Regulation and Production of Extracellular Signaling Molecules in *Bacillus subtilis*

by

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ABSTRACT

Many bacteria use chemical signals for intercellular communication. These signals accumulate extracellularly and are sensed at threshold concentrations to alter gene expression. *Bacillus subtilis* uses cell-cell signals to control genetic competence, sporulation, degradative enzyme production and antibiotic synthesis.

A family of peptide signals controls gene expression in *B. subtilis* by regulating transcription factors. I showed that the transcription of these peptide regulators is in turn controlled in part by the alternate sigma factor sigma-H. The activity of sigma-H is stimulated by nutrient starvation

I determined that *B. subtilis* produces a putative interspecies signaling activity, AI-2, that depends on the conserved protein LuxS. To determine the effect of *luxS* on regulation of transcription in *B. subtilis*, I performed experiments to compare the transcriptional profiles of *B. subtilis* when *luxS* was deleted or overexpressed. I found no significant change in transcriptional profiles, indicating that *B. subtilis* does not use *luxS*/AI-2 for signaling under these conditions.

Thesis Advisor: Alan D. Grossman

Title: Professor of Biology

Acknowledgments

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Lastly, I would like to thank my parents for always believing in me and encouraging me to follow my heart, even all the way to Cambridge, Massachusetts. Without their love, support, and guidance, none of this would have been possible. This thesis is dedicated to them.

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Thesis Plan

Chapter 1 is an introduction to cell-cell signaling in bacteria. Along with providing a general overview, this chapter focuses on the *Bacillus* spp. family of Rap/Phr regulators and work investigating the putative interspecies signaling system comprised of LuxS and AI-2.

Chapter 2 was published in *Journal of Bacteriology*, Volume 183, pages 4905-4909, as "Control of a family of phosphatase regulatory genes (*phr*) by the alternate sigma factor sigma-H of *Bacillus subtilis*," by Ryan S. McQuade, Natalia Comella, and Alan D. Grossman. Chapter 2 describes the regulation of verified and putative signaling peptides by the alternate sigma factor of *B. subtilis* that regulates sporulation initiation and stationary phase processes. I was responsible for strain creation, the primer extension analyses of the *phr* transcripts, and approximately half of the β-galactosidase assays of the *phr-lacZ* fusions. Natalia Comella performed the remainder of the β-galactosidase assays.

In **Chapter 3** I describe my work characterizing the function of the *luxS* gene in *B. subtilis*. I found that *B. subtilis* accumulates AI-2 activity, and that this activity is not produced in a *luxS* null mutant. However, I detected no other phenotypes of a *luxS* null mutant, and using transcriptional profiling I found no genes significantly differentially expressed when *luxS* expression is changed. I performed all of the AI-2 assays and all of the transcriptional profiling experiments and analysis. The microarrays used were from a common laboratory pool; creation of the original *luxS* mutant strain and the comG-lacZ experiment were performed by Natalia Comella.

Chapter 4 is a discussion of the work presented in this thesis. I discuss the regulation of production of cell-cell signals in bacteria in response to environmental stresses, including starvation. I also discuss my work with *luxS* of *B. subtilis* in relation to work from other groups.

Chapter 1

Introduction to cell-cell signaling in *Bacillus subtilis* and other bacteria

Cell-cell signaling

Cell-cell signaling is prevalent in all forms of life. Multicellular organisms use chemical signals to organize cells during the development of organs and complex structures. Hormones are important for coordinating responses between cells and tissues in both plants and animals. Many unicellular organisms use pheromones to communicate between individuals for mating and other processes. Cell-cell signaling is important in bacteria for the regulation and coordination of cellular processes.

Bacteria regulate several different processes by cell-cell signaling, including bioluminescence, pathogenesis, symbiosis, production of antibiotics and degradative enzymes, formation of dormant spores, genetic transfer, and the development of competence for DNA uptake (Fuqua et al., 2001; Bassler, 2002; Sturme et al., 2002). Bacterial cell-cell signaling is widespread and occurs in both Gram-negative and Gram-positive bacteria. Many bacteria use cell-cell signaling to sense population density in a process known as quorum sensing (Fuqua et al., 1994). The bacteria produce signaling molecules that accumulate in the extracellular medium during growth. When the signaling molecules reach a critical concentration, the cells respond by altering gene expression. By controlling gene expression based on population density, bacteria are able to coordinate gene expression at high cell density.

Three primary types of signaling molecules have been identified in bacteria (Fig. 1-1). Gram-negative species primarily use acylated homoserine lactones for signaling (Fuqua et al., 2001). The predominant signaling molecules in Gram-positive species are small peptides (Sturme et al., 2002). Recently, many bacteria have been shown to

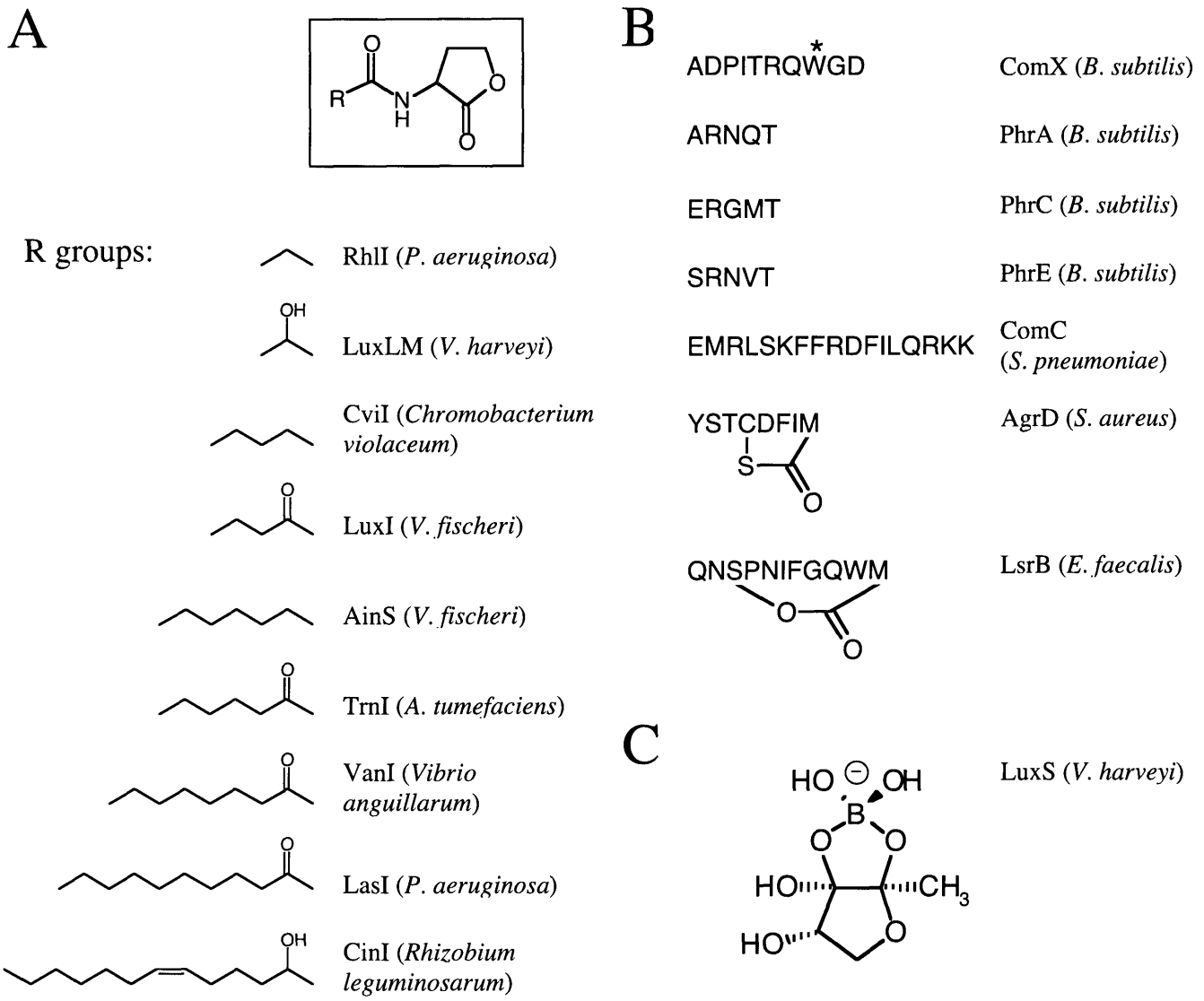


Figure 1-1. Bacterial cell-cell signaling molecules. Various signals are shown along with the species in which they are found and the protein primarily responsible for their production. (A *upper*) AHL signals contain a common homoserine lactone moiety. (A *lower*) Different R groups of AHLs. (B) Peptide signaling molecules. The asterisk above the tryptophan residue in ComX indicates a farnesyl modification. (C) The structure of *V. harveyi* AI-2.

produce an activity that can induce luminescence in *Vibrio harveyi* (Xavier and Bassler, 2003). This conserved signaling activity has been linked to the molecule VhAI-2, and is widely viewed as a potential interspecies signaling molecule.

Detection and response to the extracellular signaling molecules often occur at the cell surface by interaction with a membrane receptor and signal transduction across the plasma membrane. Alternatively, signaling molecules may cross the plasma membrane by diffusion or active transport for interaction with cytoplasmic receptors. Interestingly, there are examples of both intracellular and extracellular detection mechanisms for all three types of signaling molecules.

AHL signaling in Gram-negative bacteria

Gram-negative bacteria primarily use a family of small molecules known as acylated homoserine lactones (AHLs) for cell-cell signaling (Fig. 1-1). In this system, the AHLs are presumed to diffuse freely across the plasma membrane and interact with an intracellular receptor to activate transcription. AHL signaling is found in many Gram-negative species and controls such processes as luminescence in *Vibrio fischeri* (Eberhard et al., 1981; Kaplan and Greenberg, 1985; Devine et al., 1989), plant pathogenesis in *Erwinia* spp. (Jones et al., 1993; Pirhonen et al., 1993), plasmid conjugation in *Agrobacterium tumefaciens* (Piper et al., 1993; Zhang et al., 1993), and pathogenesis in *Pseudomonas aeruginosa* (Jones et al., 1993; Latifi et al., 1995; Pearson et al., 1995; Winson et al., 1995; Latifi et al., 1996).

The mechanics of AHL signaling were first worked out in *V. fischeri*. The circuit consists of an AHL synthase (LuxI) and an AHL-dependent transcriptional activator

(LuxR) (Engebrecht and Silverman, 1984; Devine et al., 1989; Schaefer et al., 1996). In the presence of the AHL, LuxR activates transcription of *luxI* along with other genes, including the luminescence machinery (Fig. 1-2) (Hanzelka and Greenberg, 1995; Callahan and Dunlap, 2000). The activation of transcription of *luxI* in the presence of autoinducer creates a self-reinforcing loop that increases the production of autoinducer at a threshold concentration and induces a strong, coordinated response.

Homologs of LuxR and LuxI have been identified in more than 70 Gram-negative bacteria (Swift et al., 1993; Fuqua et al., 2001). In all cases, the LuxI homolog is predicted to produce an AHL that activates transcription in conjunction with the LuxR homolog. All characterized LuxI homologs catalyze the condensation of an acyl chain with a homoserine derived from S-adenosylmethionine (Schaefer et al., 1996). Known AHL molecules differ in the length and substitutions of the acyl chain (Fig. 1-1) (Fuqua et al., 2001). All acyl chains reported consist of an even number of carbons ranging from four to fourteen. Each LuxR responds predominantly to one AHL, leading to species specificity, although some species produce and respond to more than one AHL (Brint and Ohman, 1995; Gilson et al., 1995; Ochsner and Reiser, 1995; Lupp et al., 2003). Cross-species induction has only been observed when the bacteria produce identical AHLs.

Peptide signaling in Gram-positive bacteria

Gram-positive bacteria primarily use peptides for cell-cell signaling. Peptides, often modified, are produced from larger propeptides and secreted to the extracellular medium. These peptides affect gene expression by interaction with receptors followed by

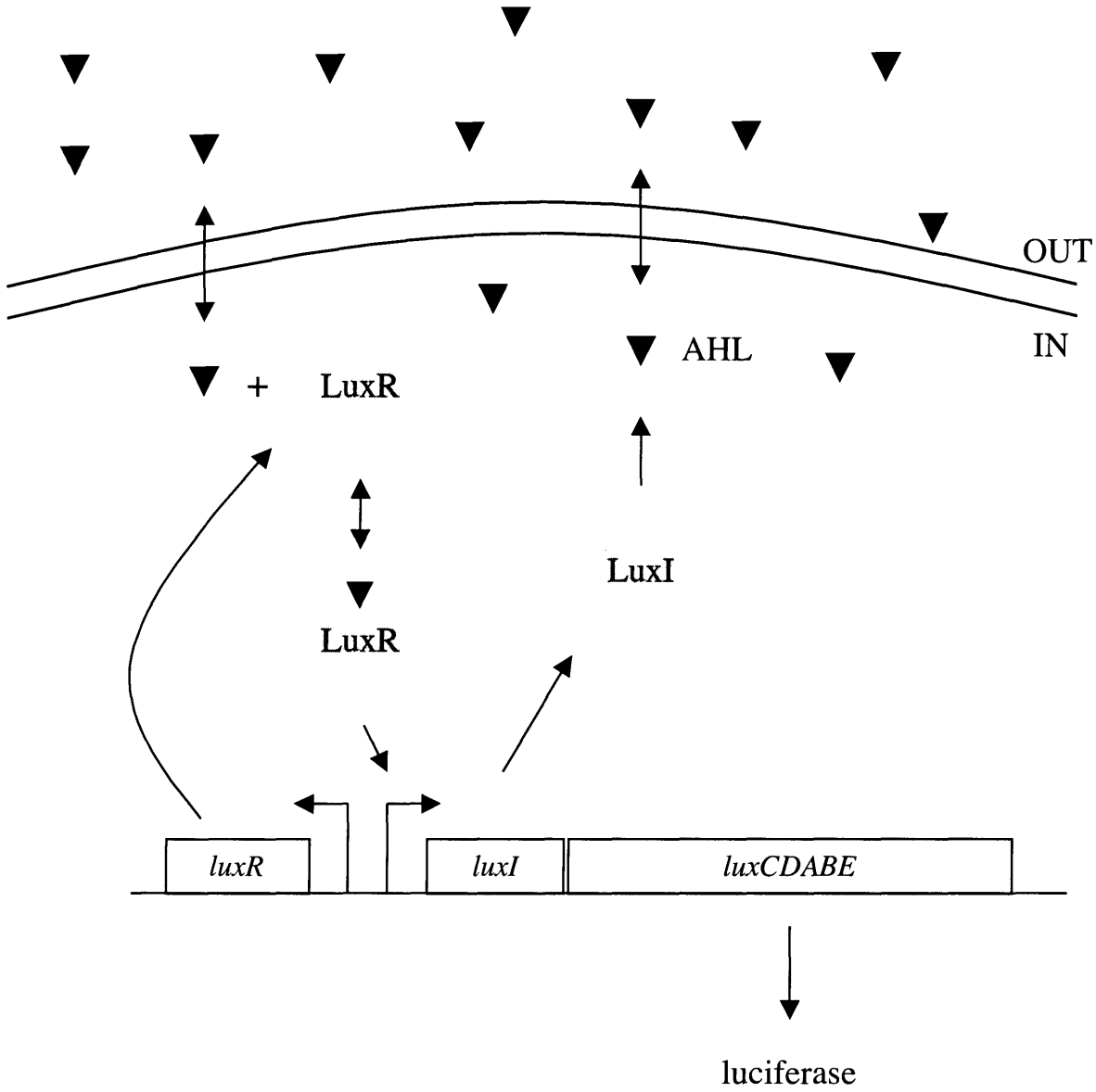


Figure 1-2. Simplified LuxIR circuit of *V. harveyi*. LuxI is an AHL synthase that produces the *V. fischeri* AHL. LuxR binds AHL, activating it to increase transcription of *luxI* and the genes for luciferase. Small triangles indicate AHL molecules.

signal transduction to transcriptional regulators. Response to these signals occurs by one of two methods: extracellular detection followed by transduction of the signal across the cell membrane by a two-component system, or import of the peptide into the cell where it interacts with a cytoplasmic receptor (Sturme et al., 2002). Cell-cell signaling in Gram-positive bacteria controls pathogenesis, competence, sporulation, antibiotic production, and conjugative plasmid transfer (Sturme et al., 2002).

Signaling using two-component systems

Many of the Gram-positive peptide signals act through two-component signal transduction systems. Two-component systems are widespread in bacteria, where they regulate diverse processes (Hoch, 2000). Two-component systems have also been identified in some eukaryotes (Ota and Varshavsky, 1993; Maeda et al., 1994; Kakimoto, 1996; Posas et al., 1996; Inoue et al., 2001). The typical two-component system consists of two proteins: a histidine kinase and a response regulator. In response to stimuli, the histidine kinase autophosphorylates on a conserved histidine. The phosphoryl group is then passed to the cognate response regulator on a conserved aspartic acid residue. The response regulator usually consists of two domains, the phosphorylatable domain and an output domain, often a transcriptional activator domain. Some two-component systems are linked in series into a four-component phosphorelay consisting of a histidine kinase followed by single domain response regulator with only the aspartyl phosphate domain, a histidine phosphotransfer protein, and finally a response regulator-transcription factor (Burbulys et al., 1991). In a phosphorelay system, the phosphoryl group is transferred in the order His-Asp-His-Asp, instead of the usual His-Asp series of a two-component

system. This organization allows for multiple points of regulation along the response pathway.

Cell-cell signaling mechanisms using two-component signaling systems for response are found in a variety of organisms, including *B. subtilis* (Magnuson et al., 1994), *Staphylococcus aureus* (Peng et al., 1988; Ji et al., 1995), *Enterococcus faecalis* (Qin et al., 2000; Nakayama et al., 2001; Qin et al., 2001), *Streptococcus pneumoniae*.(Havarstein et al., 1995; Havarstein et al., 1996; Pestova et al., 1996), and *Lactobacillus* sp.(Diep et al., 1995; Diep et al., 1996) (Fig. 1-3). In these cases, the genes required for production of and response to the signal are linked on the chromosome in a peptide signaling cassette (Lazazzera et al., 1999b). These cassettes consist of genes encoding: a peptide precursor, followed by a receptor histidine kinase required for sensing of the signal, and a response regulator transcription factor. If the signaling peptide is modified, as in *B. subtilis*, *S. aureus* and *E. faecalis*, the peptide precursor is preceded by a gene required for the production and most likely involved in the modification (Weinrauch et al., 1991; Magnuson et al., 1994; Ji et al., 1995; Qin et al., 2001; Bacon Schneider et al., 2002; Zhang et al., 2002). Identified peptide signaling cassettes regulate such processes as competence, pathogenesis, and antibiotic production.

