bs1* (ICE*Bs1*^{0}). We found that *xis-lacZ* expression was derepressed in ICE*Bs1*^{0} cells and that expression of *xis-lacZ* could be restored to the levels observed in wild-type ICE*Bs1*$^+$ cells by ectopic expression of *immR* (Fig. 2C). Although de-repressed expression of *xis-lacZ* is ~10-fold higher in ICE*Bs1*^{0} cells than in Δ*immR* cells, we think this difference is likely due to the effects of the Δ*immR* mutation on the health of the cells and not due to the presence of a second ICE*Bs1*-encoded repressor, as deletion of all the genes in ICE*Bs1* except for *immR, immA*, and *int* has no effect on *xis-lacZ* expression (data not shown). In combination, these genetic analyses

indicate that ImmR is both necessary and sufficient to repress transcription from the *xis* promoter, thereby inhibiting expression of *xis* and downstream ICE*Bs1* genes.

ImmR binds to the *xis* promoter. The results of the genetic analyses did not distinguish whether ImmR acted directly on the *xis* promoter or regulated a chromosomally-encoded regulator of *xis* expression. We reasoned that if ImmR were able to bind specifically to *xis* DNA in the absence of other cellular factors, it was likely a direct repressor of *xis* expression. Therefore, we overexpressed and purified recombinant ImmR-his_6 from *E. coli* cells. This recombinant ImmR protein has near wild-type levels of function *in vivo*; it is able to repress *xis-lacZ* expression in *B. subtilis* cells but has a slightly higher basal level of *xis-lacZ* expression due to the presence of the His-tag than cells expressing non-recombinant *immR* (data not shown).

We tested the ability of this recombinant protein to bind to DNA from the *xis* promoter region DNA through electrophoretic mobility shift assays. We found that ImmR binds specifically to the DNA from the *xis* promoter but not to control DNA from the *rapI* promoter (Fig. 2C), indicating that ImmR likely represses *xis* expression directly. In addition, we observed increased gel retardation of the ImmR-*xis* promoter DNA complex at higher ImmR concentration, indicating that ImmR likely binds at multiple sites in the intergenic region between *immR* and *xis*.

Consistent with the hypothesis that ImmR binds to multiple sites in the *xis-immR* intergenic region, we identified four putative ImmR binding sequences (Fig. 3A & 3B). Some repressors bind to DNA as homodimers, with monomers recognizing nearly identical sequences on complementary strands of DNA (inverted repeat sequences) (19, 31, 51). The four putative ImmR binding sequences we identified are imperfect inverted repeats (Fig. 3B); the positions of

these sequences indicate that they may function in repression of *xis* transcription and activation and repression of *immR* transcription.

Identification of the *immR* promoter. The approximate location of the *immR* promoter was identified through cloning and sequence analysis. The *immR* promoter was initially localized to the region 268 bp upstream of *immR*. We introduced *immR* along with 268 bp of upstream sequence into an ectopic chromosomal locus in ICE*Bs1*^{0} cells containing an *xis-lacZ* fusion and assayed *xis-lacZ* expression. We found that P*immR-immR* was sufficient for expression of ImmR and repressed *xis-lacZ* expression to the same low levels as in wild-type cells (Fig. 1D).

Further refinement of the location of the *immR* promoter came through analysis of two additional *immR* expression constructs (Fig. 3A). We used the P*spank-immR*Ω27 fusion, which contains the *immR* ORF and 27 bp of upstream sequence downstream of the inducible promoter P*spank* for the complementation experiments described in Fig. 2. Expression of *immR* from P*spank-immR*Ω27 required addition of inducer (IPTG); P*spank-immR*Ω27 containing cells were unable to restore repression of *xis-lacZ* in Δ*immR* or ICE*Bs1*^{0} cells in the absence of inducer (data not shown). These results indicated that the 27 bp upstream of *immR* did not contain a functional promoter.

However, Δ*immR* or ICE*Bs1*^{0} cells that contained the P*spank-immR*Ω142 fusion (Fig. 3A), which includes 142 bp of sequence upstream of *immR*, did not require addition of inducer to repress *xis-lacZ* expression (data not shown). These results indicated that the functional *immR* promoter is present in the longer P*spank-immR*Ω142. Analysis of this DNA sequence revealed a near consensus match to an extended –10 type recognition motif for Eσ^A (34, 40). This sequence, TG(N)TATTAT, which differs from consensus at one position (underlined), was present in the P*spank-immR*Ω142 fusion and absent in the P*spank*Ω27 fusion. Based on this