Many of these peptide signaling cassettes have also been implicated in strain-specific signaling. Different strains of a bacterial species may produce unique peptide signals. In these instances, a hypervariable region of the chromosome encodes the peptide signal and the portion of the histidine kinase receptor gene encoding the N-terminal (sensor) domain of the protein (Havarstein et al., 1997; Ji et al., 1997; Tortosa et al., 2001; Dufour et al., 2002). Each strain responds only to signals from its own kind or

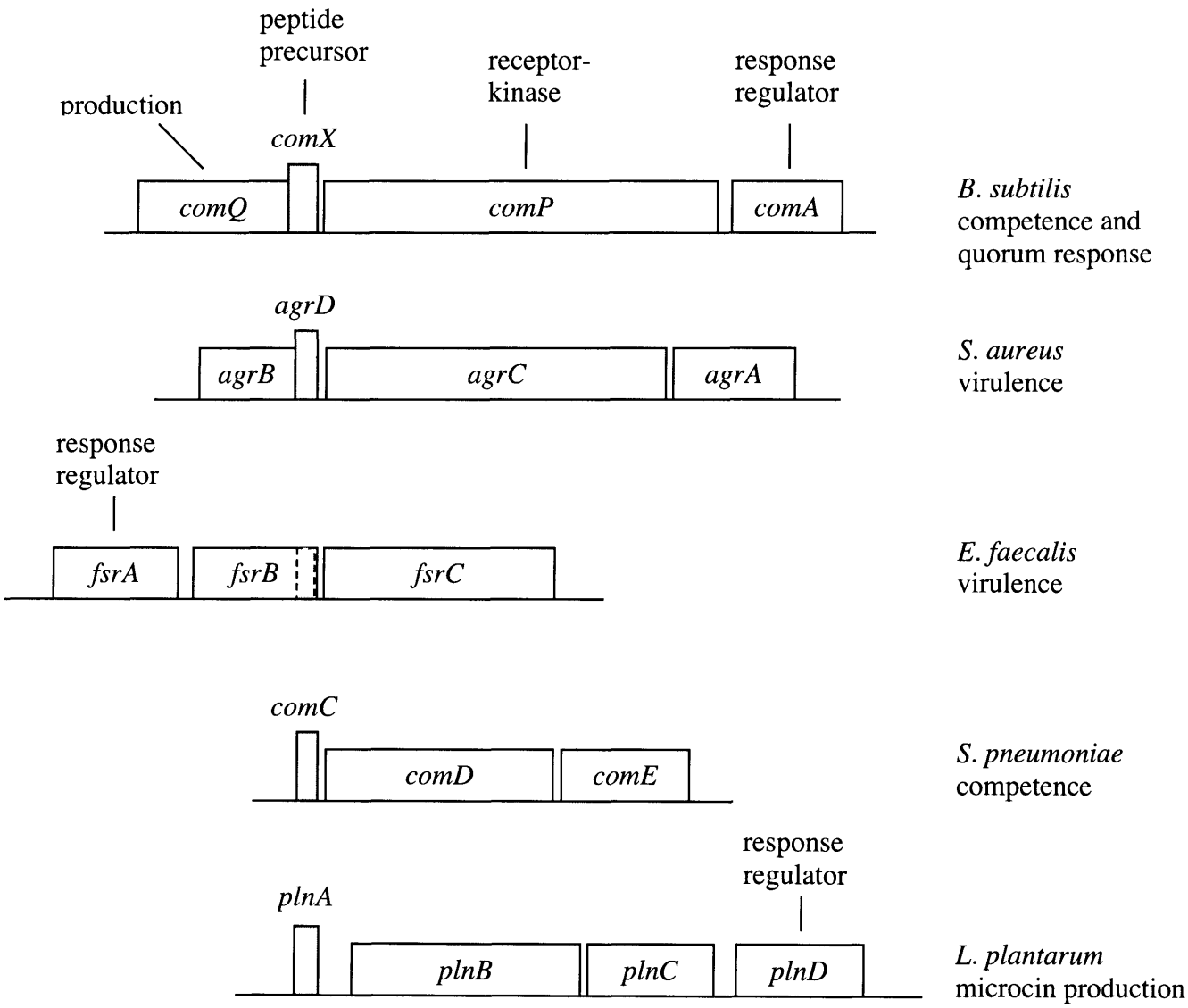


Figure 1-3. Peptide signaling cassettes in Gram-positive bacteria. In each case, the gene encoding the peptide precursor (shaded) is immediately upstream of the gene for the histidine kinase. If the protein is modified, the peptide precursor gene is preceded by a gene required for its production. The *E. faecalis* FsrB protein is homologous to the *S. aureus* AgrB protein required for peptide production and also contains the peptide precursor (shaded). The direction of transcription is from left to right.

closely related strains, with no response to heterologous signals. In *S. aureus*, the peptides can inhibit the peptide signaling response of heterologous strains (Ji et al., 1997; Jarraud et al., 2000).

Signaling by import of peptides

Other Gram-positive peptide systems use a system of export and reimport of a peptide signal to alter gene expression. In these systems, the active peptide signals are cleaved from a larger propeptide, probably after export via the protein secretory system. After maturation, the peptide signals are imported into the cell, usually by an oligopeptide permease ABC transporter, where they interact with intracellular effectors to alter transcription, either directly or indirectly. *Bacillus* spp. use imported peptides to regulate sporulation, competence, and degradative enzyme production (Perego et al., 1996; Solomon et al., 1996; Jiang et al., 2000a; Koetje et al., 2003; Ogura et al., 2003). *Enterococcus faecalis* regulates transfer of a family of conjugative plasmids using imported peptides (Clewell, 1999). A similar peptide import system has also been found to control the pattern of heterocyst formation in the cyanobacterium *Anabaena* sp. (Yoon and Golden, 1998). Also, the oligopeptide permease of *Streptomyces coelicolor* has been implicated in cell-cell signaling for regulation of aerial mycelium formation via a putative modified peptide (Nodwell et al., 1996; Nodwell and Losick, 1998).

Signaling in *Bacillus subtilis*

B. subtilis is a Gram-positive soil bacterium that has at least two adaptive developmental states in response to nutrient stress. *B. subtilis* forms metabolically dormant endospores that can resist many assaults, including desiccation, radiation, and

temperature extremes (Grossman, 1995; Piggot, 1996; Sonenshein, 2000). *B. subtilis* can also become genetically competent to take up exogenous DNA and incorporate homologous DNA into its chromosome (Grossman, 1995; Tortosa and Dubnau, 1999; Hamoen et al., 2003). Although these processes are controlled in part by nutrient status, they occur most efficiently at high cell density (Grossman and Losick, 1988; Ireton et al., 1993). Early studies implicated an extracellular factor involved in the induction of competence (Akrigg et al., 1967; Akrigg and Ayad, 1969; Akrigg and Ayad, 1970).

Regulation of the quorum response by a peptide signaling cassette

B. subtilis controls the development of competence and the general quorum response using the *comQXPA* peptide signaling cassette (Fig. 1-3). *comX* encodes the precursor of a peptide signal and requires *comQ* for peptide maturation. *comP* and *comA* code for a two-component signal transduction system: a transmembrane receptor histidine kinase and a response regulator transcriptional activator, respectively.

The ComX peptide signal is a decapeptide with the sequence ADPITQR(W)GD, where (W) denotes a modified tryptophan residue (Magnuson et al., 1994). The ComX pheromone was purified as an extracellular factor that, when added to *B. subtilis* at low cell density, could activate transcription from a promoter that is normally active at high cell density (Magnuson et al., 1994). The purified peptide corresponds to the C-terminal 10 amino acids of the *comX* open reading frame. The tryptophan modification is isoprenoid in origin based on metabolic labeling (Ansaldi et al., 2002), and is most likely a farnesyl group based on its mass of 203 Da (Magnuson et al., 1994).

Production of mature ComX peptide depends on *comX*, as well as *comQ*, the gene immediately upstream (Magnuson et al., 1994; Bacon Schneider et al., 2002). *comQ* and

comX are sufficient in *Escherichia coli* to produce and export mature ComX pheromone (Tortosa et al., 2001). Production of mature ComX peptide from the 55-amino acid ComX propheromone requires at least three activities: the propheromone must be cleaved to the C-terminal 10 amino acids, modified on the tryptophan residue, and exported from the cell. ComQ is required for ComX production and is predicted to be involved in the tryptophan modification. ComQ has similarity to isoprenyl synthetases, and alanine substitution of conserved residues involved in isoprene binding eliminate production of ComX pheromone activity (Bacon Schneider et al., 2002).

The factors that mediate the cleavage and export activities of ComX pheromone have yet to be been identified. A genetic screen for mutations that eliminate production of ComX pheromone in *B. subtilis* identified 18 mutations that all fell within *comQ* and *comX*, implying that any other factors involved are either essential or redundant (Palmer, 1999). ComQ may be involved in the peptide cleavage but is unlikely to be responsible for export on its own since the protein is cytoplasmic.

The ComPA two-component system is required for the response to ComX pheromone (Fig. 1-4). ComP is a membrane protein with 5-7 predicted transmembrane domains (Piazza et al., 1999). In response to ComX pheromone, ComP autophosphorylates and donates its histidyl phosphate to ComA. ComA~P is the transcriptionally active form, which regulates a number of genes involved in degradative enzyme production, antibiotic synthesis, and other processes (Comella and Grossman, unpublished data). Although ComA~P does not directly activate transcription of the competence machinery, ComA~P does serve to induce competence in *B. subtilis* by directing transcription of a small open reading frame, *comS*, that encodes an activator of

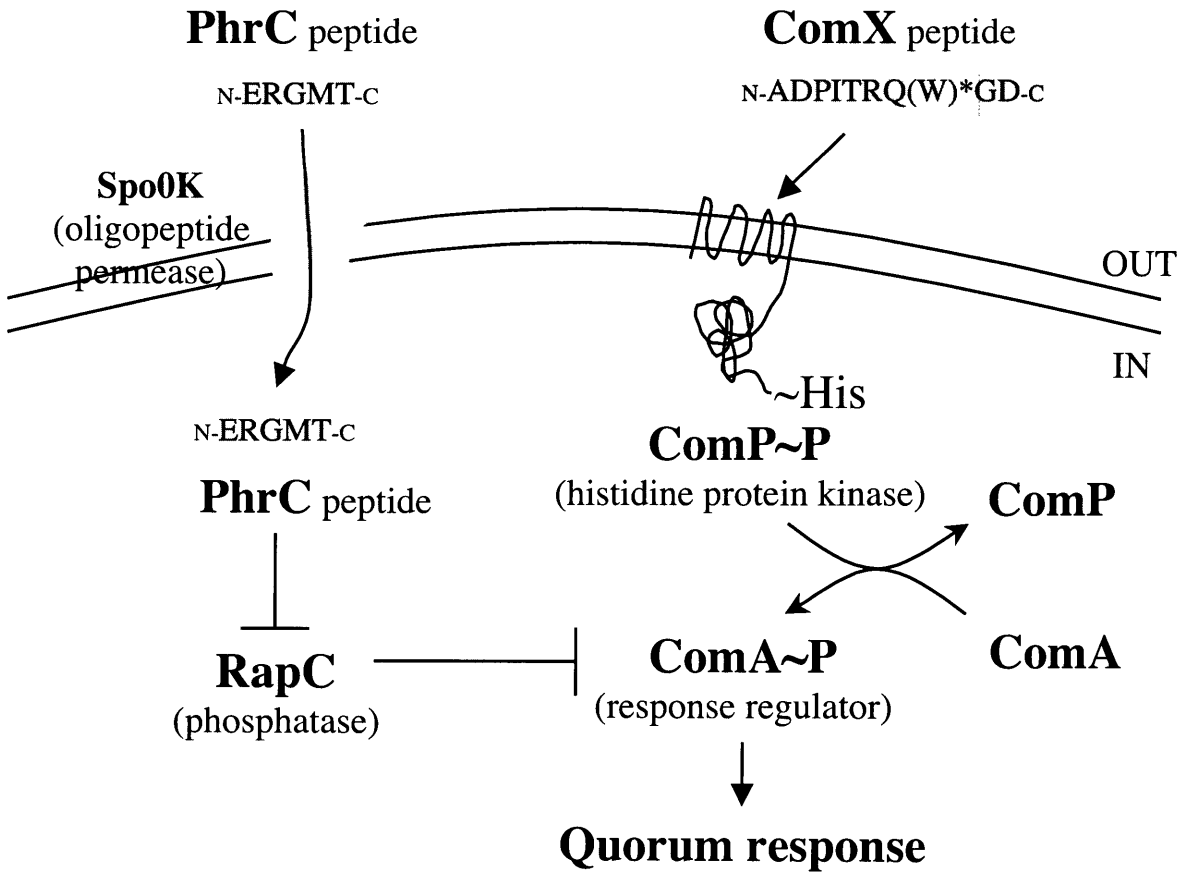


Figure 1-4. Regulation of the *B. subtilis* quorum response by two extracellular peptides. ComP autophosphorylates on binding to ComX peptide and donates the phosphoryl group to ComA, which then is able to direct transcription of the genes involved in the quorum response. RapC binds to ComA and segregates it from DNA binding. PhrC peptide enters the cell via the oligopeptide permease and prevents RapC from binding ComA, releasing it for transcription.

the competence transcription factor, ComK (D'Souza et al., 1994; van Sinderen et al., 1995).

Regulation by imported peptides

B. subtilis possesses a number of confirmed and putative imported peptide signaling systems. Eleven *rap* genes are predicted to encode negative regulators of various transcription factors (Perego et al., 1994; Reizer et al., 1997). Seven *phr* genes that encode putative and confirmed peptide regulators of the Rap proteins are cotranscribed in operons with their cognate *rap* genes (Fig. 1-5) (Perego and Hoch, 1996; Lazazzera et al., 1999a). In this system, the Phr peptides are secreted and reenter the cell via the oligopeptide permease (Opp [also known as Spo0K]) where they interfere with regulation by the cognate Rap proteins. The Rap proteins bind directly to their targets, leading to either dephosphorylation of the target protein or elimination of the ability of the target protein to bind to its target promoter sequences (Perego et al., 1996; Tzeng et al., 1998; Jiang et al., 2000a; Core and Perego, 2003; Ogura et al., 2003). Each Rap protein is predicted to be inhibited by a specific Phr peptide, although individual Phrs may act on more than one Rap. For example, the PhrC peptide inhibits both RapB and RapC (Solomon et al., 1996; Core and Perego, 2003).

Phr peptide regulators. The seven *phr* genes are small open reading frames predicted to encode propeptides of 38-44 amino acids that are subsequently processed to the active Phr pentapeptides. In the model for production of the mature Phr peptides, each Phr protein is exported via the secretory system, where it undergoes at least one proteolyitc processing step to release the active pentapeptide form. The enzymatic activity responsible for processing of the Phr propeptides has not been identified, and the

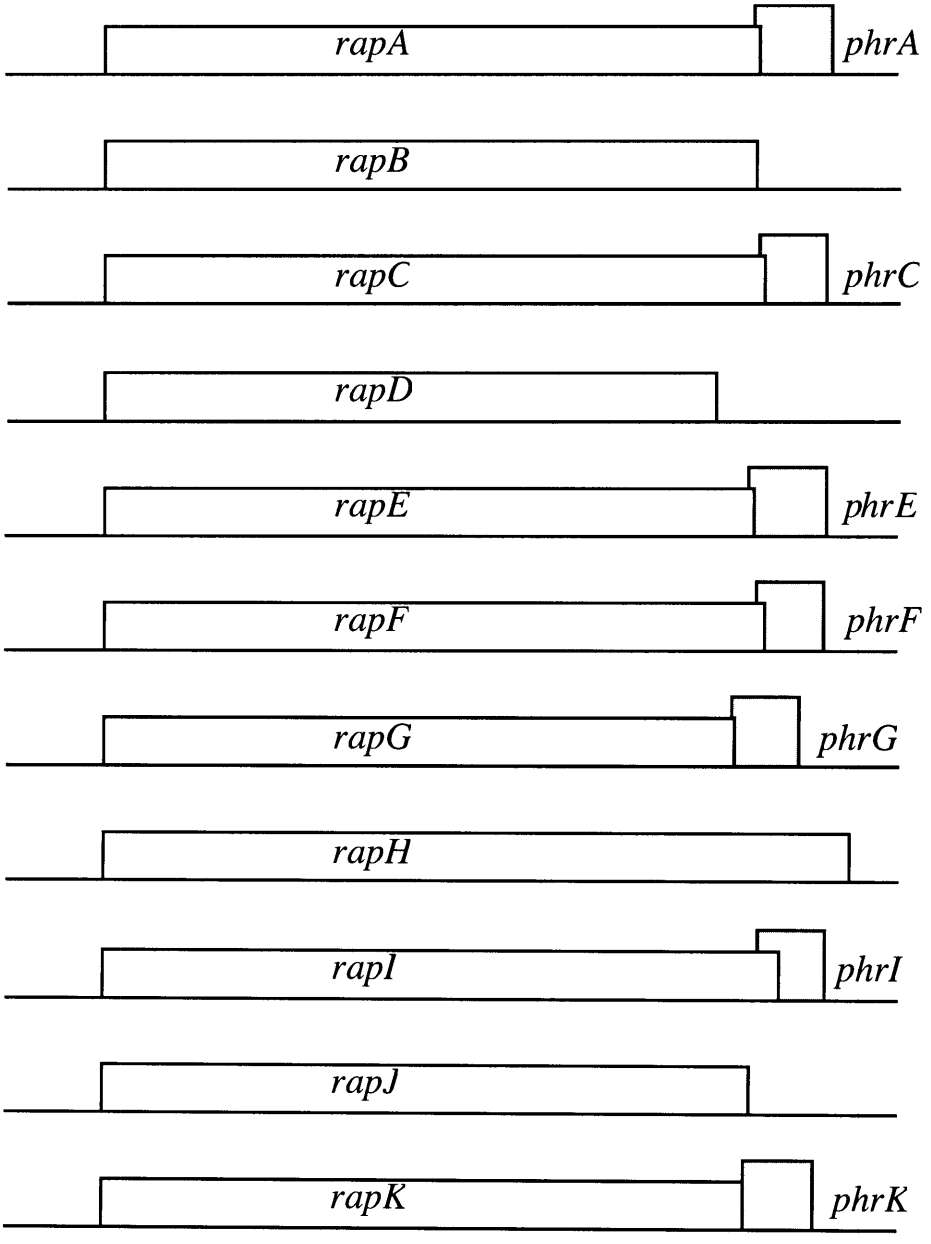


Figure 1-5. The *B. subtilis* family of *rap* and *phr* genes. Eleven genes encoding Rap homologs have been identified in the B. subtilis genome. Seven of these are followed by genes each encoding the precursor of a putative Phr peptide regulator.

final processing step does not appear to involve the signal peptidases (Stephenson et al., 2003).

After processing, the Phr peptides are then reimported into the cell via the oligopeptide permease (Opp), where they interact with their cognate Rap proteins to regulate their cellular targets (Lazazzera et al., 1997; Perego, 1997). Intracellular expression of the pentapeptides PhrC and PhrA is sufficient for inhibition of their cognate Rap proteins (Lazazzera et al., 1997; Stephenson et al., 2003). The Phr peptides are also functional for inhibition of the Rap proteins in vitro (Perego, 1997; Jiang et al., 2000a; Core and Perego, 2003; Ogura et al., 2003).

At least one Phr peptide, PhrC peptide (CSF, the competence and sporulation stimulating factor), has been found to accumulate in the extracellular medium and increase in concentration with growth (Solomon et al., 1995; Solomon et al., 1996). The result of this arrangement is to increase the ratio of Phr peptide to its cognate Rap regulator within the cell and to inhibit the Rap protein. It is unclear whether all the Phr peptides accumulate extracellularly in this manner, as no other Phr peptides have been characterized as accumulating in conditioned medium. At this time, there has been no comprehensive search for Phr peptides in conditioned medium produced by *B. subtilis*. It is clear that all the peptides characterized are functional for regulation when provided extracellularly as synthetic peptides (Perego and Hoch, 1996; Solomon et al., 1996; Jiang et al., 2000a; Ogura et al., 2003), strengthening the possibility that many Phr peptides are used for cell-cell signaling.

Perego presents a model where Phr peptides remain associated with the cell that produced them between processing and reimport (Perego, 1999). In this model,

regulation by Phr peptides is primarily cell-autonomous and not cell-cell signaling. Since the *rap* and *phr* genes are transcribed together, Perego postulates that Phr peptides may be used as a "timing" mechanism in that after transcription and translation of the *rap/phr* genes, a window of time occurs when the Rap is present intracellularly but the active peptide is not. This model is predicated in part on the inability to detect PhrA peptide activity in conditioned medium. However, the PhrC peptide definitely accumulates extracellularly. PhrA peptide has been shown to accumulate extraceullarly in a strain lacking the oligopeptide permease (Opp) (Perego and Hoch, 1996), indicating that PhrA peptide is not necessarily cell-associated. The ability of active Phr peptides to accumulate extracellularly indicates that they are likely to be used as cell-cell signaling molecules, and not simply cell-autonomous signals.