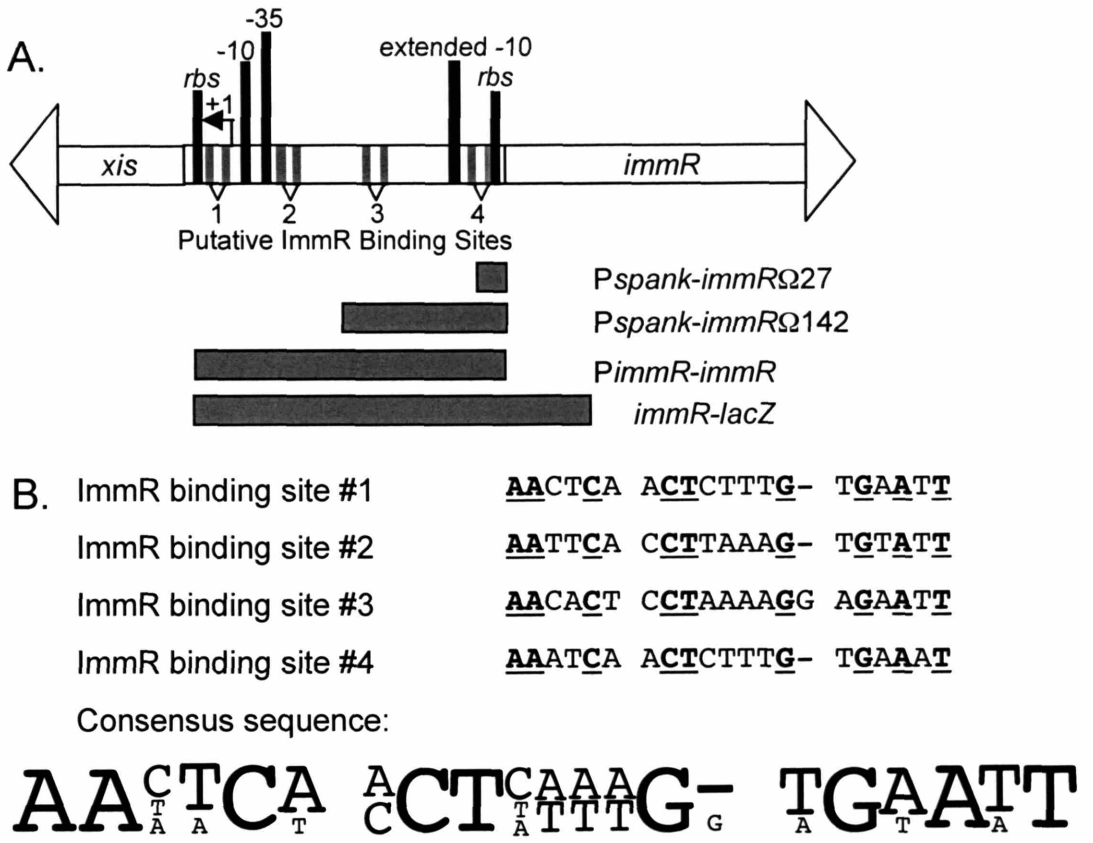

Figure 3. Characterization of the *immR-xis* intergenic region.

A. The *xis* and *immR* genes (arrows) and the shared intergenic region are shown in the diagram. The locations of the putative ribosome binding sites (*rbs*), the extended –10 promoter of *immR*, and the ImmR binding sites are indicated as well as the +1 of *xis* transcription and the –10 and –35 regions identified in Fig. 1. The boxes underneath the diagram indicate the sequences of DNA present in the indicated *immR* constructs.

B. The sequences of the four putative ImmR binding sites are shown. The two half sites are separated by a spacer region that contains some conserved sequences and varies in length from 8-9 base pairs. The nucleotide positions that are conserved in all four sequences are in bold-face type and underlined. The consensus sequence is indicated below the four putative binding sites, with the size of the letter corresponding to the frequency with which that nucleotide is found in the potential binding sites.

evidence, it is likely that *immR* transcription is dependent upon this putative extended –10 sequence.

Autoregulation of *immR* transcription. We also assayed the role of ImmR in regulating its own transcription. We monitored expression of *immR* using a fusion of a portion of *immR* and its upstream sequence to *lacZ* expressed from an ectopic chromosomal locus (Fig. 3A). *immR-lacZ* was expressed throughout growth in wild-type cells (Fig. 4). In Δ*immR* cells, *immR-lacZ* expression decreased, indicating that ImmR activates expression of *immR-lacZ* (Fig. 4). This defect in *immR-lacZ* expression in Δ*immR* cells could be suppressed by ectopic expression of *immR* (Fig. 4). Restoration of *immR-lacZ* to near wild-type levels in Δ*immR* P*spank-immR*Ω27 cells was dependent upon a low concentration of inducer (25 μM IPTG). When Δ*immR* P*spank-immR*Ω27 cells were grown in the presence of a 40-fold higher concentration of inducer (1 mM IPTG), *immR-lacZ* expression was slightly lower than that observed in wild-type cells (Fig. 4). Although this decrease was small, it was observed in multiple experiments.

These results clearly demonstrate ImmR activates its own transcription. These data also provide evidence that ImmR represses its own transcription at higher concentrations. Autoregulation of *immR* transcription is similar to the autoregulation observed for λ repressor, which activates its own transcription at low concentration and represses its own transcription at high concentration (reviewed in 21, 35). In the case of λ regulation, negative autoregulation is needed to maintain low enough concentrations for proper lysogenic induction (20, 21); this may also be true for regulation of *immR* transcription.

ImmR mediates immunity from acquisition of a second copy of ICE*Bs1*. We previously observed that transfer of ICE*Bs1* into cells that lack ICE*Bs1* occurred at ~50-fold higher frequency than into cells that contained ICE*Bs1* (5). Although intercellular peptide signaling

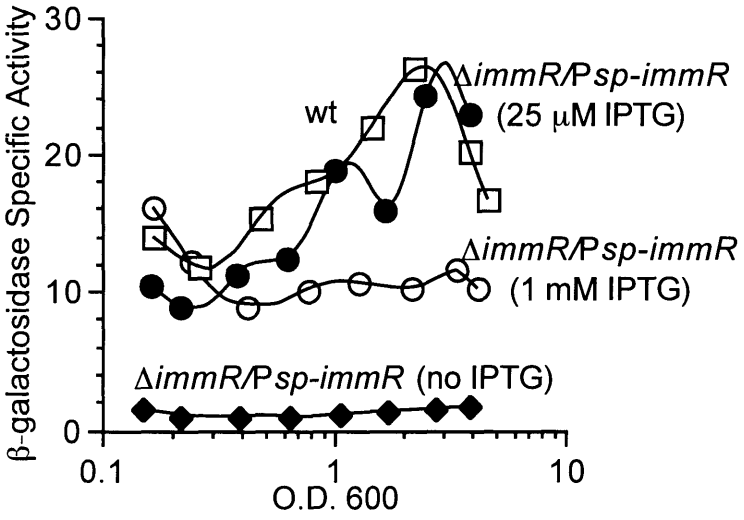

Figure 4. Expression of *immR* is autoregulated.

Expression of an *immR-lacZ* fusion was monitored throughout exponential growth in minimal medium in otherwise wild-type cells (JMA309, □), and in Δ*immR* P*spank-immR*Ω27 cells (JMA310) grown in the absence of IPTG (◆, Δ*immR*/P*sp-immR*), or in the presence of 25 μM IPTG (●) or 1 mM IPTG (○). IPTG at the indicated concentrations was present throughout growth. β-galactosidase specific activities are plotted relative to the O.D. 600 measurements of the cultures.

inhibits the ability of donor cells to transfer ICE*Bs1* in the presence of recipient cells that produce the peptide, this regulation occurs at the level of transcription of *xis* and the ICE*Bs1* conjugation genes. The ~50-fold inhibition we observed was likely not a result of peptide signaling as expression of *xis* and the ICE*Bs1* conjugation genes was stimulated by overexpression of *rapI* in the donor cells prior to exposure to recipient cells. Therefore, we looked for other ICE*Bs1* encoded proteins that would inhibit transfer of ICE*Bs1* into recipient cells.