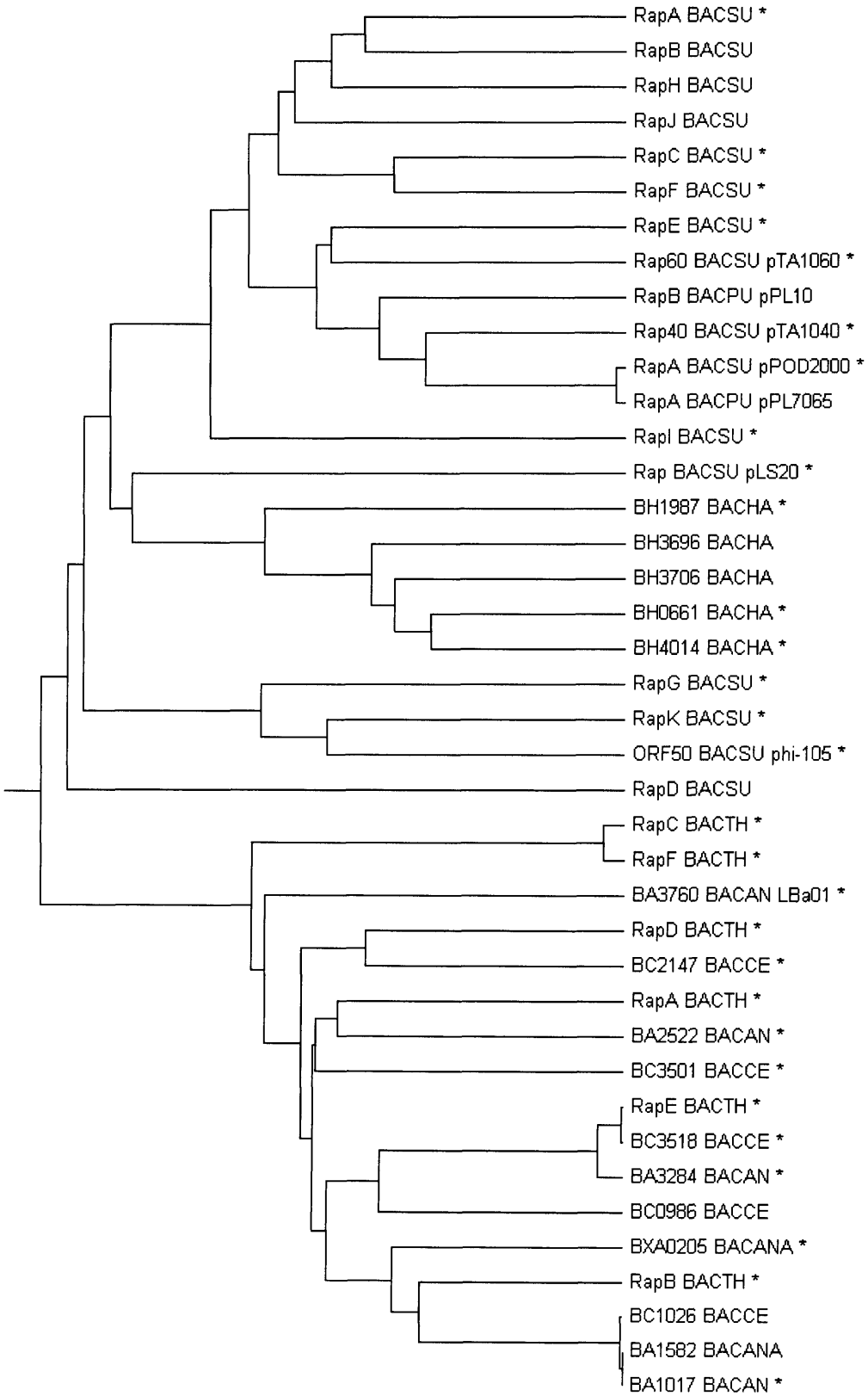
Rap regulatory proteins. The Rap proteins are members of the tetratricopeptide repeat (TPR) domain family of proteins. TPR domains consist of at least three TPR motifs, which are highly degenerate 34 amino acid repeats (Lamb et al., 1995). Each TPR motif consists of two antiparallel alpha helices, and multiple TPR motifs stack into a superhelical structure predicted to form a channel for binding target proteins (Blatch and Lassle, 1999). TPR domains mediate protein-protein interaction in a number of different systems, including molecular chaperone complexes, the anaphase promoting complex, transcription repression and protein transport (Blatch and Lassle, 1999). The Rap family members each contain 6 tandem TPR motifs (Perego and Brannigan, 2001) predicted to be important for binding both their target proteins and their Phr peptide regulators. Alteration of conserved residues in the TPR domains of RapA and RapC render the proteins insensitive to regulation by their cognate Phr peptides (Core and Perego, 2003).

Along with the 11 chromosomally encoded Rap proteins in *B. subtilis*, there are currently 30 additional uncharacterized Rap homologs in the sequence database. The additional Raps are all found in species closely related to *B. subtilis*: *Bacillus pumilis*, *Bacillus halodurans*, *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus anthracis* A2012, and *B. anthracis* strain Ames (NCBI) (Fig. 1-6). Of the 41 identified *rap* genes, 29 are followed by potential *phr* genes, a frequency similar to that of the *B. subtilis rap/phr* genes (Fig. 1-6).

Regulatory targets of Rap proteins. Most of the targets of the Rap proteins are members of the two-component system family of signal transduction pathways. RapC inhibits the transcription activation activity of the quorum sensing response regulator ComA, leading to lower expression of ComA-regulated genes in the absence of *phrC* (Solomon et al., 1996; Lazazzera et al., 1999a; Core and Perego, 2003). Unlike RapA, RapB, and RapE, RapC has not been shown to have any phosphatase activity in vitro. Instead, RapC forms a complex with ComA independent of ComA's phosphorylation state (Core and Perego, 2003). PhrC peptide can replace ComA in this complex, freeing ComA to bind to its target promoter sequences. The transcription of *rapC* and *phrC* is also regulated by ComA~P, leading to an autoregulatory loop where RapC negatively regulates its own expression and PhrC positively regulates its own expression (Lazazzera et al., 1999a). This may lead to positive reinforcement of activation of ComA~P at high cell densities due not only to high ComX concentration, but also high concentrations of the PhrC peptide.

RapA, RapB, and RapE inhibit the sporulation phosphorelay in *B. subtilis* (Fig. 1-7) (Perego et al., 1996; Jiang et al., 2000a). The sporulation phosphorelay consists of at

Figure 1-6. Phylogenetic tree of the *B. subtilis* Rap proteins and homologs in other species. Rap homologs were found by homology searches (NCBI). Sequences were aligned using ClustalW (Thompson et al., 1994), and a phylogenetic tree was constructed using MEGA 2.1 (Kumar et al., 2001). Asterisks indicate that the gene encoding the Rap homolog is followed by a small open reading frame predicted to encode a Phr peptide precursor. Species abbreviations are as follows: BACSU, *B. subtilis*; BACPU, *Bacillus pumilis*; BACHA, *Bacillus halodurans*; BACTH, *Bacillus thuringiensis*; BACCE, *Bacillus cereus*; BACAN, *Bacillus anthracis* strain Ames; BACANA, *Bacillus anthracis* strain A2012.


RapA BACSU *
RapB BACSU
RapH BACSU
RapJ BACSU
RapC BACSU *
RapF BACSU *
RapE BACSU *
Rap60 BACSU pTA1060 *
RapB BACPU pPL10
Rap40 BACSU pTA1040 *
RapA BACSU pPOD2000 *
RapA BACPU pPL7065
RapI BACSU *
Rap BACSU pLS20 *
BH1987 BACHA *
BH3696 BACHA
BH3706 BACHA
BH0661 BACHA *
BH4014 BACHA *
RapG BACSU *
RapK BACSU *
ORF50 BACSU phi-105 *
RapD BACSU
RapC BACTH *
RapF BACTH *
BA3760 BACAN LBa01 *
RapD BACTH *
BC2147 BACCE *
RapA BACTH *
BA2522 BACAN *
BC3501 BACCE *
RapE BACTH *
BC3518 BACCE *
BA3284 BACAN *
BC0986 BACCE
BXA0205 BACANA *
RapB BACTH *
BC1026 BACCE
BA1582 BACANA
BA1017 BACAN *

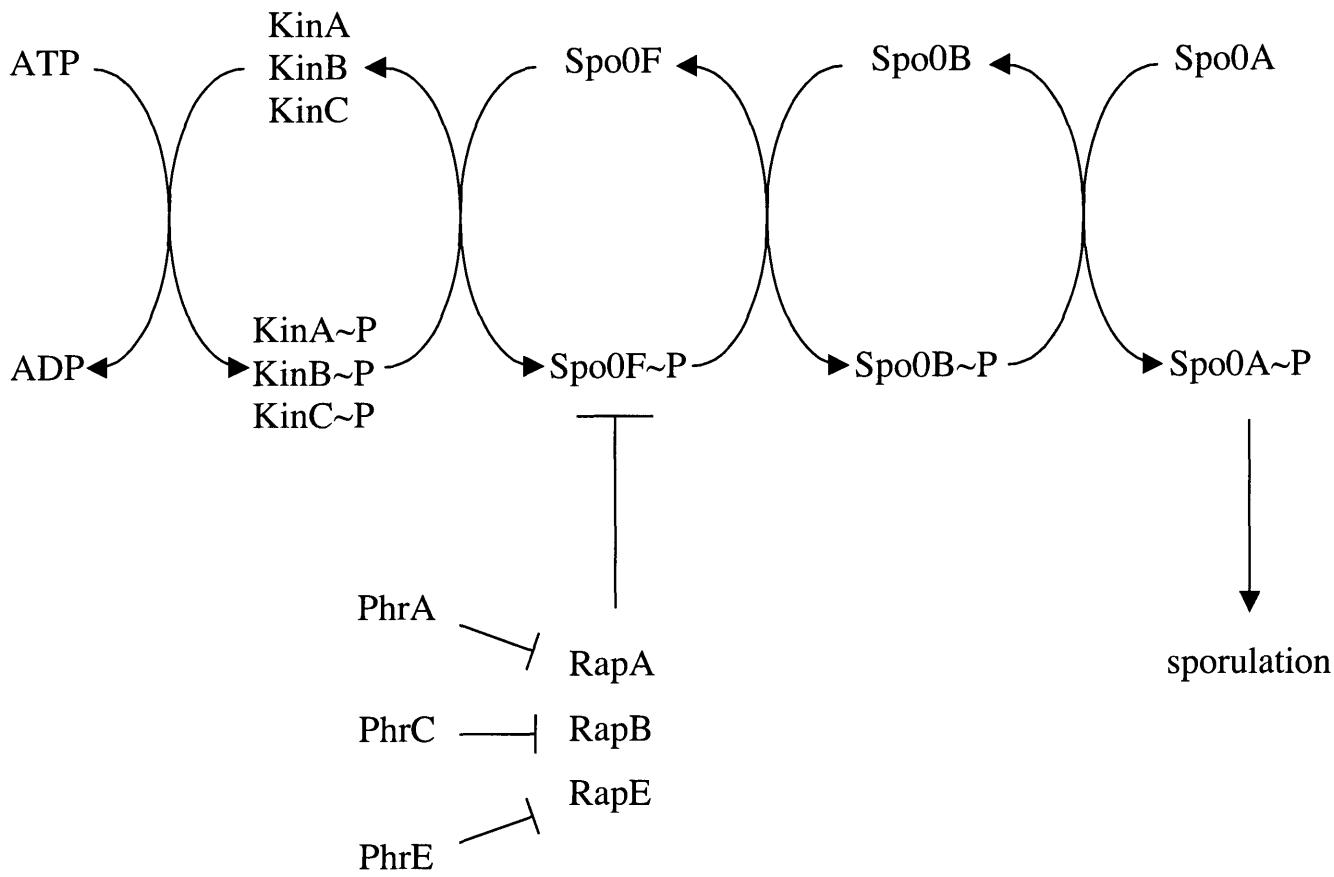


Figure 1-7. The *B. subtilis* sporulation phosphorelay is regulated by Rap proteins. The histidine kinases KinA, KinB and KinC autophosphorylate in response to unknown signals. The phosphoryl group is then passed in turn to the response regulator Spo0F, the histidine phosphotransfer protein Spo0B, and the response regulator transcription factor Spo0A. Spo0A~P directs transcription of genes required for the initiation of sporulation. Three Rap proteins (RapA, RapB and RapE) bind Spo0F~P and cause its dephosphorylation. Binding of these Rap proteins to Spo0F is inhibited by their cognate Phr peptides.

least 5 histidine kinases KinA-KinE (Perego et al., 1989; LeDeaux and Grossman, 1995; LeDeaux et al., 1995; Jiang et al., 2000b), that pass phosphate through the phosphotransfer proteins Spo0F and Spo0B to the sporulation response regulator transcription factor Spo0A (Burbulys et al., 1991). RapA, RapB and RapE all cause the dephosphorylation of Spo0F in vitro (Perego et al., 1996; Jiang et al., 2000a). However, it is unclear whether the Rap proteins act as phosphatases or simply activate an endogenous phosphatase activity of Spo0F. RapA, RapB and RapE are inhibited by the PhrA, PhrC and PhrE peptides, respectively. RapA binds to phosphorylated Spo0F in vitro, and PhrA peptide can actively displace Spo0F by binding RapA (Ishikawa et al., 2002).

The activities of two other Rap/Phr systems have been studied. RapG binds to and inhibits DegU, a response regulator transcription factor involved in regulation of degradative enzyme production (Ogura et al., 2003). This binding is relieved in vitro by the addition of the PhrG peptide. Overexpression of RapI activates excision of a novel conjugative element in *B. subtilis*, most likely by inhibiting the transcription factor that prevents expression of the conjugation machinery under most conditions (Auchtung, Lee and Grossman, unpublished data). This inhibitor, however, is not a member of the two-component family.

Many of these species with Rap homologs also contain homologs of known Rap targets that may also be regulated by Rap/Phr systems. Homologs of Spo0F are found in *B. halodurans*, *B. cereus*, *B. thuringiensis*, and *B. anthracis*. *B. cereus* contains homologs of *B. subtilis* ComP and ComA; however, *B. cereus* does not contain a homolog of ComQ, and only an unannotated ORF nearby that may encode a ComX analog. DegU homologs are found in *B. halodurans* and *B. cereus*. Interestingly, a

number of Rap homologs are encoded on mobile genetic elements, including plasmids in *B. subtilis* (Koetje et al., 2003) and *B. pumilis*, bacteriophage φ-150 in *B. subtilis*, prophage LambdaBa01 in *B. anthracis*, and a conjugative transposon in *B. subtilis* (Auchtung, Lee and Grossman, unpublished data). This mechanism of cell-cell signaling using imported peptides may be widespread among *B. subtilis* and its relatives.

Rap-like proteins in other Gram-positive bacteria. NprA of *B. stearothermophilus*, PreL of *Lactobacillus* sp., and NprR and PlcR of *B. thuringiensis* comprise another family of TPR domain proteins with significant homology to the *B. subtilis* Rap proteins but contain not only a TPR domain homologous to the Raps, but also a transcriptional activation domain (Lereclus et al., 1996; Ishikawa et al., 2002). Homology searches identified three additional members of this protein family in *Oceanobacillus iheyensis* (NCBI). PlcR activates the expression of a number of virulence factors in *B. thuringiensis* (Lereclus et al., 1996). DNA binding and activation by PlcR require a peptide product of the *papR* gene transcribed adjacent and downstream of PlcR (Slamti and Lereclus, 2002). Similar to the Phr proteins, PapR is predicted to be secreted and the C-terminus cleaved to the active peptide, probably a pentapeptide. Response to the PapR peptide requires the oligopeptide permease, and intracellular expression of the last 5 amino acids of PapR is sufficient to activate PlcR in a *papR* null mutant (Slamti and Lereclus, 2002). As with the Rap/Phr system, binding of the regulatory PapR peptide to PlcR is predicted to be mediated by the PlcR TPR domain. The other members of the NprA/PlcR family are also likely to be regulated by peptide binding to their TPR domains. Small open reading frames have been found downstream

of each of the these genes, suggesting that they all may be regulated by small peptides (Core and Perego, 2003).

AI-2/LuxS signaling

Recently, a third signaling paradigm has emerged from work on regulation of bioluminescence in *V. harveyi*. In this system, *V. harveyi* produces a signaling molecule termed AI-2 which induces expression of the luciferase operon that encodes the enzymes responsible for light production (Bassler et al., 1994). Production of AI-2 requires the enzyme LuxS (Surette et al., 1999). Homologs of LuxS have been found in several bacterial species, many of which produce an activity that can induce *V. harveyi* luminescence via the same mechanism as AI-2 (Bassler et al., 1997). These findings have led to the hypothesis that AI-2 and LuxS constitute an interspecies bacterial cell-cell signaling system.

Identity and production of AI-2 signals

The *V. harveyi* AI-2 (VhAI-2) is a furanosyl borate diester, 3α-methyl-5,6-dihydrofuro-[2,3-*d*][1,3,2]dioxaborole-2,2,6,6α-tetraol (Fig. 1-1) (Chen et al., 2002). The structure of VhAI-2 was determined by the serendipitous co-crystallization of the molecule with its periplasmic receptor protein, LuxP (Chen et al., 2002). The exact nature of the AI-2 signals from other organisms has not been elucidated. AI-2 activity has been defined as a substance which can stimulate the induction of luminescence in a *V. harveyi* tester strain (Bassler et al., 1997). This strain has one of the pathways for light induction deleted such that it produces light only through the pathway responsible for detection of VhAI-2 (Fig. 1-8). It is formally possible that different bacterial species

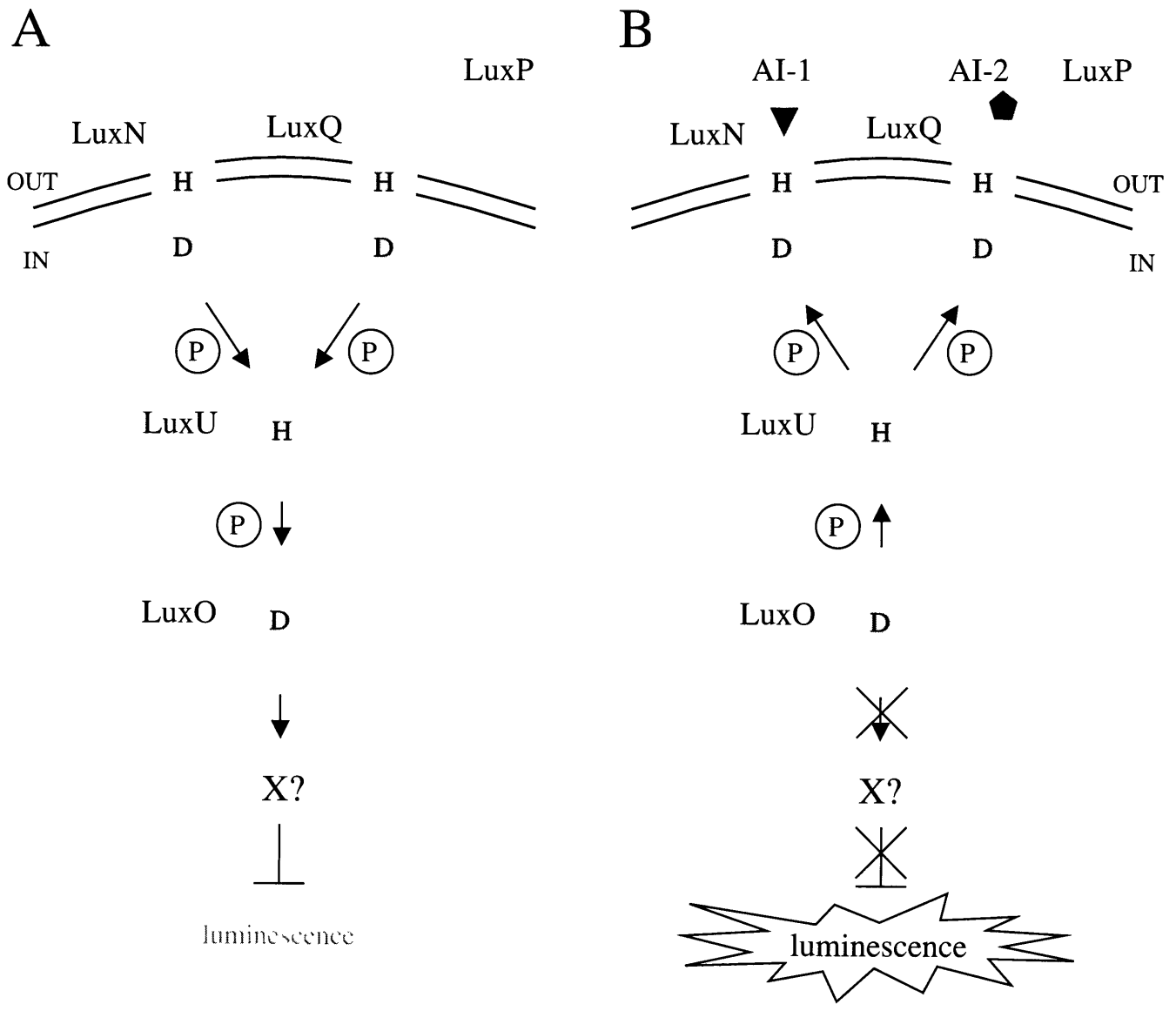


Figure 1-8. In *V. harveyi*, a phosphorelay system regulates luminescence in response to two signaling molecules. (A) In the absence of autoinducers 1 and 2, LuxN and LuxQ act as phosphoryl donors to LuxU, which then passes phosphate to LuxO. LuxO~P directs the transcription of a putative negative regulator of luminescence (X). (B) In the presence of autoinducers, LuxN ad LuxPQ act as phosphatases on LuxU, which in turn removes phosphate from LuxO. The putative negative regulator of luminescence is not produced, and *V. harveyi* produces light. D and H indicate phosphorylatable domains.

produce different and interconvertible AI-2 molecules. These molecules would all be predicted to be similar, since they all require the LuxS enzyme for production.