Table 2: Frequency of ICE*Bs1* mating from P*spank(hy)-rapI* Δ*rapIphrI* donor cells (JMA168) into recipient cells.

Recipient:	Mating Frequency[1]:
ICE*Bs1*$^{+}$ *comK* (JMA174)	$1.3 \times 10^{-4} \pm 1.0 \times 10^{-5}$
ICE*Bs1*^{0} *comK* (REM10)	$1.9 \times 10^{-2} \pm 1.7 \times 10^{-2}$
ICE*Bs1*^{0} *amyE*::P*spank-immR comK*(JMA368)	$4.6 \times 10^{-4} \pm 4.2 \times 10^{-4}$

[1] Mating assays were performed as described (5). The mating frequency is the mean number of transconjugants per donor cell (± SEM).

In some bacteriophage, the phage-encoded repressor mediates immunity to superinfection with another copy of a similar bacteriophage (64). As the ImmR protein is closely related to bacteriophage repressors (5), we thought it was likely that the presence of ImmR in recipient cells could inhibit acquisition of a second copy of ICE*Bs1*. Therefore, we compared transfer of ICE*Bs1* from donor cells into ICE*Bs1*^{0} recipient cells that either did or did not express *immR*. We found that transfer of ICE*Bs1* into ICE*Bs1*^{0} cells that expressed *immR* occurred at a similar frequency to transfer into ICE*Bs1*$^{+}$ cells. These results were observed when *immR* was expressed from the inducible P*spank* promoter (Table 2) or from its native promoter (data not shown). These data indicate that ImmR is the only ICE*Bs1*-encoded protein required for inhibition of acquisition.

These assays measure the final endpoint of mating, successful integration of the element into the chromosome, and do not indicate at which point ImmR acts to inhibit acquisition of ICE*Bs1*. Based on the analysis of other mobile elements, at least three different points of regulation are possible – inhibition of contact between donor and recipient cell (surface exclusion, 1, 3), inhibition of uptake of DNA into the cell (entry exclusion, 1, 3), and inhibition of expression of genes required for integration (22, 64). In cells lysogenic for bacteriophage λ, the λ cI repressor prevents lytic development of a superinfecting λ phage by repressing transcription of the late gene promoters (reviewed in 64). As *int* expression is directly and indirectly controlled by late gene promoters (reviewed in 22), integrase is not expressed in λ lysogens, which limits integration of the newly infecting phage. Based on its role as a regulator of gene expression, it is likely that ImmR also prevents acquisition of a second element by regulating ICE*Bs1* transcription.

Induction by RapI and the global DNA damage response requires both ImmR and ImmA. Although *xis-lacZ* expression was repressed in ICE*Bs1*^{0} cells expressing *immR*, *xis-lacZ* expression was not de-repressed in response to overexpression of *rapI* (Fig. 1C) or treatment of cells with the DNA-damaging agent MMC (Fig. 1D). These results indicated that an additional ICE*Bs1* protein or proteins is required for de-repression of *xis* expression in response to inducing signals.

Insights into the additional ICE*Bs1*-encoded protein required for de-repression of *xis* expression were provided through analysis of ICE*Bs1* deletion derivatives. In these experiments, we found that a minimal element containing *int*, *xis*, *immR* and an uncharacterized gene, *immA*, was capable of excision and de-repression of *xis-lacZ* in response to either *rapI* overexpression or treatment with the DNA damage response (C.A.L., J.M.A., R.E.M. and A.D.G., manuscript in

preparation), indicating that these four ICE*Bs1* genes were sufficient for appropriate regulation of *xis* expression.

As Int and Xis mediate site-specific recombination, we thought the most likely candidate to regulate *xis* expression was *immA*. We assayed *xis-lacZ* expression in cells that co-expressed *immR* and *immA* from their native promoter (P*immR-immR immA*). We found that expression of *immR* and *immA* was sufficient for repression of *xis-lacZ* expression under non-inducing conditions and de-repression of *xis-lacZ* in response to *rapI* overexpression (Fig. 1C) or treatment with MMC (Fig. 1D). Expression of *immA* alone in ICE^0 cells had no effect on *xis-lacZ* expression (data not shown). These results indicate that ImmR represses *xis* expression and that ImmA is needed to antagonize ImmR activity in response to inducing signals.

Δ*immA* cells do not respond to inducing signals. Consistent with these results, we also found that in ICE*Bs1* cells that lacked *immA*, *xis-lacZ* expression was not de-repressed in response to treatment with mitomycin C (Fig. 5) or overexpression of *rapI* (data not shown). This defect in de-repression of *xis-lacZ* could be partially suppressed by ectopic expression of *immA* from the inducible promoter P*spank* (Fig. 5). These results provide further evidence that ImmA is required to antagonize ImmR's activity in response to inducing conditions.

Incomplete suppression of the Δ*immA* phenotype by the P*spank-immA* construct is likely due to inappropriate levels of *immA* expression from this construct, as we also observed only partial de-repression of *xis-lacZ* expression in ICE*Bs1*^{0} P*immR-immR* cells that expressed P*spank-immA* *in trans* (data not shown). *immR* and *immA* are normally encoded together on a single transcript with the initiation codon of *immA* (GTG) overlapping the termination codon of *immR* (TGA) in the sequence GTGA. This overlap in sequence may indicate that translation of *immA* is normally coupled to *immR*, as an overlap of initation and termination codons is observed with other