The LuxS enzyme required for production of AI-2 activity catalyzes the final step of the pathway involved in recycling the byproducts of methylation using S-adenosylmethionine (SAM) (Fig. 1-9) (Schauder et al., 2001; Winzer et al., 2002a). SAM is an essential methyl donor involved in the methylation of several molecules, including DNA, RNA, proteins and quinones (Matthews, 1996). Methylation by SAM yields the toxic metabolite S-adenosylhomocysteine (SAH), which is a potent inhibitor of methylation reactions that utilize SAM. In eukaryotes and some prokaryotes, SAH is detoxified by and recycled in one step by the enzyme S-adenosylhomocysteine hydrolase (SAHH) to form adenosine and homocysteine. In other prokaryotes, two steps are utilized for the detoxification and recycling of SAH. First, SAH is cleaved to adenine and S-ribosylhomocysteine (SRH) by the nucleosidase enzyme Pfs (5-methylthioadenosine/S-adenosylhomocysteine nucleosidase) (Cornell and Riscoe, 1998). Next, LuxS converts SRH to homocysteine and 4,5-dihydroxy 2,3-pentanedione (DPD) (Chen et al., 2002; Winzer et al., 2002a). DPD is unstable and predicted to quickly rearrange into a furanone structure, which can then complex with borate to form VhAI-2 (Chen et al., 2002).

The rearrangement and borate addition are not well understood and may require at least one additional enzyme. AI-2 activity that can activate luminescence in *V. harveyi* has been produced in vitro using SRH and purified LuxS protein from *Salmonella typhimurium* (Chen et al., 2002). However, no product matching the measured mass of VhAI-2 was detected by mass spectrometry of the reaction products. Presumably, the

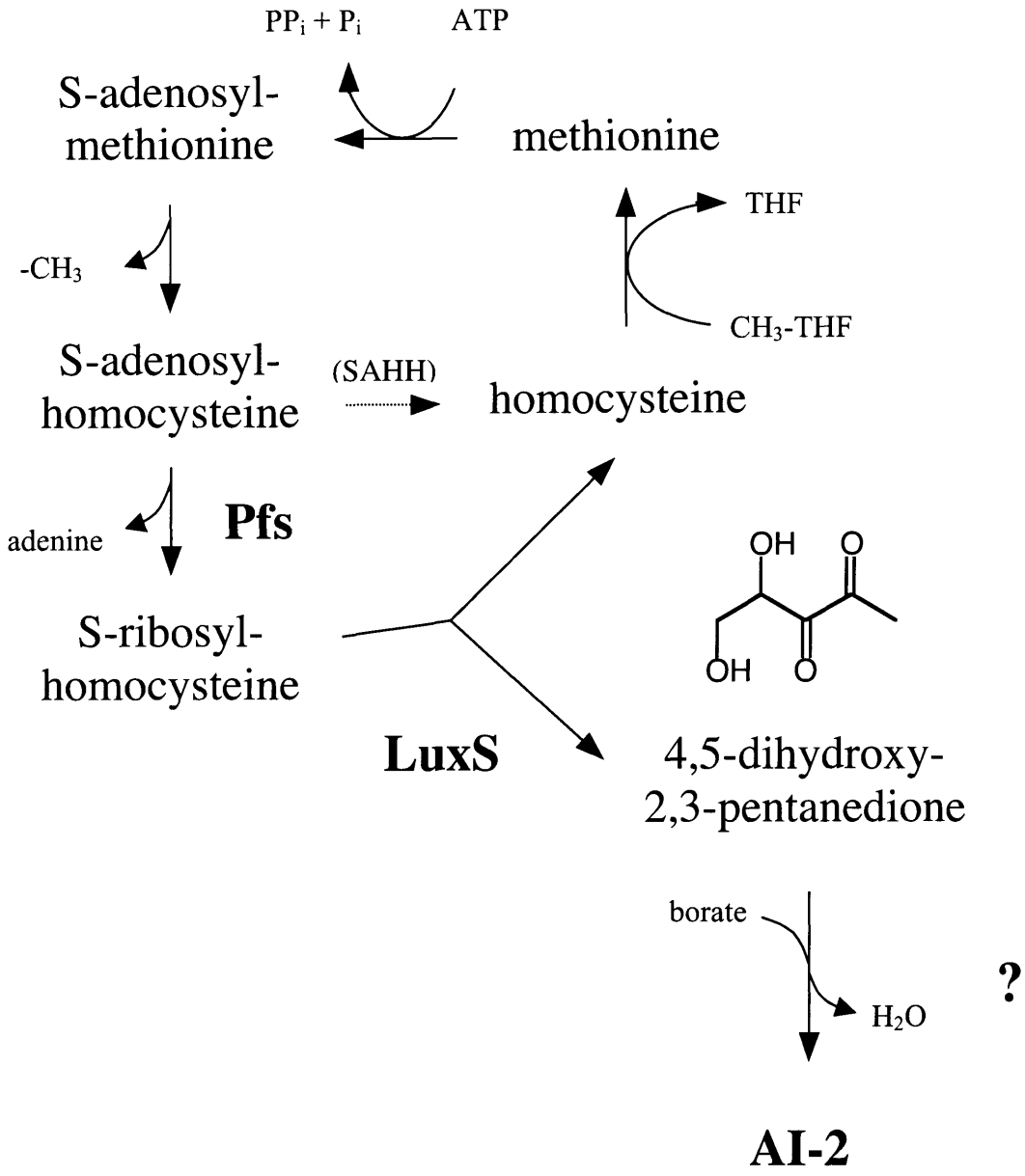


Figure 1-9. The synthesis of AI-2 activity is linked to S-adenosylmethionine metabolism. The usage of S-adenosylmethionine in methylation reactions produces S-adenosylhomocystein. The enzyme Pfs acts to cleave the adenine base, resulting in S-ribosylhomocysteine, the substrate for LuxS. LuxS cleaves S-ribosylhomocysteine to homocysteine and 4,5-dihydroxy-2,3-pentanedione, which is converted to AI-2 by an unknown mechanism. S-adenosylhomocysteine is converted directly to homocysteine and adenosine in eukaryotes and some prokaryotes (dotted arrow).

conversion of the AI-2 activity to VhAI-2 in this system occurs after addition of the reaction products to the *V. harveyi* assay culture. The conversion of precursors to the VhAI-2 molecule may occur spontaneously or may require an additional enzyme. LuxP, the *V. harveyi* AI-2 receptor protein, may be involved in this catalysis (Chen et al., 2002).

At the time of this writing, 40 of the 100 bacterial species whose genomes have been sequenced contain homologs of LuxS (NCBI). Every genome that contains LuxS also contains a homolog of Pfs, although there is a minority of bacteria that expresses Pfs in the absence of LuxS (Xavier and Bassler, 2003). To date, predicted homologs of LuxS and Pfs have only been found in bacteria. Eukaryotes and archaea exclusively use SAHH for detoxification of S-adenosylhomocysteine, whereas only some bacterial species contain SAHH. Phylogenetic analysis of SAHH distribution among bacteria suggests that Pfs is the more ancient bacterial detoxification system, with SAHH being introduced into bacteria by horizontal transfer in at least three distinct events (Bujnicki et al., 2003). To date, no species contains both LuxS and a homolog of SAHH, and no species that does not contain LuxS has been shown to respond to AI-2 (Xavier and Bassler, 2003). Taken together, these findings suggest that although LuxS is common among bacteria, it is not a universal or essential component of bacterial life.

Response to AI-2/*luxS*

The effects of *luxS* and AI-2 have been investigated in many species (Table 1). Although there are many distinct effects of *luxS* in different bacteria, there are some common themes. *luxS* appears to be involved in the regulation of virulence factors in several species (Table 1-1). In addition, *luxS* affects motility and biofilm formation in some organisms (Table 1-1). Although deletion of *luxS* results in a phenotype in many

Table 1-1. Genes and functions controlled by *luxS*/AI-2 in bacteria.

Species	Functions regulated by *luxS*	Genes regulated by *luxS*	Extracellular?	References
Actinobacillus actinomycetecomitans	Virulence factors: leukotoxin, iron acquisition	*afuA*	yes	(Fong et al., 2001; Fong et al., 2003)
Borrelia burgdorferi	Expression of many proteins on two-dimensional gels		yes	(Stevenson and Babb, 2002)
Campylobacter jejuni	Motility		no	(Elvers and Park, 2002)
Clostridium perfringens	Virulence factors: alpha, kappa and theta toxins	*pfo*	yes	(Ohtani et al., 2002)
Escherichia coli W3110	Cell division, DNA processing, cell shape and morphology	242 genes (microarray)	yes	(DeLisa et al., 2001)
Escherichia coli EHEC (O157:H7)	Virulence factors: type-III secretion, Shiga toxin, flagella, motility, cell division	LEE operon, *stx*, *ptsN*, *sulA*, *flhD*, *fliA*, *fliC*, *motA*, *qseA*, *qseBC* 404 genes (microarray)	yes	(Sperandio et al., 1999; Sperandio et al., 2001; Sperandio et al., 2002a; Sperandio et al., 2002b)
Escherichia coli EPEC (O127:H6)	Motility		no	(Sperandio et al., 1999; Giron et al., 2002)
Neisseria meningitidis	Bacteremic infection		no	(Winzer et al., 2002; Dove et al., 2003)
Photorhabdus luminescens	Carbapenem biosynthesis	*cpm*	no	(Derzelle et al., 2002)
Porphorymonas gingivalis	Virulence factors: protease, hemagglutinin activities, hemin acquisition	*uvrB*, *hasF*	yes	(Chung et al., 2001; Burgess et al., 2002; McNab et al., 2003)
Salmonella typhi	Biofilms		no	(Prouty et al., 2002)
Salmonella typhimurium	AI-2 ABC transport system	*lsrACDBFGE*	yes	(Taga et al., 2001)
Shigella flexneri	Transcription factor involved in controlling virulence	*virB*	yes	(Day and Maurelli, 2001)
Streptococcus gordonii	Biofilms		yes	(Blehert et al., 2003; McNab et al., 2003)
Streptococcus mutans	Biofilms		no	(Merritt et al., 2003)
Streptococcus pneumoniae	Virulence		no	(Stroeher et al., 2003)
Streptococcus pyogenes	Internalization, virulence factors: secreted protease, hemolysin	*speB*, *sagA*, *emm3*	no	(Lyon et al., 2001; Marouni and Sela, 2003)
Vibrio cholerae	Virulence factors: Cholera toxin, toxin-coregulated pilus	*tcpP*, *tcpA*, *ctxAB* ~70 virulence genes (microarray)	yes	(Zhu et al., 2002)
Vibrio harveyi	Light production, colony morphology, siderophore production	*luxCDABE*	yes	(Lilley and Bassler, 2000)
Vibrio vulnificus	Virulence factors: secreted protease, hemolysin	*vvpE* and *vvhA*	yes	(Kim et al., 2003)

organisms, in some systems researchers have not tested whether this phenotype is a cell-autonomous defect or bona fide cell-cell signaling through AI-2 or an AI-2 like molecule. Although in some cases there is an extracellular effect of *luxS*, with the exception of *Vibrio* spp. and *S. typhimurium*, the molecule or molecules that are responsible for the regulation and the mechanisms involved have not been characterized. Most of the functions that appear to be regulated by *luxS*/AI-2 are primarily controlled by other mechanisms, and *luxS* appears to play a secondary role in their regulation.

Is *luxS*/AI-2 acting as a cell-cell signaling system in multiple organisms, and potentially an interspecies cell-cell signaling system? Winzer et al. have defined the properties required of a specific bona fide cell-cell signaling molecule (CCSM):

1. The production of the CCSM occurs during specific stages of growth, under certain physiological conditions, or in response to changes in the environment.
2. The CCSM accumulates extracellularly and is recognized by a specific receptor.
3. Accumulation of the CCSM generates a concerted response, once a critical threshold concentration has been reached.
4. The cellular response extends beyond physiological changes required to metabolize or detoxify the CCSM. (Winzer et al., 2002b)

For the majority of the species in which *luxS* signaling has been investigated, the specific molecule involved in regulation of gene expression has not yet been identified, although it is expected to be an AI-2-like molecule. However, since *luxS* is involved in cellular metabolism, it is plausible that the difference between medium conditioned by wild-type and *luxS* bacteria is not simply AI-2. The systems in which *luxS* response has been linked to a specific AI-2 molecule, *Vibrio* spp. and *S. typhimurium,* use differing mechanisms to alter gene expression in response to AI-2.

AI-2 response in *V. harveyi*

V. harveyi controls light production using AI-2 and another signaling molecule termed AI-1. The two signaling molecules are sensed concurrently and the signals integrated to induce light production (Fig. 1-8). *V. harveyi* AI-1 (VhAI-1) is an acylated homoserine lactone (3-hydroxybutanoylhomoserine lactone) that is produced by a non-canonical AHL synthetase system consisting of the proteins LuxL and LuxM (Cao and Meighen, 1989; Bassler et al., 1994). LuxL and LuxM share no homology with the LuxI family of AHL synthetases responsible for AHL production in most Gram-negative signaling systems (Bassler et al., 1993). Also unusually, AI-1 is detected by a membrane sensor histidine kinase, LuxN, instead of an intracellular LuxR homolog as in the closely related *V. fischeri* (Bassler et al., 1994). This sensing mechanism more closely resembles the two-component signal transduction system of Gram-positive bacteria.

The response to AI-2 occurs through the proteins LuxP and LuxQ (Bassler et al., 1994; Chen et al., 2002). The AI-2 receptor LuxP is a periplasmic protein with significant homology to the periplasmic binding protein of a ribose importer. This is unsurprising, as AI-2 is predicted to be formed from the ribosyl group of S-ribosylhomocysteine. LuxP bound to AI-2 is predicted to interact with the membrane histidine kinase LuxQ for transduction of the AI-2 signal across the plasma membrane.

The two parallel sensor kinases LuxN and LuxPQ begin the convergent phosphorelay system controlling luminescence in *V. harveyi.* Each sensor is a multifunctional protein consisting of a membrane histidine kinase domain as well as an aspartate phosphorelay domain (Bassler et al., 1993; Bassler et al., 1994). The kinase function of each sensor is active only in the absence of inducer, when both proteins pass

phosphate to the convergent histidine phosphorelay protein LuxU (Freeman and Bassler, 1999b; Mok et al., 2003). The phosphate is then passed to an aspartate on the response regulator LuxO, which activates transcription of a number of genes together with the alternate sigma factor sigma-54 (Freeman and Bassler, 1999a; Lilley and Bassler, 2000). In the presence of autoinducers, the sensors act as phosphatases to remove phosphate from LuxU. This reverses the flow of phosphate in the phosphorelay, resulting in the dephosphorylation and deactivation of LuxO (Freeman and Bassler, 1999a).

Since LuxO is active and phosphorylated in the absence of autoinducers, i.e. when cells are dark, the activation of light is presumed to be indirect through the LuxO-dependent activation of a repressor of luminescence. This hypothesis is supported by genetic data that a *luxO* null mutant is constitutively bright, whereas a mutation that causes LuxO to be constitutively active is unable to produce light even in the presence of autoinducers (Freeman and Bassler, 1999a). The predicted luminescence repressor has yet to be identified.

AI-2 in *Vibrio* spp. meets all the criteria of a bona fide cell-cell signaling molecule. It accumulates throughout exponential growth and is sensed by the receptor kinase LuxPQ. The response to AI-2 (luminescence or expression of virulence factors) is both concerted and separate from functions required for metabolism of AI-2.

AI-2 response in *S. typhimurium*

S. typhimurium was one of the first non-*Vibrio* species shown to produce an activity that could activate luminescence in *V. harveyi* through the AI-2 signaling system. Accumulation of AI-2 activity in *S. typhimurium* occurs during the exponential phase when grown in the presence of glucose (Surette and Bassler, 1998; Surette and Bassler,

1999). Following the late exponential phase, the amount of AI-2 activity in the medium decreases due to the removal of AI-2 by an active transport system (Taga et al., 2001).

In order to identify genes controlled by AI-2/*luxS* in *S. typhimurium*, researchers performed a screen for insertional reporter fusions that were differentially expressed in a *luxS* null mutant compared to wild-type, and confirmed that the differential expression was non-cell autonomous by testing for complementation with conditioned medium (Taga et al., 2001). All of the positive fusions were located in the *lsr* operon, which is homologous to the *rbs* operon of *E. coli* and *S. typhimurium* (Fig. 1-10). The *rbs* genes encode proteins required for the uptake and utilization of ribose (Iida et al., 1984). *lsrACDB* encode a predicted ABC transporter that is required for the decrease in AI-2 activity observed in late exponential phase cultures of *S. typhimurium* (Taga et al., 2001). Mutations in any of these genes eliminate the ability of *S. typhimurium* to remove AI-2 activity from the medium.

Selection for mutants that overexpress the *lsr* operon in the absence of *luxS* identified a negative transcriptional regulator of the operon, *lsrR*, located immediately upstream of the *lsr* operon and transcribed divergently (Taga et al., 2001). LsrR is a predicted transcriptional regulator homologous to SorC, a transcriptional regulator of genes involved in sorbose metabolism in *Klebsiella pneumoniae* (Wehmeier and Lengeler, 1994). Based on similarity to other sugar uptake systems, LsrR most likely represses transcription of the *lsr* operon in its free state and this repression is relieved on binding to AI-2 or an AI-2 derivative.