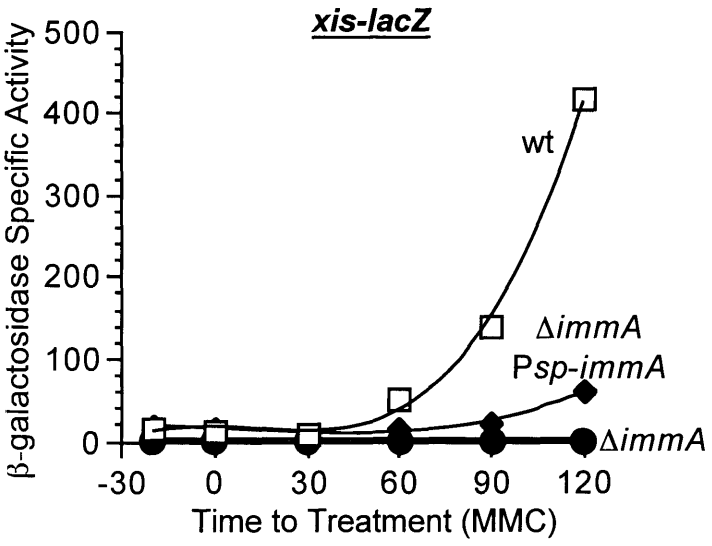

Figure 5. ImmA is required for derepression of *xis* expression. Expression of *xis-lacZ* was monitored throughout exponential growth in minimal medium in Δ*int* control cells (CAL16, □, wt), Δ*immA* Δ*int* cells (JMA726, ●, Δ*immA*), and Δ*immA* Δ*int* P*spank-immA* cells (JMA840, ◆, Δ*immA* P*spank-immA*). MMC was added at the indicated time, and β-galactosidase specific activity is plotted relative to the time of MMC addition.

proteins that are translationally coupled (37, 50). Translational coupling can be important for appropriate levels of expression of the coupled proteins (37, 50, 66) or folding of the downstream protein (7). Translational coupling could explain the observation that *immA* expressed *in cis* with *immR* is able to restore wild-type levels of de-repression to *xis-lacZ*, while *immA* expressed *in trans* results in ~10-fold lower level of *xis-lacZ* expression under inducing conditions (compare Fig. 1 to Fig. 5).

ImmA interacts directly with ImmR. We reasoned that if ImmA antagonizes the activity of ImmR directly, then these two proteins should interact. We looked for interaction of these proteins through yeast two-hybrid analysis by introducing fusion proteins of ImmR to the Gal4 activation domain (AD) and ImmA to the Gal4 DNA binding domain (BD) into *S. cerevisiae* cells that expressed the *ADE2* gene, a gene required for adenine synthesis, under the control of a Gal4-activated promoter. Growth of these cells on medium lacking adenine requires an interaction between ImmR and ImmA to unite the two domains of Gal4 and activate transcription of *ADE2*.

Cells that contained the ImmR and ImmA fusion proteins were able to grow on medium lacking adenine (Table 3), indicating that ImmR and ImmA interact. We also detected self-interaction between ImmR fusion proteins, indicating that this protein likely acts as a dimer or higher order multimer to regulate gene expression (Table 3). This is not surprising as several phage repressors act as dimers or multimers to regulate gene expression (19, 31, 68, 74, 77). The interaction of ImmR was specific for ImmR and ImmA, as we did not detect interaction of ImmR fusion proteins with RapI-Gal4-BD (Table 3) or RapI-Gal4-AD fusion proteins (data not shown). We were also unable to detect interaction between ImmA and RapI (Table 3), indicating that if these two proteins normally interact in *B. subtilis*, this interaction may either be transient or

Table 3: Characterization of interactions between ImmR, ImmA, and RapI through yeast two-hybrid assays

Fusion proteins[1]	Growth on -ura -leu -ade[2]	Growth on -ura -leu[3]
AD - ImmR BD - ImmR	+	+
AD - ImmR BD - ImmA	+	+
AD - ImmR BD - RapI	--	+
AD - RapI BD - ImmA	--	+

[1] *immR*, *immA*, and *rapI* were fused in-frame to either the GAL4 DNA binding domain (BD) or activation domain (AD) and transformed into *Saccharomyces cerevisiae* strain PJ69-4A (39), which contains the *ADE2* gene under the control of the Gal4-dependent *GAL2* promoter.

[2] Interaction of the fusion proteins is indicated by growth of transformants on synthetic complete medium lacking uracil (-ura) and leucine (-leu), which selects for maintenance of the fusion protein encoding plasmids, and lacking adenine (-ade), which selects for Gal4-dependent transcription from *ADE2*. + : growth; -- : no growth

[3] Growth of cells on medium lacking uracil and leucine is shown as a control for maintenance of the plasmids encoding the fusion proteins.

require the presence of an additional protein, such as ImmR. These results indicate that the ImmR and ImmA proteins interact and support the hypothesis that ImmA is able to modulate the activity of the ImmR protein through direct interaction.

Discussion

The results of these experiments have shown that ImmR is the ICE*Bs1* immunity repressor: ImmR inhibits expression of genes that mediate excision and transfer of ICE*Bs1* and mediates immunity against acquisition of a second copy of ICE*Bs1*. ImmR also regulates the transcription of its own promoter, which drives transcription of *immR, immA*, and *int*. This auto-regulation likely allows ImmR to maintain levels of ImmR sufficient for repression of *xis* and the ICE*Bs1* conjugation genes while keeping the levels of ImmR and ImmA at the appropriate concentrations

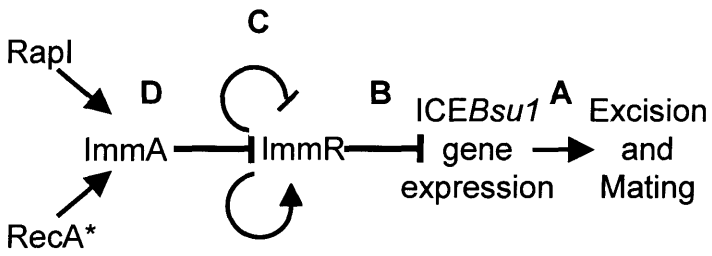

Figure 6. Model for two-protein regulatory mechanism governing ICE*Bs1* gene expression, excision, and transfer.