At this point in time, AI-2 does not appear to meet the requirements for a cell-cell signaling molecule in *S. typhimurium* (Winzer et al., 2002b). Although AI-2 does

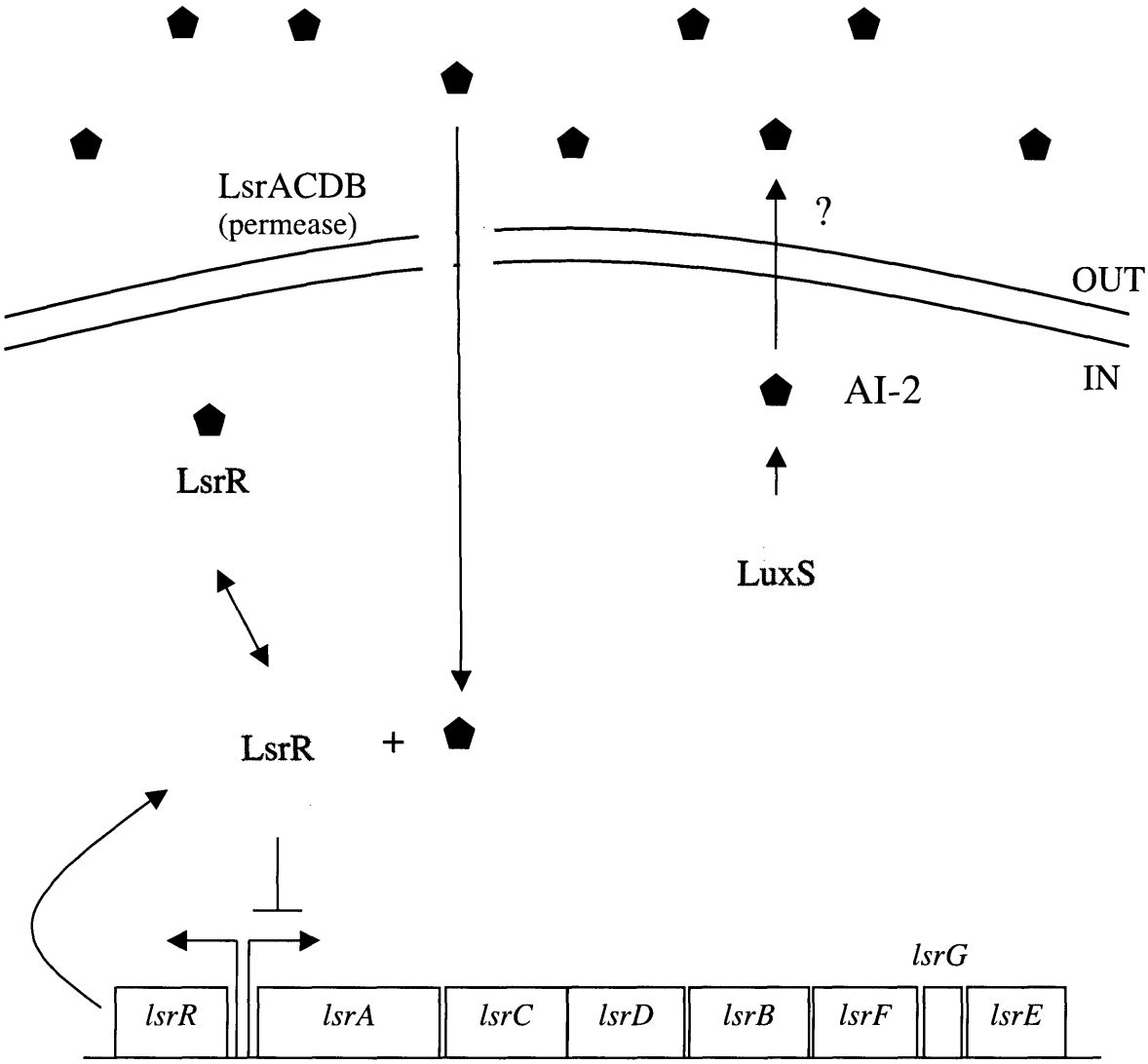


Figure 1-10. A model for control of an operon required for uptake of AI-2 activity in response to AI-2 in *S. typhimurium*. LuxS produces AI-2 in S. typhimurium. An active permease (LsrACDB) imports AI-2 into the cell, where it is predicted to be further modified by the products of the remaining lsr genes. Some derivative of AI-2 binds LsrR and prevents its repression of the lsr operon. Small pentagons indicate AI-2 molecules.

accumulate during exponential growth and elicits a specific response, the only identified response to AI-2 is the expression of the *lsr* operon, and all the genes of the *lsr* operon are predicted to encode proteins required for the uptake and utilization of AI-2. This calls into question the model that AI-2 is a cell-cell signal that can elicit a response in multiple bacteria. It remains to be seen what the mechanism and response to AI-2 will be for other bacterial species.

Summary

The Gram positive soil bacterium *Bacillus subtilis* uses cell-cell signaling to regulate multiple cellular processes, including sporulation and the development of genetic competence (Grossman, 1995). This thesis presents my work investigating the regulation of a family of putative peptide signaling molecules by an alternate sigma factor involved in stationary phase processes and sporulation (Chapter 2). I also present evidence that *B. subtilis* produces AI-2 activity produced during exponential growth (Chapter 3).

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Chapter 2

Control of a family of phosphatase regulatory genes (*phr*) by the alternate sigma factor sigma-H of *Bacillus subtilis*

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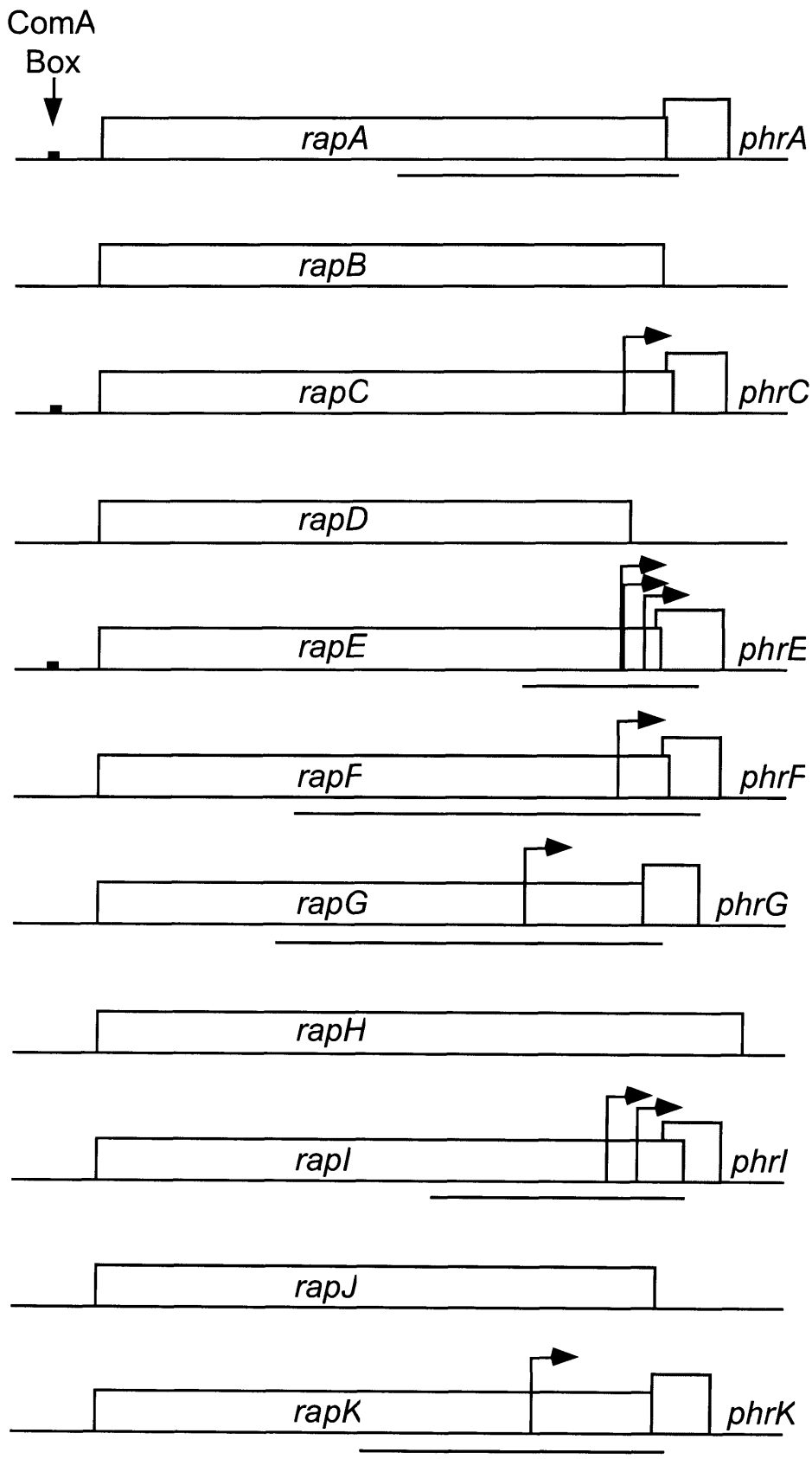
A family of 11 phosphatases can help to modulate the activity of response regulator proteins in *Bacillus subtilis*. Downstream of seven of the *rap* (phosphatase) genes are *phr* genes, encoding secreted peptides that function as phosphatase regulators. Using fusions to *lacZ* and primer extension analysis, we found that six of the seven *phr* genes are controlled by the alternate sigma factor sigma-H. These results expand the potential of sigma-H to contribute to the output of several response regulators by controlling expression of inhibitors of phosphatases.

A family of phosphatases and cognate regulators (Fig. 2-1) modulate the output of two-component signal transduction systems in *Bacillus subtilis* (Perego et al., 1994; Perego and Hoch, 1996b; Lazazzera et al., 1999b). Two-component systems generally consist of a histidine protein kinase that autophosphorylates on a histidine residue and a response regulator, often a transcription factor, whose activity is controlled by phosphorylation of an aspartate residue (Hoch and Silhavy, 1995; Hoch, 2000). The response regulator obtains phosphate from its cognate kinase. Many cellular processes are controlled by two component systems; the *B. subtilis* genome encodes 37 histidine kinases and 34 response regulators (Kunst et al., 1997; Fabret et al., 1999).

The phosphorylation state of many response regulators is negatively regulated by phosphatases. *B. subtilis* has a family of eleven genes encoding phosphatases (or putative phosphatases) that are homologous to each other (Perego and Hoch, 1996b; Kunst et al., 1997; Perego, 1999). Several of these response regulator aspartyl phosphatases (Rap phosphatases) have been characterized. RapA and RapB (and to a lesser extent RapE) negatively regulate the initiation of sporulation (Mueller and Sonenshein, 1992; Perego et

Figure 2-1. The family of *rap* phosphatases and *phr* peptide regulators. Putative transcriptional start sites, as determined by mapping 5' ends by primer extension analysis, are indicated by arrows. To analyze expression, DNA fragments (denoted by solid lines underneath the genes) were cloned upstream of *lacZ* and integrated into the chromosome at *amyE*. The fragments were amplified by PCR using Vent polymerase and primers with restriction sites (*Eco*RI and *Bam*HI) at the ends. These fragments were subcloned between the *Eco*RI and *Bam*HI sites of pKS2 (Magnuson et al., 1994). The resulting plasmids were linearized and transformed into wild-type *B. subtilis*, selecting for Neo^r transformants. Transformants were screened for an amylase-deficient phenotype to confirm the plasmid had integrated into the chromosome at the *amyE* locus. For each *phr* gene, the fragment endpoints are indicated relative to the translation start site: *phrA*, 530 bp upstream, 30 bp downstream; *phrE*, 275 bp upstream, 73 bp downstream; *phrF*, 749 bp upstream, 62 bp downstream; *phrG*, 745 bp upstream, 30 bp downstream; *phrI*, 465 bp upstream, 37 bp downstream; *phrK*, 583 bp upstream, 22 bp downstream.


ComA
Box
rapA
phrA
rapB
rapC
phrC
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rapE
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rapF
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rapJ
rapK
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al., 1994; Perego et al., 1996; Jiang et al., 2000a). They do so by dephosphorylating the response regulator protein Spo0F, which is part of the phosphorelay (Burbulys et al., 1991) that is required to activate the sporulation transcription factor Spo0A in response to multiple signals, including starvation (Ireton et al., 1993; Perego et al., 1994; Grossman, 1995).

The activities of RapA, RapB, and RapE are negatively regulated by specific pentapeptides. RapA and RapE are inhibited by the pentapeptides produced from precursors encoded by *phrA* and *phrE*, the genes downstream from *rapA* and *rapE*, respectively (Perego and Hoch, 1996a; Perego, 1997; Jiang et al., 2000a). RapB is inhibited by the pentapeptide that is produced from the precursor encoded by *phrC*, the gene downstream from *rapC* (Perego, 1997). These inhibitory peptides are exported, and at least some of them accumulate in culture supernatant. They are then imported by the oligopeptide permease (Opp [also known as Spo0K]) (Perego et al., 1991; Rudner et al., 1991; Lazazzera et al., 1997). In this way, the peptides can be indicators of population density (Perego and Hoch, 1996a; Solomon et al., 1996; Lazazzera et al., 1997; Lazazzera and Grossman, 1998; Lazazzera et al., 1999a), as well as the intracellular conditions necessary for their production (Perego, 1997; Lazazzera et al., 1999a; Perego, 1999).

The pentapeptide produced from the *phrC* gene product, called CSF (competence and sporulation-stimulating factor) was initially purified from culture supernatant as an activity that stimulated expression of genes activated by the transcription factor ComA (Solomon et al., 1995; Solomon et al., 1996). *comA* encodes a response regulator required for the development of genetic competence (Nakano and Zuber, 1989; Weinrauch et al., 1989) and the general quorum response (Lazazzera and Grossman,

1998; Lazazzera et al., 1999b). ComA is active in the phosphorylated form and obtains phosphate from the histidine kinase ComP (Weinrauch et al., 1990; Roggiani and Dubnau, 1993; Piazza et al., 1999; Jiang et al., 2000b). RapC inhibits expression of genes activated by ComA~P, most likely by dephosphorylating ComA~P (Solomon et al., 1996). Expression of genes activated by ComA~P is increased by two different extracellular peptides, the ComX pheromone, which activates the kinase ComP (Magnuson et al., 1994; Solomon et al., 1995; Piazza et al., 1999), and CSF, which appears to inhibit the phosphatase RapC (Solomon et al., 1995). In this way, two different extracellular signaling molecules contribute to activate a general quorum response controlled by ComA.

Transcription of *phrC* is controlled by two promoters: P1, which is upstream of *rapC* and directs transcription of *rapC* and *phrC* (Lazazzera et al., 1999a); and P2, which is internal to *rapC* and directs transcription of *phrC* (Carter et al., 1990; Lazazzera et al., 1999a). Transcription from P2 is controlled by the alternate sigma factor sigma-H (the product of *sigH* [also known as *spo0H*]) and increases during entry into stationary phase (Carter et al., 1990; Lazazzera et al., 1999a). *phrE* is also dependent on sigma-H for maximal expression (Jiang et al., 2000a).

A *phr* gene, encoding a putative or bona fide secreted peptide that functions as a phosphatase regulator, is found downstream from 7 of the 11 *rap* genes (Fig. 2-1). We have found that, with the exception of *phrA*, all of the *phr* genes have sigma-H promoters upstream of the gene and internal to the cognate *rap* gene. These findings indicate that the alternate sigma factor sigma-H controls production of a family of phosphatase

regulators and that synthesis of these regulators is controlled by the nutritional conditions that modulate sigma-H.

Expression of *phr-lacZ* fusions

A search of the *B. subtilis* genome found putative sigma-H promoters upstream of each *phr* gene, except *phrA* (Lazazzera et al., 1999b). To measure expression from the potential promoters upstream of the *phr* genes and to test the dependence on sigma-H, we constructed transcriptional fusions of the regions upstream of the *phr* genes to *lacZ* (Fig 2-1 and Table 2-1). We amplified the putative promoter regions by PCR and cloned the fragments upstream from *lacZ*, and the resulting fusions were integrated into the chromosome at a heterologous site (*amyE*). We measured β-galactosidase specific activity for each fusion during growth and after entry into stationary phase in nutrient broth sporulation medium (Fig. 2-2) and defined minimal medium (data not shown) at 37°C . The patterns of expression were similar in both media. All of the fusions had an initially low level of expression that increased at or shortly before the transition to stationary phase (T_0). The peak of β-galactosidase specific activity was between 1 and 3 h after the end of exponential growth. No expression was observed from a fusion made to the region upstream of *phrA* (data not shown), indicating that under the conditions tested, there is not a functional promoter in this region.

A *sigH* null mutation reduced the maximal level of expression to less than 1% of that of the wild type for all fusions, except *phrE* and *phrI* (Fig. 2-2). The *phrE-* and *phrI-lacZ* fusions retained a significant level of expression in a *sigH* null mutant (Fig 2-2A, D), presumably due to transcription from the sigma-A promoters described below. The

Table 2-1. *B. subtilis* strains used in this study.

Strain	Relevant genotype[a]
JH642	*trpC2 pheA1*
IRN238	*amyE::(phrC-lacZΩ2 neo)*
IRN243	*amyE*::(*phrC-lacZΩ2 neo*) *sigH*::*cat*::*spc*
RSM106	*amyE*::(*phrE-lacZΩ2 neo*)
RSM114	*amyE*::(*phrE-lacZΩ2 neo*) *sigH*::*cat*::*spc*
RSM154	*amyE*::(*phrE-lacZΩ2 neo*) *spo0AD56N-cat*::*spc*
RSM139	*amyE*::(*phrE-lacZΩ2 neo*) *abrB*::Tn*917*
RSM147	*amyE*::(*phrE-lacZΩ2 neo*) *spo0AD56N-cat*::*spc abrB*::Tn*917*
RSM128	*amyE*::(*phrF-lacZΩ7 neo*)
RSM135	*amyE*::(*phrF-lacZΩ7 neo*) *sigH*::*cat*::*spc*
RSM132	*amyE*::(*phrF-lacZΩ7 neo*) *spo0AD56N-cat*::*spc*
RSM140	*amyE*::(*phrF-lacZΩ7 neo*) *abrB*::Tn*917*
RSM148	*amyE*::(*phrF-lacZΩ7 neo*) *spo0AD56N-cat*::*spc abrB*::Tn*917*
RSM129	*amyE*::(*phrG-lacZΩ8 neo*)
RSM136	*amyE*::(*phrG-lacZΩ8 neo*) *sigH*::*cat*::*spc*
RSM133	*amyE*::(*phrG-lacZΩ8 neo*) *spo0AD56N-cat*::*spc*
RSM141	*amyE*::(*phrG-lacZΩ8 neo*) *abrB*::Tn*917*
RSM149	*amyE*::(*phrG-lacZΩ8 neo*) *spoT0A* (D56N)-*cat*::*spc abrB*::Tn*917*
NC131	*amyE*::(*phrI-lacZΩ2 neo*)
RSM165	*amyE*::(*phrI-lacZΩ2 neo*) *sigH*::*cat*::*spc*
RSM166	*amyE*::(*phrI-lacZΩ2 neo*) *spo0AD56N-cat*::*spc*
RSM167	*amyE*::(*phrI-lacZΩ2 neo*) *abrB*::Tn*917*
RSM168	*amyE*::(*phrI-lacZΩ2 neo*) *spo0AD56N-cat*::*spc abrB*::Tn*917*
RSM130	*amyE*::(*phrK-lacZΩ9 neo*)
RSM137	*amyE*::(*phrK-lacZΩ9 neo*) *sigH*::*cat*::*spc*
RSM134	*amyE*::(*phrK-lacZΩ9 neo*) *spo0AD56N-cat*::*spc*
RSM142	*amyE*::(*phrK-lacZΩ9 neo*) *abrB*::Tn*917*
RSM150	*amyE*::(*phrK-lacZΩ9 neo*) *spo0AD56N-cat*::*spc abrB*::Tn*917*

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

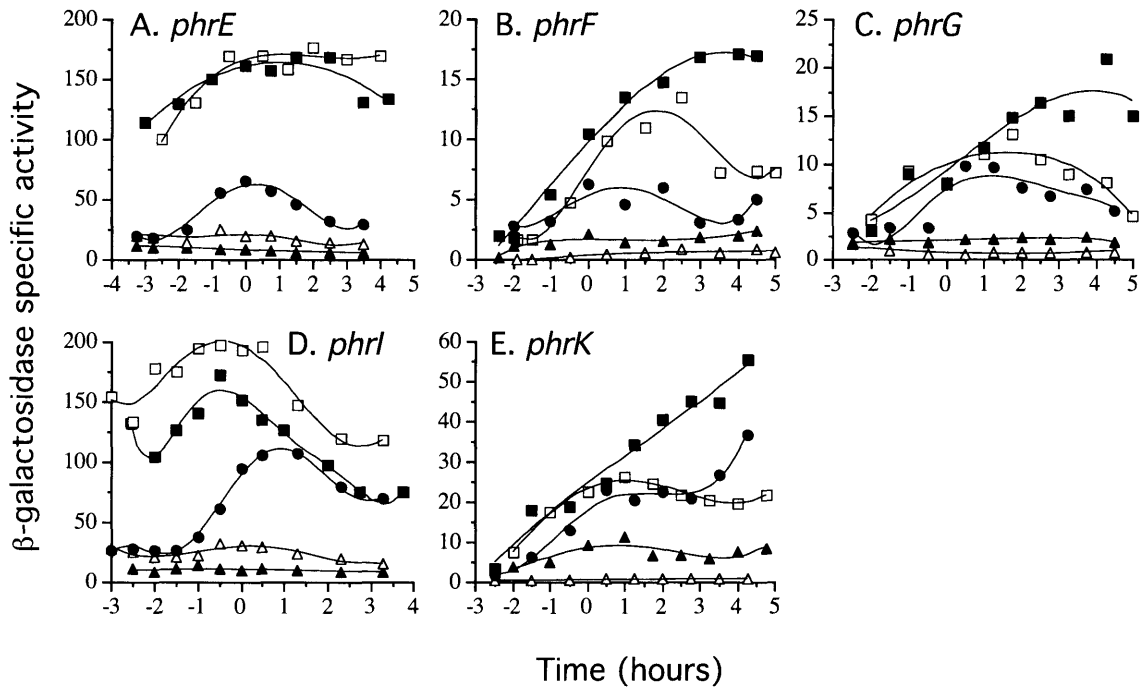


Figure 2-2. Expression of the *phr-lacZ* transcriptional fusions. ß-galactosidase specific activity was assayed as described previously (Miller, 1972; Jaacks et al., 1989). Time indicated is relative to the transition to stationary phase (T_0). (A) *phrE-lacZ*; (B) *phrF-lacZ*; (C) *phrG-lacZ*; (D) *phrI-lacZ*; (E) *phrK-lacZ.* Symbols: filled circles, wild-type; open triangles, *sigH*; filled triangles, *spo0A*; filled squares, *abrB*; open squares, *spo0A abrB*. Strains are indicated in Table 2-1.

pattern of expression seen for the *phrE-lacZ* fusion is consistent with that described previously (Jiang et al., 2000a).

Spo0A and AbrB affect expression of many sigma-H-regulated promoters (Zuber and Losick, 1987; Perego et al., 1988; Weir et al., 1991; Grossman, 1995; Hahn et al., 1995; Strauch, 1995). In a *spo0A* null mutant, transcription of *phrC*P2 is down ~5-fold during stationary phase. This effect is relieved by a null mutation in *abrB* (Carter et al., 1990; Lazazzera et al., 1999a). Similar effects were observed with the other *phr-lacZ* fusions (Fig. 2-2). In all cases, a *spo0A* null mutation caused a decrease in activity compared to that of the wild type, and this effect was relieved by a null mutation in *abrB*.

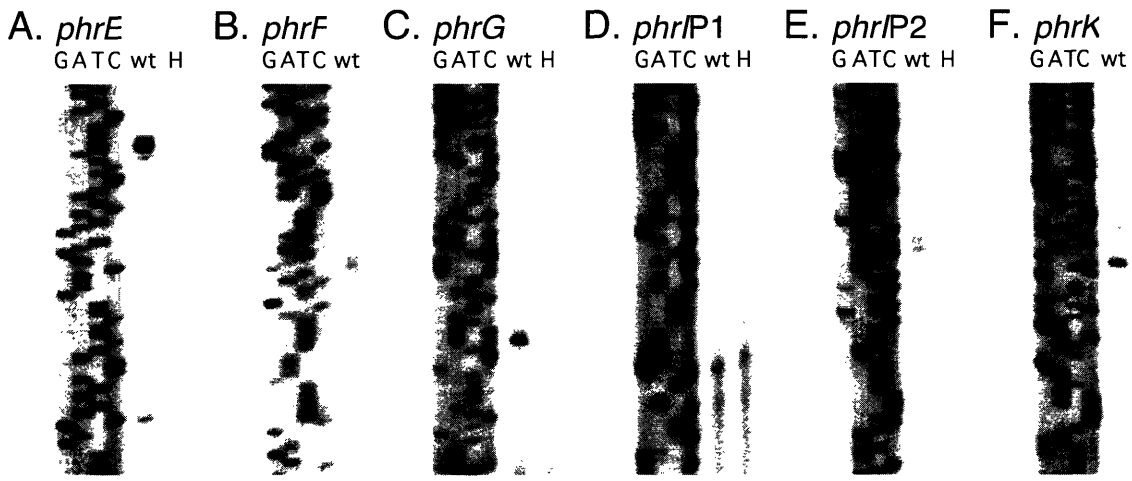
The expression of the *phrE-lacZ* and *phrI-lacZ* fusions was significantly higher in the *abrB* null mutant than in the wild type (Fig 2-2A, D). There are sequences in these promoter regions that resemble known AbrB binding sites (Strauch, 1995) and we suspect that AbrB acts directly to repress expression from *phrE*P1 and/or P2, and *phrI*P1.

Primer extension mapping of *phr* promoters

The *lacZ* fusions confirmed the existence of promoters upstream of the *phr* genes. To determine if the location of the promoters correlated with the sequences resembling sigma-H-dependent promoters, we used primer extension to map precisely the 5' ends of transcripts of *phrE*, *phrF*, *phrG*, *phrI*, and *phrK* (Fig. 2-3). Cells were grown in sporulation medium (Schaeffer et al., 1965) at 37°C, and RNA was prepared from cells 2 to 3 h after the end of exponential growth. Primer extension was performed essentially as described previously (Triezenberg, 1992). The major primer extension products for all the genes correlated to start sites downstream from the putative sigma-H promoters (Fig.

Figure 2-3. Primer extension mapping of the 5' ends of *phr* mRNA and sequence of the promoter regions. (A-F). RNA for primer extension was prepared using the Qiagen RNeasy protocol. Approximately 50 μg RNA was used per primer extension reaction. Each primer extension reaction is shown alongside a sequencing ladder (lanes labeled G, A, T, C) produced using the same end-labeled primer. RNA was prepared from wild type (wt) cells (IRN238 or RSM128) and a *sigH* mutant (H) (IRN243 or RSM135). (A) *phrE*, primer phrE4 (5'-CCAATTAAAACGGCGGATAAACTGA-3'); (B) *phrF*, primer phrF5 (5'-GAGCCAGACAAGAGAGTAATAGTTTAGA-3'); (C) *phrG*, primer RM36 (5'-CTTACACTGTCATACTCTTTCTCGGAC-3'); (D) *phrI*P1, primer phrIPEU (5'-CGTCTTGCTTCAATACTCATACG-3'); (E) *phrI*P2, primer phrIPE (5'-CTATGCCCCTACCCGATCTGCAGC-3'); (F) *phrK*, primer RM38 (5'-GCTCGTTGCTTCTTCAAAAGCACC-3').

(G) Sigma-H promoters identified by primer extension analysis. Promoters were aligned by the –10 region with spaces added to align the –35 regions. Residues with identity to the sigma-H consensus sequence are indicated in bold. The transcription start sites (+1) as determined by primer extension are indicated by bold, lower-case letters. The sigma-A consensus site in *phrE*P1-2 is underlined.



G.

	-35 region	-10 region	+1
phrC	TAG**AGGA**TT**T**AGCCCTAGAAGTAGCAA**AAT**ATTACTATGA**a**CA		
*phrE*P1-2	G<u>TT**AGGA**</u>GGCAACCTTTTCGAT--<u>TAT**AAT**</u>GGGAA**a**AT**a**GAGG		
*phrE*P3	TAG**AGGA**TAGTATCATCTGTTTC-AA**GAA**GATGGTATAT**g**CTC		
phrF	TTG**AA**G**A**TT**T**CGCTATTGATATGGCAA**AAT**ATTATC**a**TGAACG		
phrG	TGA**AGGA**AAAATTGACTGCTGAG-CC**GAAT**AGAATATGT**g**AGG		
*phrI*P2	ACA**AGGA**AA**T**TTAATGCTCTCTA-AT**GAAT**ATTATCGT**a**TG**a**T		
phrK	CAC**AGGA**AAGACTCATATTAATG-GA**GAAT**AAAGTATAC**g**AAG		
SigH consensus	**AGGA**NN**T**.....N15-17.....**GAAT**		

2-3). Furthermore, these products were not observed in a *sigH* null mutant (Fig. 2-3 and data not shown). *phrE* and *phrI* also had primer extension products that were not dependent on sigma-H (Fig. 2-3A, D), consistent with the pattern of expression of the *phrE-lacZ* and *phrI-lacZ* fusions (described above).

phrE transcripts had three detectable 5' ends (Fig. 2-3A). The level of each was reduced in a *sigH* null mutant, with the two downstream ends reduced below the limit of detection. The most upstream end (P1) corresponds to a potential sigma-A promoter and the two downstream ends (P2 and P3) correspond to potential sigma-H promoters. We suspect that the effect of the *sigH* null mutation on P1 is due to the effects of *sigH* on expression of *spo0A*, which is required for full expression of *phrE* (Fig. 2-2A and Jiang et al., 2000a).

phrI had two apparent 5' ends corresponding to promoters of two different sigma factors (Fig. 2-3D to E). The upstream product was independent of sigma-H and corresponds to the start site for a potential sigma-A promoter (P1). The downstream product was not observed in a *sigH* null mutant and matches the start site of the sigma-H promoter (P2) predicted by sequence analysis.

Sigma-H and regulation of response regulators

The characterized Phr peptides act as extracellular regulators of gene expression by inhibiting Rap phosphatases that act on response regulators (Solomon et al., 1996; Lazazzera et al., 1997; Perego, 1997; Jiang et al., 2000a). The remaining *phr* genes are predicted to encode similar extracellular peptides that regulate the activity of other Rap phosphatases (Perego and Hoch, 1996b). The finding that all of the *phr* genes (except

phrA) are transcribed by RNA polymerase holoenzyme containing sigma-H implies that many cellular processes and response regulators are regulated indirectly by sigma-H.

Sigma-H is regulated by many diverse signals, and this regulation occurs at the transcriptional and post-translational levels. The activity of sigma-H is regulated by growth phase (Healy et al., 1991; Weir et al., 1991), pH (Cosby and Zuber, 1997), members of the Clp protease family (Nanamiya et al., 1998; Liu et al., 1999; Liu and Zuber, 2000), and perhaps the stringent response (Eymann et al., 2001). As cells experience conditions that increase activity of *sigH*, the amount of *phr* transcription relative to its upstream *rap* will increase. This will enhance the regulation (inhibition) of the Rap phosphatases by their respective Phr peptides.

The effects of sigma-H on controlling expression of the *phr* genes most likely contributes to the effects of cell culture density on sporulation. Sporulation is more efficient at high rather than low cell densities (Vasantha and Freese, 1980; Grossman and Losick, 1988; Ireton et al., 1993; Lazazzera et al., 1997), due to the accumulation of signaling peptides in culture supernatant (Grossman and Losick, 1988; Lazazzera et al., 1997). *sigH* mutants are defective in production of at least some of the signaling peptides (Grossman and Losick, 1988; Lazazzera et al., 1997). We suspect that several other cellular responses are modulated by population density and that sigma-H contributes to these via its role in expression of the *phr* genes.

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Chapter 3

luxS in *Bacillus subtilis*: production of AI-2 activity with no detectable transcriptional response

Abstract

LuxS is a protein conserved among many bacterial species that is responsible for production of the putative interspecies signaling molecule AI-2. Some bacteria have been shown to modulate gene expression in response to *luxS* levels, and in some cases this response may be cell-cell signaling. We investigated the function of *luxS* in *Bacillus subtilis*. Production of AI-2 activity by *B. subtilis* in defined minimal medium was dependent on *luxS*. *luxS* of *B. subtilis* is also functional for AI-2 production in an *Escherichia coli* strain missing the endogenous *luxS* (DH5α). A *luxS* null mutant of *B. subtilis* had no apparent phenotype, and no significant change in transcriptional profile compared to the wild type. Overexpression of *luxS* in *B. subtilis* also had no significant effect on the transcriptional profile. *luxS* is not involved in signaling in *B. subtilis* under the conditions tested.

Introduction

Many bacteria regulate gene expression in response to cell density in a process known as quorum sensing. These bacteria produce signaling molecules that accumulate extracellularly as the density of the bacterial population increases. The bacteria sense and respond to threshold concentrations of these molecules by altering gene expression. This process allows bacteria to optimize gene expression at high cell density. Gram-positive bacteria primarily use small peptides for signaling, whereas Gram-negative bacteria primarily use acylated homoserine lactones (AHLs). Many processes are regulated by quorum sensing in bacteria, including bioluminescence, pathogenesis, degradative enzyme production, antibiotic synthesis, conjugative plasmid transfer, competence and sporulation (Taga and Bassler, 2003).

Although the majority of bacterial signaling molecules are species-specific, several bacteria produce an activity capable of inducing the quorum response of *Vibrio harveyi* (Bassler et al., 1997; Surette and Bassler, 1998). These findings led to the hypothesis that many bacteria produce and respond to a signaling molecule identical or similar to a quorum signal of *V. harveyi.*

The quorum response of *V. harveyi* controls light production through a convergent two-component phosphorelay (Bassler et al., 1994b; Freeman and Bassler, 1999) with two receptor histidine kinases, one of which senses and responds to the AHL VhAI-1 (Cao and Meighen, 1989; Bassler et al., 1993), the other of which senses and responds to a small molecule termed AI-2 (Bassler et al., 1994a; Chen et al., 2002) (Fig. 3-1). Cross-

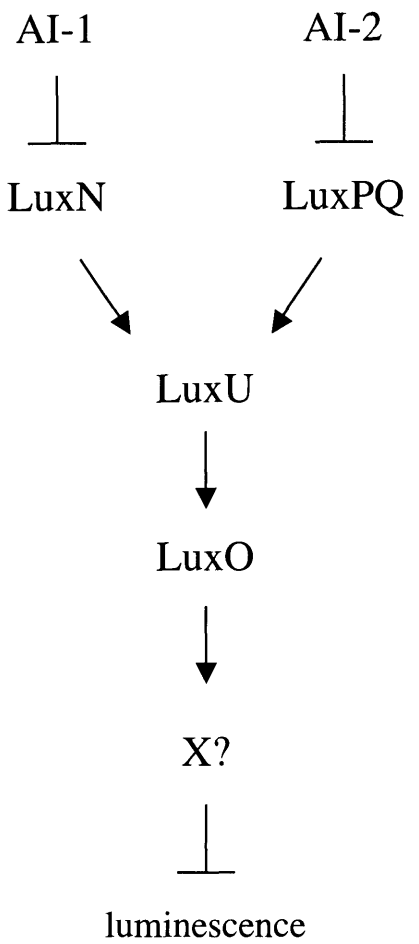

Figure 3-1. Two autoinducers control bioluminescence in *V. harveyi.* AI-1 inhibits the activity of the receptor kinase LuxN, whereas AI-2 inhibits the activity of the receptor kinase LuxPQ. In the absence of autoinducer, these two kinases donate phosphate to LuxU, which in turn donates phosphate to the response regulator transcription factor LuxO. LuxO activates the transcription of an unknown inhibitor of luminescence.

species induction of luminescence is specific to the AI-2 pathway (Bassler et al., 1997), and this activity has come to be known as AI-2.

Besides *Vibrio* spp., only *Salmonella typhimurium* has an identified mechanism of transcriptional response to AI-2. A genetic screen identified a single operon, *lsr*, upregulated in response to conditioned medium containing AI-2 activity (Taga et al., 2001). The *lsr* operon encodes an ABC transporter that is required for uptake of AI-2 activity from the medium. A transcriptional repressor of the *lsr* operon, LsrR, was also identified. Both the *lsr*-encoded ABC transporter and LsrR have high similarity to proteins involved in sugar utilization. By analogy to these systems, AI-2 most likely interacts with LsrR to relieve repression of the *lsr* operon.

Production of AI-2 activity is dependent on *luxS* in all species tested (Surette et al., 1999). Homologs of *luxS* are found in many diverse bacterial species. LuxS converts S-ribosylhomocysteine to homocysteine and AI-2 activity in vitro (Schauder et al., 2001; Winzer et al., 2002a). This enzymatic function is downstream of reactions in the recycling of products of methylation using S-adenosylmethionine (SAM), an essential process in bacteria.

luxS has been shown to be involved in the regulation of various processes in different bacteria (Xavier and Bassler, 2003), although the mechanism of regulation is unknown in nearly all cases. Furthermore, in many cases it is not known if the effect of deletion of *luxS* is cell-autonomous or if the effect is due to cell-cell signaling.

Bacillus subtilis is a Gram-positive soil bacterium that controls several processes by multiple peptide signaling. The modified peptide signal ComX pheromone controls development of competence as well as a more general quorum response involving

degradative enzyme production and antibiotic synthesis (Magnuson et al., 1994; Solomon et al., 1995). The Rap/Phr family of regulators and secreted peptides is involved in the control of competence, sporulation, degradative enzyme production and the excision of an integrative conjugative element in response to cell density and stationary phase (Perego and Hoch, 1996; Solomon et al., 1996; McQuade et al., 2001; Ogura et al., 2003).

Along with the characterized peptide signaling systems, *B. subtilis* also possesses a gene homologous to *luxS*, but does not produce AI-2 in LB medium (Bassler et al., 1997). We hypothesized that *B. subtilis luxS* may function in cell-cell signaling through AI-2 or an AI-2-like molecule in media other than LB. We found that *B. subtilis* produces AI-2 activity in defined minimal medium containing glucose, and that this activity is not produced in a *luxS* null mutant. However, a *luxS* mutant had no other discernible phenotype, and transcriptional profiling analyses found no genes whose expression changed significantly upon altered *luxS* expression. These data indicate that *B. subtilis* does not use *luxS* for cell-cell signaling under the conditions tested.

Materials and Methods

Strains and media. The *B. subtilis* strains used are listed in Table 3-1. All are derived from the parent strain JH642 (Perego et al., 1988) and contain the *trpC2* and *pheA1* mutations. Standard procedures were used for genetic transformation and strain construction (Harwood and Cutting, 1990). All optical densities were measured at 600 nm using a spectrophotometer.

Table 3-1. Bacterial strains used.

Strain	Relevant characteristic(s)
B. subtilis[a]	
AG174 (JH642)	*trpC2 pheA1*
AG522	*kinA*::Tn*917*
AG1046	*amyE*::(*comG-lacZ neo*)
NC107	*luxS*::*spc*
NC110	*amyE*::(*comG-lacZ neo*) *luxS*::*spc*
RSM187	*amyE*::(Pspank-hy *spc*)
RSM188	*amyE*::(Pspank-hy-*luxS spc*)
E. coli	
DH5α	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 luxS*
RSM184	DH5α pDR111 (Pspank-hy *amp spc*)
RSM185	DH5α pRM14 (Pspank-hy-*luxS amp spc*)
V. harveyi	
BB170	*luxN*::Tn*5* (AI-2 tester)
BB152	*luxL*::Tn*5* (AI-1$^-$ AI-2$^+$)

[a] All *B. subtilis* strains used are derived from JH642 and contain the *trpC2* and *pheA1* mutations.