A. Expression of *xis* and the ICE*Bs1* conjugation genes results in excision and conjugal transfer of ICE*Bs1*.

B. ImmR represses transcription of *xis* and the ICE*Bs1* conjugation genes, thereby preventing excision and transfer.

C. Transcription of ImmR is also autoregulated

D. When ImmA is stimulated by the inducing signals, either RapI or RecA bound to single-stranded DNA (RecA*), ImmA antagonizes the activity of ImmR. This leads de-repression of *xis* and ICE*Bs1* conjugation gene transcription and results in increased excision and transfer.

for rapid derepression by inducing signals. Furthermore, ImmR is the only ICE*Bs1*-encoded gene required to repress ICE*Bs1* gene expression and mediate immunity (Fig. 6). However, de-repression of ICE*Bs1* gene expression in response to RapI or RecA requires a second ICE*Bs1*-encoded gene, *immA* (Fig. 6). ImmA interferes with the activity of ImmR, likely through a direct interaction with ImmR, as yeast-two hybrid analysis revealed that these two proteins interact.

Models for the regulation of ImmR activity by ImmA. ImmA could utilize several mechanisms to antagonize ImmR, such as binding to ImmR and interfering with DNA binding or ImmR oligomerization. ImmA could also mediate proteolysis of ImmR, either directly or indirectly. Comparative sequence analysis using the conserved domain architecture retrieval tool (C-DART, 27) indicated that ImmA contains conserved residues found in the active sites of Zinc-dependent metalloproteases (25, and references therein), and it is possible that this domain is a functional protease domain that could mediate proteolysis of ImmR.

Several repressor antagonists have been characterized; many of these proteins are antirepressors that function by direct interaction with their repressor proteins. Tum, an antirepressor from coliphage 186, binds to the coliphage 186 cI repressor and inhibits DNA binding (68). The P1 antirepressor Coi forms an equimolar complex with the c1 repressor of P1 that inhibits binding of c1 to DNA (33). E, an antirepressor from the satellite phage P4, forms a multimeric complex with the C repressor from phage P2; this complex prevents C repressor from binding to DNA and repressing transcription (46). The satellite phage RS1 encodes the RstC protein, an antirepressor that promotes aggregation of the RstR repressor of the CTXø phage (18). The P22 antirepressor also binds to the c2 repressor and inhibits its ability to repress transcription (72). In contrast, another type of repressor antagonist is typified by the λ cro protein, which binds directly to DNA and competes with the λ cI repressor for its binding sites

(31). ImmA is likely more analogous to the characterized antirepressors than to Cro, as it interacts directly with ImmR and lacks an obvious DNA-binding motif. However, further work will be needed to distinguish between these models.

Models for activation of ImmA by RapI and RecA. These results also demonstrated that ImmA antagonizes the activity of ImmR in response to increased RapI activity or induction of the RecA-dependent DNA damage response. It is currently unclear how these inducing conditions stimulate the activity of ImmA, although it is unlikely that these conditions regulate *immA* transcription, as *immA* transcription and translation appear to be coupled to transcription and translation of *immR*. In both *E. coli* and *B. subtilis*, RecA, when bound to ssDNA, stimulates autocleavage of the global SOS repressor, LexA (4, and references therein). RecA also stimulates autoproteolysis of the λ cI, 434 cI, and P22 c2 repressors through a similar mechanism; this autoproteolysis results in de-repression of lytic gene expression (49, 78), and references therein). Autocleavage of *E. coli* LexA, λ cI, 434 cI, and P22 c2 is dependent upon the presence of catalytic residues in the C-terminus (49, 70). These residues are also present in *B. subtilis* LexA and are likely required for autocleavage (54). However, these conserved residues do not appear to be present in the ImmA protein, indicating that RecA likely stimulates its activity through an alternative mechanism.

Other RecA-dependent mechanisms for inducing expression of bacteriophage genes in response to DNA damage are found in coliphage 186 and the CTXø phage of *Vibrio cholerae*. In the CTXø phage, lytic gene expression is partially repressed by the LexA repressor, which is inactivated during the global DNA damage response (62). In coliphage 186, LexA inhibits transcription of the Tum antirepressor (68). However, a LexA-dependent mechanism does not regulate induction of ICE*Bs1* gene expression, as ICE*Bs1* gene expression is not repressed by

LexA and gene expression is still induced by the RecA-dependent DNA damage response in *lexA*- mutants (A.I. Goranov and A.D.G., manuscript in preparation).

Other Rap proteins are known to interact with response regulator proteins and either interfere with the ability of these proteins to bind to DNA (9, 16, 57) or stimulate auto-dephosphorylation of these proteins (41,60). The primary feature of Rap proteins that are thought to be important for activity is the presence of several TPR domains, which are thought to mediate protein-protein interaction (41). Although ImmA is not a response-regulator type protein, RapI may still bind to this protein and cause a new antagonistic interaction between ImmA and ImmR to occur.

The putative Zinc metalloprotease domain present in ImmA could be important for activation of ImmA by RapI and RecA. If ImmA is capable of autoproteolysis mediated by the Zinc-metalloprotease domain, both RapI and RecA could bind to ImmA and stimulate its autoproteolytic activity. If RapI binds to ImmA to stimulate its activity, this interaction is likely transient or requires the presence of ImmR, as yeast two-hybird assays failed to detect a direct interaction between ImmA and RapI. This new form of ImmA could then antagonize the activity of ImmR. Alternatively, RapI and RecA could potentially stimulate proteolytic activity of ImmA against ImmR. Further investigation will be needed to explore these hypotheses about the molecular mechanisms of ImmA activation by RapI and RecA as well as ImmA inhibition of ImmR.

Signaling through ImmR and ImmA-like proteins may regulate other mobile genetic elements. We identified several homologs of ImmA and ImmR through comparative sequence analysis (BLAST (2) and CDART (27)), and found that many of these proteins are encoded in

Table 4: ImmR and ImmA homologs in mobile genetic elements and putative mobile genetic elements

Organism	Element	ImmR[1] (%Id/%Sim)	ImmA[2] (%Id/%Sim)	Damage Inducible	Ref
Known mobile elements:					
Bacillus clarkii	BCJA1c phage	gp5 (29/48)	gp4 (39/59)	No	(43)
B. subtilis	ø105 phage	cø105 (24/50)	Orf2 (32/52)	Yes	(53)
B. subtilis	PBSX phage	Xre (34/50)	XkdA (N.S.)	Yes	(52)
B. subtilis	*skin* element[3]	YqaE (34/52)	YqaB (N.S.)	No	(42)
B. thuringiensis	MZTP02 phage[4]	AAX62112.1 (N.S.)	AAX62113.1 (34/50)	--[5]	--[6]
Listeria monocytogenes	A118 phage	gp36 (33/62)	Gp35 (N.S.)	Yes	(47)
Streptococcus pneumoniae	MM1 phage	CI (25/52)	Orf2 (N.S.)	Yes	(55)
S. thermophilus	o1205 phage	Orf4 (35/43)	Orf3 (N.S.)	Yes	(71)
Putative mobile elements[7]					
Bacillus anthracis	λBa04	BA3829 (30/52)	BA3830 (33/52)	--[5]	(63)
B. halodurans C-125	--	BH3549 (22/42)	BH3550 (38/57)	--[5]	(13)
Desulfitobacterium hafniense DCB-2 ctg1078	--	DhafDRAFT_2630 (22/40)	DhafDRAFT_2631 (N.S.[2])	--[5]	--[6]
Enterococcus faecalis V583	--	EF2544 (45/68)	EF2545 (42/64)	--[5]	(59)
E. faecium DO ctg653	--	EfaeDRAFT_2195 (29/47)	EfaeDRAFT_2196 (N.S.[2])	--[5]	--[6]
Listeria innocua CLIP 11262	--	Lin1762 (31/52)	Lin1763 (28/47)	--[5]	(28)
L. innocua CLIP 11262	--	Lin1234 (32/56)	Lin1233 (35/55)	--[5]	(28)
Staphylococcus haemolyticus JSC1435	--	SH1805 (26/55)	SH1806 (N.S.[2])	--[5]	--[6]
Thermoanaerobacter tencongensis	--	TTE2125 (26/49)	TTE2126 (26/45)	--[5]	(6)

[1] All ImmR-like proteins contain a predicted phage repressor helix-turn-helix motif identified by C-DART (27). 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 (27). 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] *skin* is a defective prophage
[4] MZTP02 was identified as a phage in direct submission of sequence to NCBI
[5] Response of element to DNA damage has not been reported.
[6] Unpublished sequence deposited in NCBI.
[7] Putative mobile genetic elements were identified based on the presence of multiple genes predicted to encode proteins homologous to those found in bacteriophages, transposons, or conjugative elements.

known or putative mobile genetic elements (Table 4). Most of the characterized mobile genetic elements are induced by treatments that induce the global DNA damage response, and the mechanisms governing these responses have not been characterized. Furthermore, some of these mobile genetic elements (ø105, *skin*, and λBa04) also contain homologs of RapI and PhrI (5). Therefore, we think that the ImmR and ImmA homologs present in these elements likely regulate expression of element genes and that ImmR and ImmA represent a conserved two-protein strategy for regulating gene expression in response to the global DNA damage response and intercellular peptide signaling.

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Chapter 5: Discussion

In this thesis, I have demonstrated the roles that intercellular peptide signaling plays in regulating two mechanisms of horizontal gene transfer in the Gram-positive soil bacterium *Bacillus subtilis*. In Chapter 2, I showed the roles that multiple signaling peptides play in regulating the ComA-dependent response that leads to genetic competence. In Chapter 3, I described the role that signaling peptides play in regulating the transfer of the mobile genetic element ICE*Bs1*. Chapter 4 focused on the identification of an ICE*Bs1*-encoded two-protein regulatory system that regulates expression of ICE*Bs1* genes required for excision and transfer. Intercellular peptide signaling and the RecA-dependent DNA damage response regulate the activity of this two-protein regulatory system. In this discussion, I would like to focus on how the insights that have been gained through this thesis research are likely to influence future research.

Regulation of the ComA response by multiple intercellular signaling peptides. Multiple intercellular signaling peptides stimulate the activity of ComA, a transcription factor that activates the expression of genes involved in competence development, and production of antibiotics, degradative enzymes, and other secreted products (Chapter 2). The involvement of several signaling peptides allows for the integration of multiple layers of regulation to modulate the timing and level of the ComA response. These layers of regulation include factors that regulate the expression of genes that encode the signaling peptides and their cognate receptor proteins, and could also involve factors that influence secretion, processing, diffusion, and import of the secreted peptides.

The work described in Chapter 2 focused primarily on identifying peptide signals that regulate ComA-dependent gene expression and characterizing the roles that these signaling peptides play in modulating gene expression under a single condition. In order to fully understand how these signaling peptides modulate the activity of ComA, more extensive

characterization of their roles under a variety of conditions will be needed. This work should include characterizing the roles that *rapC, rapF,* and *rapK* play in inhibiting ComA activity under a variety of conditions, characterizing any additional factor or factors that regulate expression of the *rap*s and *phr*s, and characterizing factors that may modulate secretion, processing, and import of the secreted PhrC, PhrF, and PhrK peptides.

B. subtilis cells are known to form multicellular structures, such as biofilms (1, 4, 12) and fruiting bodies (1). Robust biofilm formation depends upon the activity of ComA, as well as other signaling proteins (1, 4, 12). As PhrC, PhrF, and PhrK peptide signaling is known to regulate the activity of ComA, it is likely that the PhrC, PhrF, and PhrK peptides provide cells within the forming biofilm information about diffusion, population density, and spatial position relative to other cells. Therefore, further work evaluating the roles that the PhrC, PhrF, and PhrK peptides play in regulating ComA-dependent gene expression should also include conditions that promote the formation of multicellular communities. As it is likely that microenvironments exist within forming biofilms and that this will result in heterogeneous expression of ComA-dependent genes, this work would be facilitated by utilizing techniques that would allow the analysis of ComA-dependent gene expression in single cells as opposed to population-based approaches.

Further exploration of the various roles that the PhrC, PhrF, and PhrK signaling peptides play in regulating the ComA-dependent response should provide insight into the diversity of information that can be integrated into Phr peptide signaling. This information, when added to the existing knowledge of the complex, overlapping regulatory networks that govern post-exponential phase processes in *B. subtilis* (2, 3), may lead to a more comprehensive understanding of how *B. subtilis* interprets cues from its environment and chooses between a

variety of physiological responses. This information will also lead to a greater understanding of the diversity of factors that can influence horizontal gene transfer through genetic competence.