Routine growth and maintenance of *B. subtilis* and *Escherichia coli* DH5α cultures were done in Luria-Bertani medium at 37°C. Defined minimal medium containing S7 salts was prepared as described (Vasantha and Freese, 1980), except that MOPS (morpholinopropanesulfonic acid) buffer was present at 50 mM (Jaacks et al., 1989). Minimal medium contained glucose (1%), glutamate (0.1%) and required amino acids (40 μg/ml tryptophan and phenylalanine). Antibiotics were used at the following concentrations: ampicillin (Amp) at 100 μg/ml, spectinomycin (Spc) at 100 μg/ml, and chloramphenicol (Cm) at 5 μg/ml. Induction of expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

Spore assays. Cells were grown in DS (Schaeffer et al., 1965) or minimal medium at 37°C, and spores were assayed 20-24 h after the end of exponential growth. The number of spores per milliliter culture was determined as the number of heat-resistant (80°C for 20 min) CFU on LB plates. Viable cells were measured as the total number of CFU (before heat treatment) under similar plating conditions.

β-Galactosidase assays. β-Galactosidase specific activity was measured essentially as described previously (Miller, 1972; Jaacks et al., 1989; Magnuson et al., 1994) and is presented as (ΔA_{420} per minute per milliliter of culture per unit of optical density at 600 nm (OD_{600}) × 1,000.

Generation of cell-free conditioned medium and *V. harveyi* luminescence assay. Cells were grown as described, and samples were taken at various points during growth. Cells were removed by centrifugation, and the culture supernatant was filtered through a 0.2 μm filter and stored at –80°C until assaying. The AI-2 bioluminescence assay was performed essentially as described (Surette et al., 1999). Briefly, the *V.*

harveyi AI-2 assay strain BB170 (kindly provided by Bonnie Bassler, Princeton U.) was grown overnight in AB (autoinducer bioassay) medium at 30°C. AB medium contains 300 mM NaCl, 100 mM $MgSO_4$, 10 mM potassium phosphate, 0.2% casamino acids, 1% glycerol, 1 mM L-arginine, 10 ng/ml riboflavin, and 1 μg/ml thiamine with a final pH of 7.5. The overnight culture was diluted 1:5000 in fresh AB medium and the sample to be tested was added to 10% of the final volume. Light production was measured over time with a MicroLumat Plus luminometer (Berthold), and relative light production was measured in relative light units (RLU).

Construction of the *B. subtilis* Δ*luxS* strain. *B. subtilis luxS* is monocistronic. To create a *luxS* null mutant, the 5' and 3' ends of the *luxS* gene were amplified by PCR and subcloned with the spectinomycin gene between the two fragments. The construct was integrated by double crossover to delete the majority of the *luxS* open reading frame. The 5' end, from 420 bp upstream and 50 bp downstream of the start codon, and the 3' end, from 75 bp upstream and 538 bp downstream of the stop codon, of *luxS* were amplified by PCR using the primers 5F2YTJB (5'-CTTGGATCCGGAGCAACAACCGCATTATG-3') and 3F2YTJB (5'-ACCGAATTCGTCCATGGCTTAGTCATTG-3'); and 5YTJBF1 (5'-GAAAAGCTTTCTTCTGACTGATTGCTGG-3') and 3YTJBF1 (5'-AAGGTCGACGATCTGGAAGGCGCTAAACG-3'), respectively. The resulting fragments were cut with *Bam*HI and *Eco*RI, and *Hin*dIII and *Sal*I, respectively and ligated in series into pGEM-cat (Youngman et al., 1989) to form pNC4. The spectinomycin resistance gene from pUS19 (Benson and Haldenwang, 1993) was cut with *Bgl*I and *Nde*I and inserted into pBluescript SK(+) (Stratagene) to form pJL74,

which was then cut with *Bam*HI and *Sal*I and inserted between the *luxS* fragments of pNC4 by ligation. The resulting plasmid, pNC5, was transformed into AG174 with selection for spectinomycin resistance. Double crossover insertions were chosen by screening for clones that were spectinomycin resistant and chloramphenicol sensitive. The construction of the final strain was confirmed by PCR. Construction of the *luxS* null strain was performed by N. Comella.

Construction of the *luxS* expression plasmid pRM14. For overexpression of *luxS* a plasmid was created with the *luxS* gene placed under control of the Pspank-hy promoter. The coding sequence of *luxS*, from 54 bp upstream of the start codon to 32 bp downstream of the stop codon, was amplified by PCR using the primers RM109 (5'-GCGTCGACCGGAAAAGTATGTTATAATG-3') and RM110 (5'-ACATGCATGCTGCTTAAGCGCAAAAAGGTC-3'). The resulting product was cut with *Sal*I and *Sph*I and inserted into pDR111 (Britton et al., 2002) by ligation. The resulting fusion placed *luxS* under control of the IPTG-inducible Pspank-hy promoter. This plasmid allows for expression of Pspank-hy-*luxS* in *E. coli* and contains sequences for insertion of the fusion into the chromosome of *B. subtilis* at the *amyE* locus by homologous recombination.

Isolation of total RNA from *B. subtilis*. Samples of *B. subtilis* cultures were collected under the indicated conditions. Each sample was harvested into an equal volume of ice-cold methanol. The cells were centrifuged and frozen at –80°C for later RNA extraction using an RNeasy Mini Kit (Qiagen). The amount of RNA isolated was quantitated based on A_{260}.

Microarray experiments and data analysis. Construction of the DNA microarrays, cDNA labeling, and hybridization conditions were as described previously except as noted (Britton et al., 2002). Strain culturing and RNA isolation were performed in quadruplicate. RNA was used to create fluorescent cDNA probes containing Cy3 or Cy5 by aminoallyl labeling. In brief, the RNA was reverse-transcribed with nucleotide mixture with final concentrations 500 μM dATP, dCTP and dGTP; 400 μM aminoallyl-dUTP; and 100 μM dTTP. After RNA degradation and cDNA purification, the pH was adjusted with 50 mM $NaHCO_3$ (pH 9.0) and monofunctional NHS-ester-Cy3 or -Cy5 (Amersham Biosciences) was added. The reaction was incubated for 60 min at room temperature and then quenched for 15 min with 1 M hydroxylamine. The products were then purified as described previously. Probes from each sample were hybridized to glass slide microarrays in comparison to a common control consisting of a mixture of all the RNAs used in that experiment. Signal intensity of each spot was normalized to the total signal intensity of all spots for that chromophore. Significance analysis of microarrays (SAM) was performed on the data pairwise to identify genes reproducibly differentially expressed in our experiments (Tusher et al., 2001). We also analyzed the data for significance using a standard t-test.

Results and Discussion

The *B. subtilis luxS* deletion mutant. To investigate the effects of *luxS* and AI-2 in *B. subtilis*, we created a strain containing a chromosomal deletion that removed the beginning of the *luxS* open reading frame. *luxS* mutants of a small number of bacteria, including *Actinobacillus actinomycetecomitans* (Fong et al., 2001), *Streptococcus*

pyogenes (Lyon et al., 2001), and *Bacillus anthracis* (Jones and Blaser, 2003) have medium-dependent growth defects. The *luxS* mutant had no obvious phenotypes on LB or minimal plates or in liquid medium (LB, DSM, defined minimal medium). Growth rate, lag phase after dilution, and density at stationary phase were all identical between *luxS* and wild-type (Fig. 3-2).

The *luxS* mutant showed no significant defects in processes known to be controlled by cell density in *B. subtilis*. There was no measurable defect in sporulation of the *luxS* null mutant (Table 3-2). There was also no defect in competence as measured by the expression of a competence-specific promoter, *comG* (Fig. 3-3).

The *B. subtilis luxS* gene is functional in *E. coli*. The *E. coli* strain DH5α does not produce AI-2 activity (Surette and Bassler, 1998) due to a frameshift mutation in its *luxS* gene (Surette et al., 1999). Transformation of *E. coli* DH5α with plasmids that express a functional *luxS* can restore production of AI-2 activity (Surette et al., 1999). We tested whether *B. subtilis luxS* could complement DH5α for AI-2 production. We cloned the *luxS* gene of *B. subtilis* under control of an IPTG-inducible promoter (Pspank-hy) and transformed DH5α with the plasmid containing *B. subtilis luxS* or the vector alone without the *luxS* insert. The *luxS* expression plasmid directed production of high levels of AI-2 activity when the strain was grown in 1 mM IPTG (Fig. 3-4). Even in the absence of IPTG, the expression strain produced significant levels of AI-2 activity. These results demonstrate that *B. subtilis luxS* can complement an *E. coli* mutant for AI-2 production and is a functional enzyme.

Production of AI-2 activity by *B. subtilis*. To determine whether *B. subtilis* produces AI-2 activity, we measured the AI-2 activity in cell-free conditioned medium of

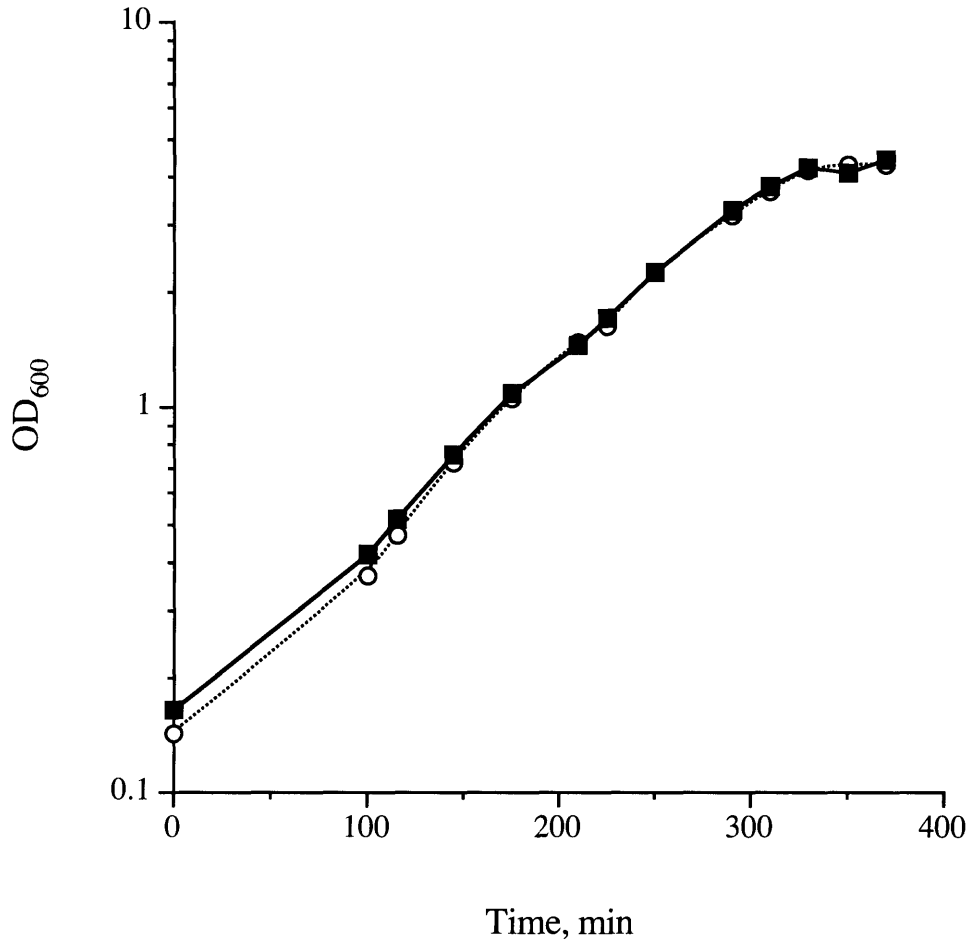

Figure 3-2. Growth of the *B. subtilis luxS* mutant. Wild-type and *luxS* mutant strains of *B. subtilis* were grown in defined minimal medium with glucose as a carbon source at 37° C. Optical density of the culture at 600 nm was measured periodically throughout growth. ■ AG174, wild type; ○ NC107, *luxS*.

Table 3-2. *luxS* and sporulation.

Genotype	Spores / ml	Viables / ml	% spores	% of wild type
$S7_{50}$ minimal				
AG174 wt	1.24×10^{8}	1.85×10^{8}	67.0	100
NC107 *luxS*	8.54×10^{7}	1.05×10^{8}	81.5	122
AG522 *kinA*	5.96×10^{7}	2.43×10^{8}	24.5	36.6
DSM				
AG174 wt	5.49×10^{8}	7.97×10^{8}	68.9	100
NC107 *luxS*	5.14×10^{8}	5.49×10^{8}	93.5	136
AG522 *kinA*	1.99×10^{7}	6.38×10^{8}	3.12	4.5

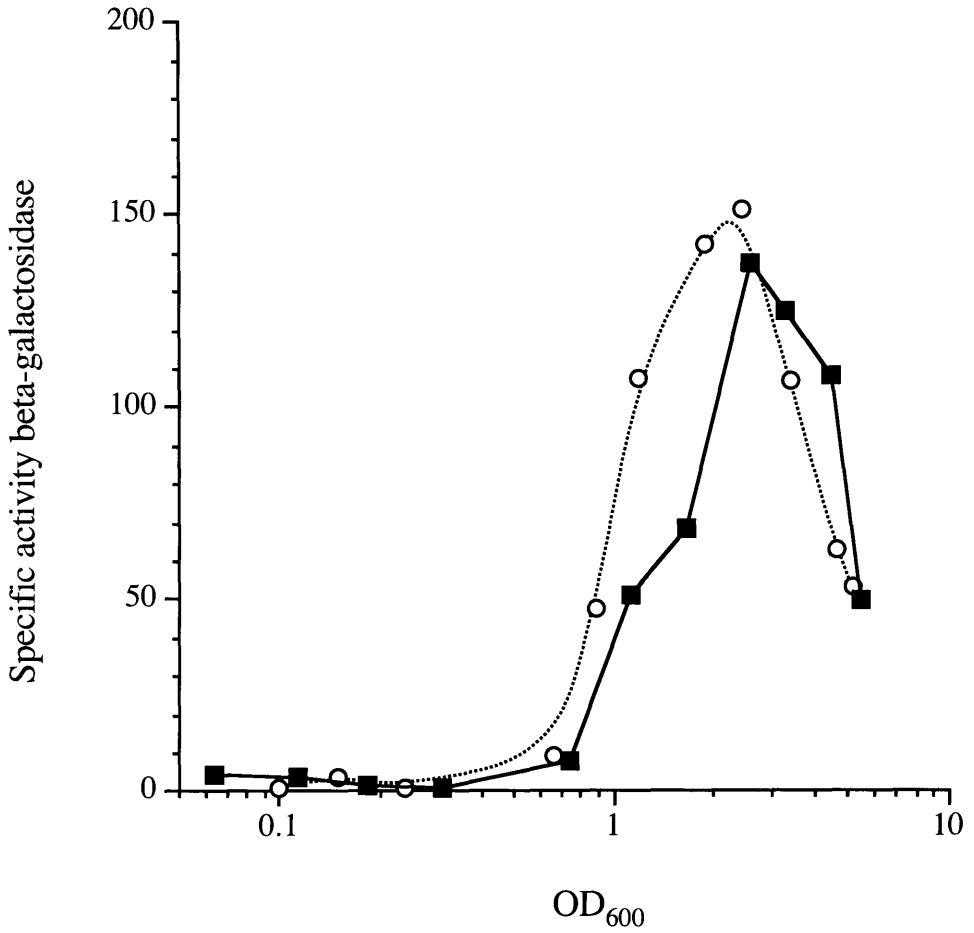

Figure 3-3. Expression of *comG-lacZ* in a *luxS* null mutant. Strains of *B. subtilis* expressing the fusion *comG-lacZ* were grown in defined minimal medium containing glucose at 37°C. Samples were taken throughout growth and the β-galactosidase activity measured as described. Specific activity is the ratio of units of activity to the OD_{600} of the culture. ■ AG1046, wild type; ○ NC110, *luxS*. Data courtesy of N. Comella.

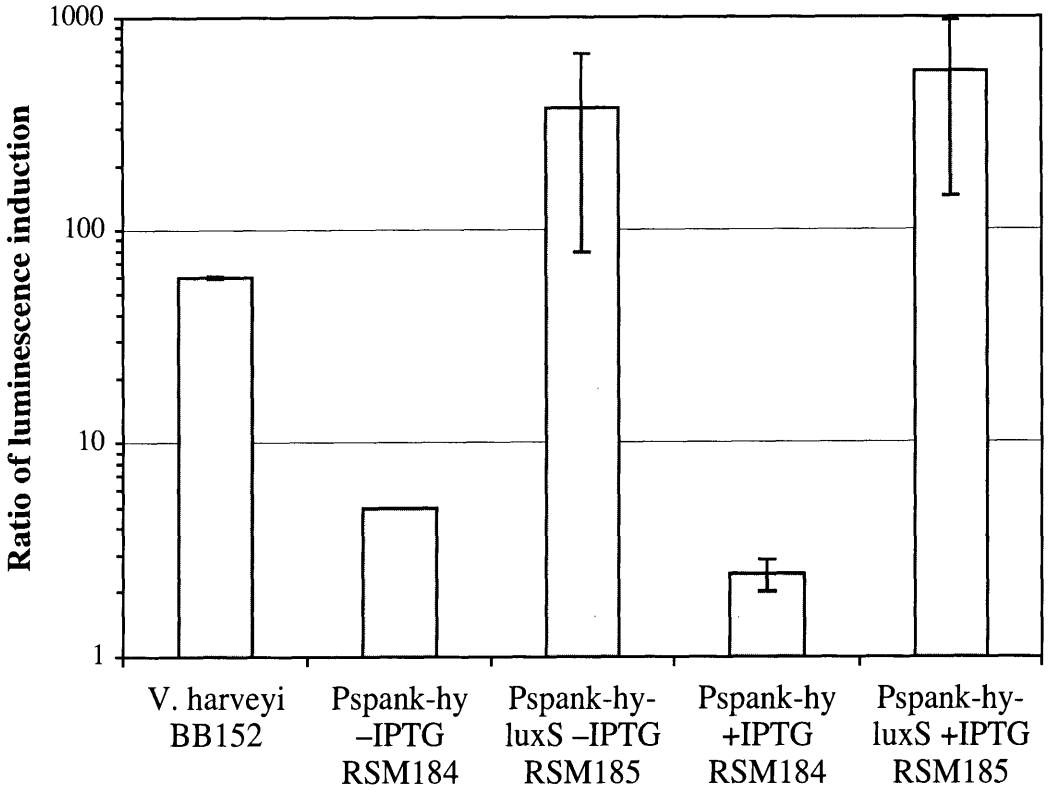

Figure 3-4. *B. subtilis luxS* complements DH5α for AI-2 production. Conditioned medium from *V. harveyi* (BB152) or *E. coli* cultures at OD_{600}=1 was assayed for AI-2 activity. For each sample, maximal ratios are presented of luminescence induction activity of conditioned medium over activity of fresh medium. RSM185 expresses the Pspank-hy-*luxS* fusion. RSM184 is the vector control. Error bars represent the standard deviation of three replicates.

wild-type and the *luxS* null mutant from multiple points during growth in defined minimal medium (Fig. 3-5). AI-2 activity accumulated approximately in proportion to cell number throughout exponential growth, peaking around the entry to stationary phase. After the entry to stationary phase, the AI-2 activity rapidly diminished. No AI-2 activity was observed in *B. subtilis* cultures grown in LB medium, consistent with results previously reported (Bassler et al., 1997).

Accumulation of AI-2 activity in many bacterial species follows a pattern similar to that of *B. subtilis,* reaching peak levels in mid to late exponential phase. When grown in LB, *E. coli* overexpressing *luxS* from *Helicobacter pylori* accumulates AI-2 activity maximally at the entry to stationary phase, and the AI-2 levels decrease following the entry to stationary phase (Hardie et al., 2003). *S. typhimurium* expresses an operon, *lsr*, encoding a putative ABC transporter that can actively remove and process AI-2 activity from culture medium (Taga et al., 2001). The sole operon in *B. subtilis* with homology to the *lsr* operon is implicated in the uptake and utilization of ribose. This operon may be involved in AI-2 uptake as well in *B. subtilis*, or *B. subtilis* may produce a heterologous importer or an enzyme that can degrade AI-2 activity. In preliminary experiments, incubation of conditioned medium containing AI-2 with conditioned medium from stationary phase *B. subtilis* did not significantly reduce activity.