Regulation of transfer of ICE*Bs1* by intercellular peptide signaling. As demonstrated in Chapter 3, intercellular peptide signaling regulates transfer of the mobile genetic element ICE*Bs1*. The element-encoded PhrI peptide serves as a signal of cells that contain ICE*Bs1*; signaling through this peptide limits ICE*Bs1* transfer to cells that already contain the element. The PhrI peptide inhibits expression of several ICE*Bs1* genes required for excision and transfer by antagonizing the activity of RapI, a regulatory protein that promotes expression of these genes. A second, chromosomally-encoded signaling system activates expression of *rapI*. *rapI* transcription is repressed during exponential growth and derepressed by signals of high population density and starvation. This combined regulation of RapI's transcription and activity ensures that expression of ICE*Bs1* genes involved in excision and transfer is activated under conditions that are most likely to promote successful dissemination to recipient cells lacking ICE*Bs1*.

***rap*s and *phr*s in other mobile genetic elements.** Comparative sequence analysis revealed that homologs of *rapI* and *phrI* are present in several other *Bacillus* mobile genetic elements (Fig. 1; Table 6, Appendix B). Further research will be needed to determine if these Rap and Phr homologs regulate the mobility of their respective mobile genetic elements. Some of these mobile genetic elements, such as the ø105 phage from *B. subtilis* and the putative λBa04 phage from *B. anthracis* contain homologs of ImmR and ImmA in addition to RapI and PhrI homologs. ImmR and ImmA were shown in Chapter 4 to be responsible for regulating expression of ICE*Bs1* genes required for excision and transfer in response to RapI activity. In addition, the global DNA damage response stimulates dissemination of ø105, which is also true for ICE*Bs1*

(Chapters 3 & 4). Therefore, it will be interesting to determine if these Rap-Phr and ImmR-ImmA-like systems work similarly in regulating dissemination of ø105 and λBa04, as these putative regulatory networks are most analogous to the regulatory network present in ICE*Bs1*.

Rap proteins and potential Phr peptides are also encoded by several plasmids (Fig. 1; Appendix B, Chapter 5). Of these plasmids, only pLS20 is known to encode all the proteins required to direct its own intercellular transfer through conjugation (6). pLS20 also mediates conjugal transfer of other plasmids (6). Further research will be needed to determine if signaling through the putative *rap-phr* system of pLS20 regulates transfer of pLS20 or its ability to transfer other plasmids intercellularly. As pLS20 has been only partially sequenced and genes encoding homologs of ImmR and ImmA, if present on pLS20, have not yet been identified, it is not possible to predict whether a regulatory system similar to ICE*Bs1* is found on pLS20.

The remaining Rap-Phr-encoding plasmids identified in Chapter 3 are not capable of self-conjugation. However, several of these plasmids may be transferred (mobilized) through the activity of another element's conjugation machinery. Mobilization of pTA1060 is dependent upon the presence of a single plasmid-encoded protein, Mob (9). pBC10987 is closely related to pXO1, a *B. anthracis* mobilizable plasmid, and is therefore thought to be mobilizable (11). pFL5 and pFL7 encode potential mobilization proteins and origins of transfer, indicating that they may also be mobilized (10). If Rap-Phr signaling modulates transfer of these mobilizable plasmids, the mechanism of regulation is likely distinct from the ICE*Bs1* regulatory mechanism, as these plasmids do not encode homologs of ImmR and ImmA.

Several of the plasmids encode Phrs that are predicted to contain the same five amino acid sequence in the mature form of the peptide (Fig. 1). This does not appear to be the result of direct descent from the same progenitor *rap-phr* signaling cassette, as the entire precursor

A.

Element	Gene	Phr pentapeptide
ICE*Bs1*	*phrI*	DRVGA[1]
skin (defective prophage)	*phrE*	SRNVT[2]
pTA1060	*phr60*	SRNAT[3]
pTA1040	*phr40*	SRKAT[4]
pPOD2000	*rapAB*	SRNAT[4]
pLS20	*orfAB*	QKGMY[4]
pFL5	*phr5*	SRNAT[5]
pBC10987	*BCEA0147*	EKIVQ[5]
pFL7	*phr7*	SRNAT[5]

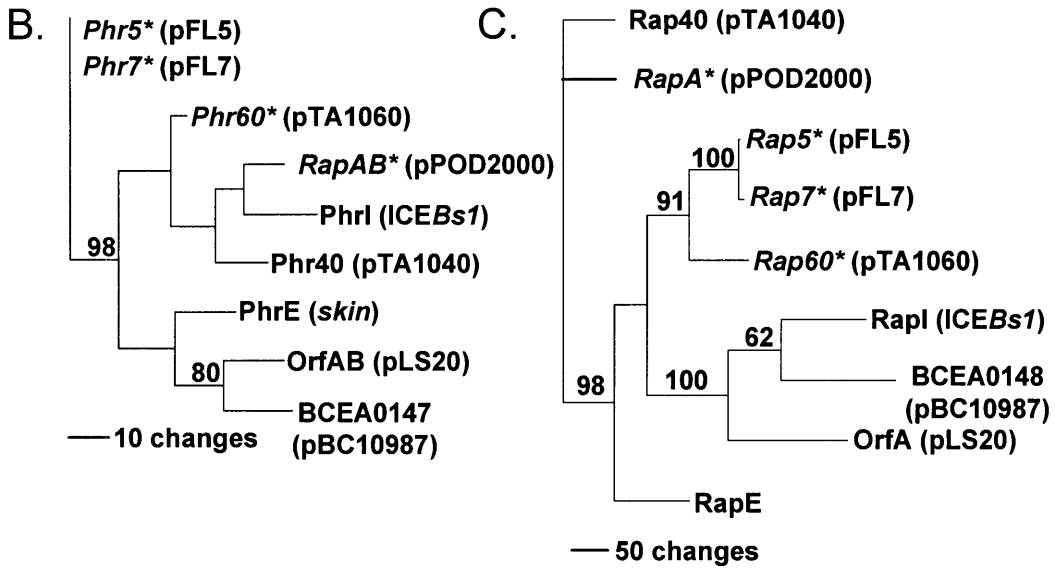

Figure 1: Comparison of Rap, Phr, and Phr pentapeptide sequences from several mobile genetic elements.

A. The known or predicted forms of the Phr peptides from several *Bacillus* mobile genetic elements are listed.

[1] Mature peptide shown to be active in Chapter 3.

[2] Mature peptide shown to be active in (5).

[3] Mature peptide shown to be active in (7).

[4] Mature peptide predicted in (8).

[5] Mature peptide predicted based on similarity to other Phr peptides.

B. Phylogram of the full-length pre-Phr peptide sequences corresponding to the mature peptides shown in part A. The tree topology and bootstrap values (numbers indicated on branches of tree) were generated by parsimony analysis of the entire amino acid sequence of the Phrs using PAUP (13). Amino acid sequences were obtained from Genbank and were initially aligned using Clustal-W (14). The sequence alignment was further refined to align the mature Phr peptide sequences. Trees were constructed and bootstrap re-sampled (100 replicates) with maximum parsimony by 1,000 heuristic random-addition sequence searches using the tree bisection reconnection (TBR) branch-swapping option. Proteins that are italicized and followed by an asterisk have the same amino acid sequence in the mature Phr peptide. The element that encodes each Phr is indicated in parentheses.

C. A phylogram of the Rap proteins that correspond to the Phr peptides shown in part A was constructed as described in part B. Proteins that are italicized and followed by an asterisk are the cognate Rap proteins to those mature Phr peptides that have the same amino acid sequence. The element that encodes each Rap is indicated in parentheses.

peptide and the cognate Rap sequence is not more similar among these plasmid-encoded sequences (Fig. 1). Those plasmids that encode the same mature forms of Phr peptides are likely to cross-regulate; further work will be needed to investigate whether signaling among these different plasmids occurs and what function it may serve.

Determining whether *rap-phr* signaling systems regulate the transfer of other mobile genetic elements, will reveal whether ICE*Bs1* regulation is unique or is a conserved mechanism used by several *Bacillus* mobile genetic elements. Although intercellular signaling is known to regulate the transfer of several conjugative plasmids (described in Chapter 1), the regulatory proteins involved are not related to RapI, PhrI, ImmR, or ImmA. The proposed research described above is necessary to understand the roles that additional *rap-phr* systems may play in regulating horizontal gene transfer.

Characterization of a two-protein system for regulating element gene expression. In Chapter 4, I described the identification and initial characterization of a two-protein regulatory system that is required to regulate expression of several ICE*Bs1* genes in response to RapI protein activity and the RecA-dependent response to DNA damage. I also identified several other potential ImmR and ImmA homologs that are present in known and putative mobile genetic elements, including some elements that encode Rap and Phr homologs, as well as other elements that are known to be activated by DNA damage. Although one of the primary paradigms for regulation of mobile genetic element gene expression by the DNA damage response is found in bacteriophage λ, alternative mechanisms of regulating mobile element gene expression in response to DNA damage have been characterized and were described in Chapter 4. It is likely that the ImmR-ImmA paradigm represents a conserved alternative mechanism utilized by mobile genetic elements to respond to the global DNA damage response.

A significant amount of information about the molecular mechanisms of ImmR and ImmA activity remains to be discovered. This will likely require a combination of *in vivo* and *in vitro* analysis of these proteins to determine how ImmA antagonizes the activity of ImmR and how ImmA responds to RapI and to RecA bound to single-stranded DNA. In addition, in order to determine whether this mechanism is conserved, several ImmR and ImmA homologs and their respective mobile genetic elements will also need to be investigated. This work will likely lead to the characterization of additional functional mobile genetic elements, provide insights into the molecular mechanisms that govern dissemination of these elements, and determine whether intercellular Phr peptide signaling regulates dissemination of bacteriophage.

Potential benefits of ICE*Bs1* regulation. Regulation of ICE*Bs1* transfer likely provides the element with several benefits. Constitutive expression of ICE*Bs1* genes required for excision and transfer in Δ*immR* mutants results in increased excision and instability of the element, which can be lost in about ~10% of cells in the population (Appendix B and Chapter 4). The Δ*immR* mutation also makes the host cells sick, likely due to the increased burden placed upon the cells because of high levels of expression of conjugation proteins. Therefore, mechanisms that limit expression of ICE*Bs1* genes required for excision and transfer provide an obvious benefit to the element, as they promote a stable association of ICE*Bs1* with the host.

Mechanisms that allow the element to respond to host cell distress and promote transfer in the presence of cells lacking the element play an important role in dissemination of the element. These strategies allow the element to ensure it is present in many cells, thereby limiting the possibility that the element will be lost due to death of a limited number of cells that contain ICE*Bs1*. The benefits provided to the element by mechanisms that limit transfer into cells that already contain a copy of the element are less clear. These mechanisms may help maintain the

genetic stability of ICE*Bs1* by limiting inter-element recombination that could occur in host cells containing multiple copies of the element. This regulation may also help to limit the metabolic burden placed on host cells due to replication of additional DNA. Further insights into the possible benefits provided by mechanisms that limit acquisition of multiple copies of ICE*Bs1* could be facilitated by further analysis of strains that contain multiple copies of the element.

Conclusions. Horizontal gene transfer plays an important role in bacterial evolution. However, as horizontal gene transfer can be detrimental as well as beneficial, mechanisms that promote horizontal gene transfer under favorable conditions and inhibit horizontal gene transfer under unfavorable conditions are advantageous to cells and mobile genetic elements. In some *Streptococcus* species as well as *B. subtilis*, intercellular peptide signaling activates competence development. This regulation limits acquisition of DNA from the environment to conditions when cells are surrounded by high concentrations of closely related cells, thereby decreasing the likelihood of acquiring foreign DNA that is detrimental to the cell. Intercellular peptide signaling also regulates transfer of conjugal plasmids in *Enterococcus* and the *B. subtilis* ICE*Bs1* element. Element transfer is inhibited when potential recipient cells already contain a copy of the element. This regulation likely provides several benefits to the element, including reducing the chance of genetic instability through inter-element recombination in the presence of multiple elements and reducing burdens imposed upon the host cell in the presence of multiple elements. These regulatory mechanisms may allow cells to minimize the potential risks of horizontal gene transfer while maximizing the potential rewards.

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