It is important to note that the detection of AI-2 activity is highly dependent on the pH of the *V. harveyi* assay medium (AB). Initial batches of the AB medium, made according to the recipe, had a much lower pH than expected (< 6.4 instead of 7.5) and were not able to allow for the detection of AI-2 activity in *B. subtilis* conditioned medium. Table 3-3 presents the results of performing the AI-2 assay as described with

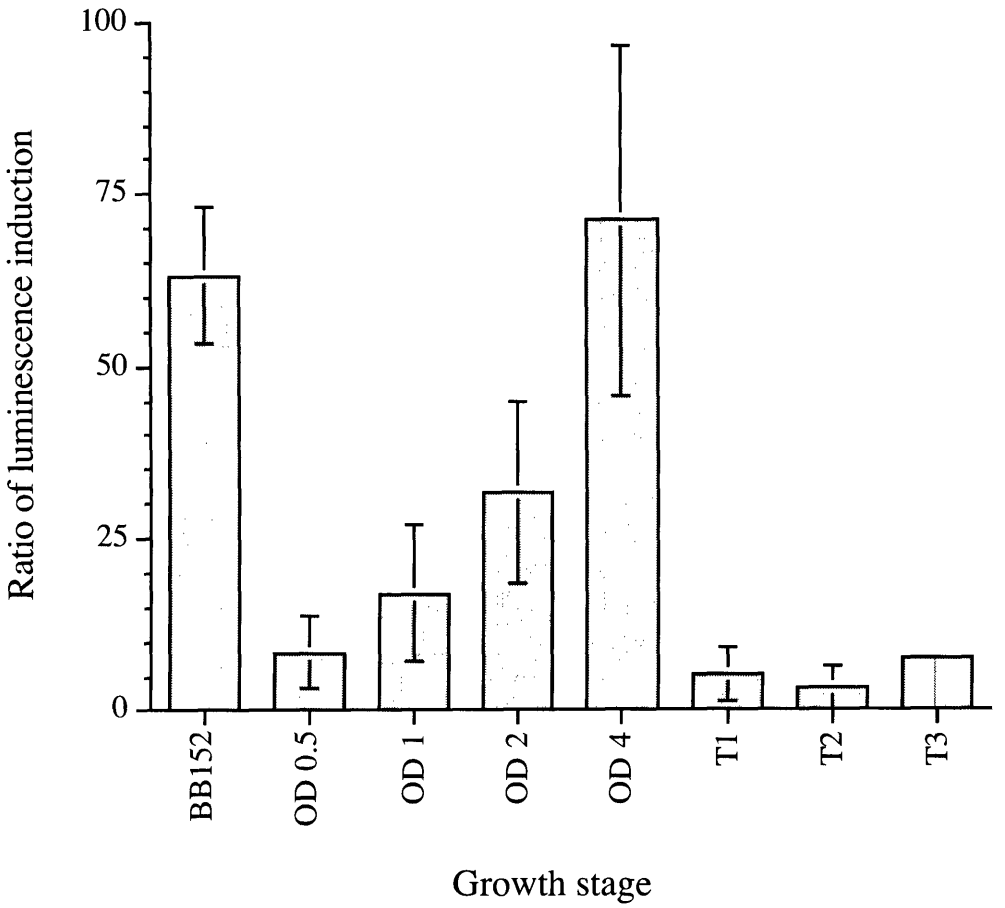

Figure 3-5. *B. subtilis* accumulates AI-2 activity during exponential growth. Conditioned medium samples from *V. harveyi* and *B. subtilis* were assayed for AI-2 activity. BB152 ratio is activity of conditioned medium over activity of fresh medium. Due to growth differences between fresh and conditioned minimal medium, ratios of luminescence induction for *B. subtilis* samples are presented as activity of wild-type conditioned medium (AG174) over activity of *luxS*$^-$ conditioned medium (NC107). T0 is defined as the end of exponential growth. T1, T2 and T3 are 1, 2 and 3 h after T0. Error bars are the standard deviation of at least three replicates.

Table 3-3. Luminescence induction ratios are dependent on the pH of the assay medium.

	V. harveyi BB152 / AB	*B. subtilis* wt / *luxS*$^-$
pH 7.4	55.8	68.5
pH 6.4	8.84	2.36

identical AB media at either pH 6.4 or pH 7.4. The ratio of luminescence induction is significantly reduced in the low-pH medium. The activity of the *V. harveyi* medium was decreased ~6-fold, whereas the activity of the *B. subtilis* medium was decreased ~30-fold. It is unclear why there is a difference in the pH effects depending on the sample source. This may be an artifact of comparing conditioned to fresh medium for the *V. harveyi* samples, whereas in the *B. subtilis* samples conditioned medium from a wild type strain is compared to that of a *luxS*$^-$ strain.

Microarray expression analysis of the *B. subtilis luxS* mutant. To determine if *luxS* has any effect on gene expression in *B. subtilis*, we compared the transcriptional profiles (RNA levels) of *luxS*$^+$ and *luxS* null mutant cells. This analysis was expected to identify genes regulated by *luxS* both cell-autonomously and through cell-cell signaling. Cells grown in defined minimal medium at 37°C were harvested at optical densities of 0.5, 1.0 and 2.0, and at 1 and 3 h after the entry to stationary phase. RNA was isolated from the samples, labeled, and hybridized to PCR product microarrays containing >99% of the protein coding genes in the *B. subtilis* genome.

The primary analysis of my microarray data was conducted using the Significance Analysis of Microarrrays (SAM) program (Tusher et al., 2001). This program determines the reproducibility of results and can identify a set of genes likely to be differentially expressed and also estimate the number of false positives present in that set. The parameters of the program may be altered to change the size of the set, while also changing the calculated estimated rate of false positive identification as more genes are included that may be less likely to be significant. Ideally, the estimated false positive rate should be as low as possible for confidence in the results. Consistently, analysis of my

data using SAM would yield two types of gene sets. In the first type, a large number of genes were identified (>1000) with an estimated false positive rate of at least 0.5. Due to the large false positive rate and the small fold changes observed (primarily less than 3-fold), this result was not found to be significant. In the second type of result, only expression of *luxS* was identified as decreased in all experiments, with a false positive rate near 1. Although this estimated false positive rate is high, the identification of *luxS* is significant, since *luxS* was deleted in these experiments. Therefore, analysis using the SAM program identifies *luxS* as the only gene differentially expressed in a *luxS* null mutant as compared to wild-type throughout exponential growth and early stationary phase.

The data was also analyzed by looking at fold changes with statistical analysis by a standard t-test. Table 3-4 presents genes changed more than 3-fold at approximately 1 h before (OD 2) and after the entry to stationary phase, when AI-2 activity is reaching peak levels and after AI-2 activity decreases, respectively. As expected, *luxS* expression was lower in the mutant under all conditions tested. Very few genes were differentially expressed consistently at more than one time point, the exceptions being the genes encoding the arginine synthesis pathway. These genes are found to be differentially expressed in many of our microarray experiments, as well as in experiments by other groups. Direct measurement of transcription of these genes has shown that the transcript levels of the *arg* genes change rapidly during the late exponential phase of growth (Comella and Grossman, unpublished data). Because of this, small differences in the time of sampling can lead to large apparent differences in *arg* transcript levels on microarrays. Thus, the *arg* genes are not expected to be significantly changed in

Table 3-4. Genes changed >3-fold in a *luxS* null mutant.

Gene	Fold change	p value[a]	Putative function
OD 2			
luxS	–9.22	5.34×10^{-6}	autoinducer 2 production
argG	13.3	0.0081	arginine biosynthesis
argD	10.1	0.096	arginine biosynthesis
argH	9.95	0.024	arginine biosynthesis
argJ	9.95	0.041	arginine biosynthesis
ytzD	7.16	0.094	unknown
carB	6.91	0.138	arginine biosynthesis
argF	6.48	0.045	arginine biosynthesis
argC	5.76	0.042	arginine biosynthesis
carA	5.32	0.250	arginine biosynthesis
argB	4.82	0.119	arginine biosynthesis
yqiX	3.64	0.034	similar to arginine ABC transporter
yqiZ	3.42	0.021	similar to arginine ABC transporter
ysxE	3.06	0.348	unknown; similar to unknown proteins
spoIVB	3.02	0.217	stage IV sporulation
cotX	3.01	0.515	spore coat protein
T1[b]			
luxS	–4.71	9.79×10^{-4}	probable autoinducer 2 production
feuA	–4.07	0.255	iron-uptake system (binding protein)
lytA	–3.86	0.372	secretion of autolysin
dhbA	–3.34	0.495	2,3-dihydroxybezoate biosynthesis
cah	–3.10	0.372	cephalosporin C deacetylase
ybbA	–3.03	0.115	unknown; similar to esterase
dhbB	–3.01	0.229	2,3-dihydroxybezoate biosynthesis
nasD	3.91	0.644	assimilatory nitrite reductase (subunit)
cotC	3.62	0.326	spore coat protein

[a] p values reported are the output of a standard t-test comparing the ratios of experimental to control for each condition (wild type or *luxS*).

[b] T1 is defined as 1 h after the end of exponential growth.

response to *luxS*. All the other genes presented have p values > 0.1 and are not likely to be significantly changed.

The lack of a concerted transcriptional response to *luxS* in *B. subtilis* is in stark contrast to responses identified by microarray analyses of *E. coli luxS* signaling. In *luxS*⁻ *E. coli* cells, >200 genes were found to be significantly differentially expressed after addition of *E. coli* conditioned medium with or without AI-2 activity (DeLisa et al., 2001). Similar experiments in *Neisseria meningitidis* gave results more similar to that seen in our experiments, with only one transcript that was differentially expressed, a putative iron siderophore receptor (Dove et al., 2003). Interestingly, this gene is similar to *feuA*, which was downregulated in the *B. subtilis luxS* null mutant 1 h after the entry to stationary phase but was not called as significant by the SAM program (Table 3-4).

Overexpression of *luxS*. We also tested for altered transcription when *luxS* was overexpressed from a strong, inducible promoter. *luxS* was placed under the control of Pspank-hy and integrated into the chromosome at the *amyE* locus. The vector containing Pspank-hy was integrated at *amyE* as a control. At an optical density of 0.2, expression was induced with 1 mM IPTG, and the cultures were sampled at 15, 30, and 120 min post induction. Microarray transcriptional profiling was performed to compare the Pspank-hy-*luxS* strain to the control strain.

Analysis using the SAM program again identified only *luxS* as significantly differentially expressed between the *luxS* overexpresser and the control. Very few genes were differentially expressed more than 2-fold in this experiment, and there were no genes whose expression consistently changed at two time points except *luxS*, which was expressed at levels 7-10 fold higher than in the wild-type (Table 3-5). There were also no

Table 3-5. Genes changed >2-fold after *luxS* overexpression.

Gene	Fold change	p value [a]	Putative function
T15[b]			
luxS	10.1	7.93×10^{-7}	autoinducer 2 production
spoIVA	2.20	0.243	stage IV sporulation
T30[b]			
luxS	7.62	2.87×10^{-7}	autoinducer 2 production
spoIIID	2.16	0.00211	stage III sporulation
iolR	–2.38	0.120	myo-inositol catabolism
ogt	–2.03	0.294	O6-methylguanine DNA alkyltransferase
T120[b]			
luxS	8.22	3.78×10^{-5}	autoinducer 2 production
ytnJ	4.53	0.314	unknown; similar to nitriloacetate monooxygenase
argJ	3.81	0.330	arginine biosynthesis
ytnL	3.48	0.298	unknown; similar to aminohydrolase
argC	2.46	0.276	arginine biosynthesis
ssuB	2.38	0.183	aliphatic sulfonate ABC transporter (binding protein)
ssuA	2.14	0.235	aliphatic sulfonate ABC transporter (binding lipoprotein)
argG	2.14	0.290	arginine biosynthesis
yxeL	–2.84	0.173	unknown; similar to unknowns in *B. subtilis*
yjcD	–2.10	0.230	unknown; similar to ATP-dependent DNA helicase
yvgM	–2.01	0.262	unknown; similar to molybdenum transport permease

[a] p values reported are the output of a standard t-test comparing the ratios of experimental to control for each condition (wild type or *luxS*).

[b] Time is minutes after induction of *luxS* overexpression.

genes in common with those found differentially expressed in the *luxS* null mutant, except again for genes involved in arginine biosynthesis. Besides *luxS*, the only gene found differentially expressed in this experiment with a p-value less than 0.05 is *spoIIID*. *spoIIID* is a transcriptional regulator of genes controlled by sporulation-specific sigma factors which are not present during exponential growth (Ichikawa and Kroos, 2000). The identification of *spoIIID* is likely to be spurious.

***luxS* overexpression does not significantly increase AI-2 accumulation.** Steady-state overexpression of Pspank-hy-*luxS* did not lead to a significant change in accumulation of AI-2 activity during growth (Fig. 3-6). The variability of the activity observed in the overexpresser (RSM188) at OD 4.0 may be due to differences in the entry to stationary phase, or the *luxS* overexpresser may initiate depletion of AI-2 activity earlier than wild type.

The lack of change in AI-2 production on *luxS* overexpression in *B. subtilis* is consistent with reports that substrate, not the *luxS* enzyme, is limiting for production of AI-2 activity in *S. typhimurium* (Beeston and Surette, 2002). AI-2 appears to have no effect on its own production, and it is predicted that every methylation using SAM and subsequent deadenylation of SAH by Pfs yields the substrates for one potential molecule of AI-2 since *luxS* is in excess.

Conclusions. The data presented do not support the model that *B. subtilis* uses *luxS* to regulate gene expression, either through cell-cell signaling or cell-autonomously. However, *B. subtilis* does produce AI-2 activity in some media and appears to have the capacity to deplete AI-2 activity. Why is this? It may be that *B. subtilis* produces and

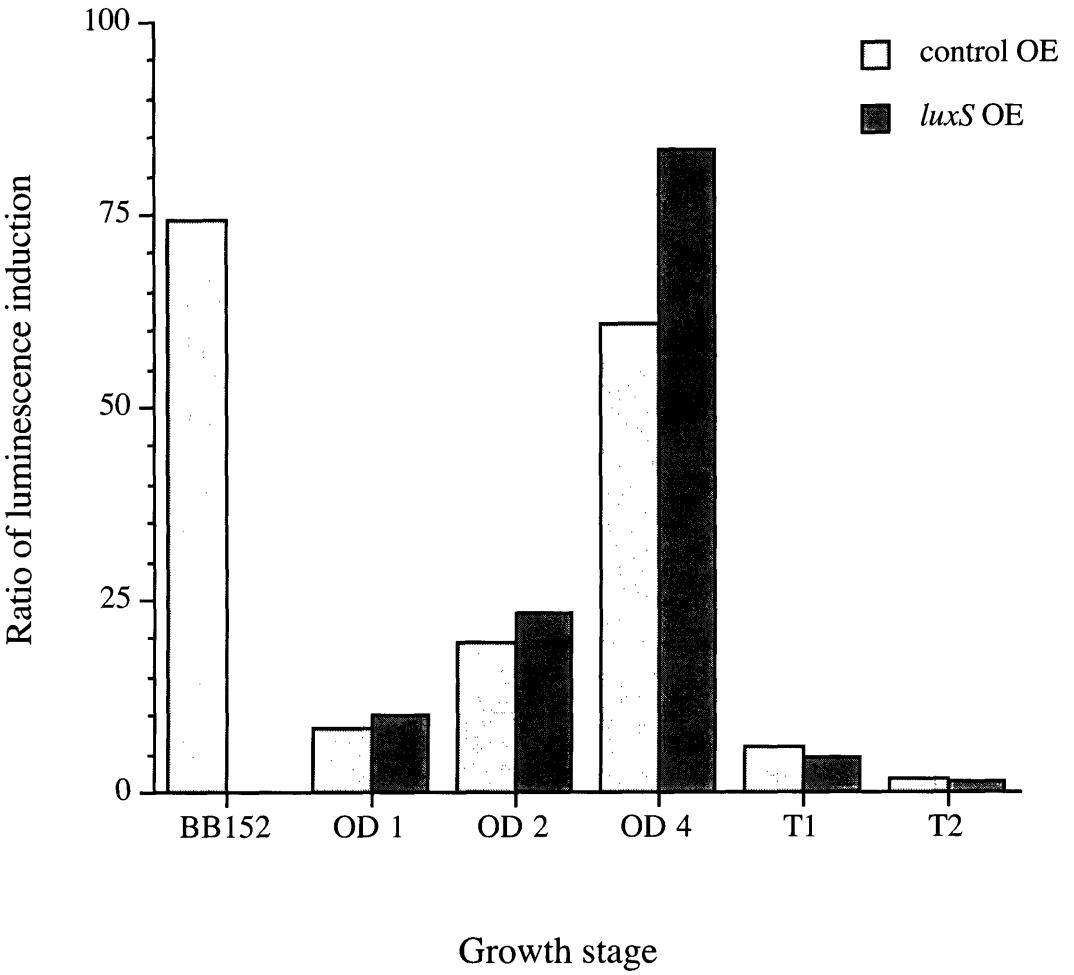

Figure 3-6. Steady-state overexpression of *luxS* in *B. subtilis* does not significantly alter the accumulation of AI-2 activity. Conditioned medium samples from *V. harveyi* and *B. subtilis* were tested for AI-2 activity. *V. harveyi* BB152 ratio is activity of conditioned medium over activity of fresh medium. *B. subtilis* ratios are presented as RSM187 (integrated vector control) activity or RSM188 (integrated *luxS* overexpresser) activity over the *luxS* null mutant (NC107). Representative data are shown.

depletes AI-2 activity for interaction and/or competition with other bacterial species. Alternately, AI-2 activity may simply be a secreted metabolite produced by *B. subtilis*.

It is unclear whether *B. subtilis* produces the same LuxS-dependent AI-2 molecule as *V. harveyi*. The enzymatic activity of LuxS generates 4,5-dihydroxy 2,3-pentanedione (DPD), which is predicted to cyclize and complex with borate to form the molecule found in complex with the *V. harveyi* AI-2 receptor. This cyclization is predicted to occur spontaneously, and may proceed through multiple intermediates, any of which could be responded to in organisms as AI-2 signals. Furthermore, the concentration of borate in aquatic and terrestrial environments (0.9-9 μM) (Fresenius et al., 1988) is much less than that found in the marine environment that *V. harveyi* inhabits (0.4 mM) (Winzer et al., 2002b). The only assay available for AI-2 is a bioassay to detect induction of luminescence in *V. harveyi*. This assay may well convert DPD-related precursors produced by *B. subtilis* to *V. harveyi* AI-2.

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Chapter 4

Discussion

In this thesis, I present work investigating the regulation and production of putative and confirmed cell-cell signaling molecules in *Bacillus subtilis*. Chapter 2 details evidence that *B. subtilis* controls a family of putative and confirmed secreted regulatory peptides by the alternate sigma factor sigma-H involved in stationary phase response and sporulation. Chapter 3 presents work that shows that *B. subtilis* can produce a putative intercellular signaling activity dependent on the conserved *luxS* gene. However, microarray analysis detected no differential expression when *luxS* is deleted, suggesting that *luxS* is not involved in signaling and transcriptional regulation in *B. subtilis*.

Interregulation of quorum sensing and stress responses

B. subtilis contains a family of putative and confirmed secreted peptides (Phrs) that inhibit the activity of their cognate regulatory proteins (Raps), which negatively regulate transcription factors. The Phr peptides are predicted to accumulate in the extracellular medium and the enter the cell by the oligopeptide permease to interact specifically with their cognate Rap proteins (Perego and Hoch, 1996; Solomon et al., 1996; Lazazzera et al., 1997). These Rap proteins serve to regulate transcription factors involved in sporulation (Perego et al., 1996; Jiang et al., 2000), competence (Solomon et al., 1996), degradative enzyme production (Koetje et al., 2003; Ogura et al., 2003), and a conjugative element (Auchtung, Lee, and Grossman, unpublished data).

Except for *phrA*, all of the *phr* genes are transcribed, in part, from promoters specific for the alternate sigma factor sigma-H (McQuade et al., 2001). Sigma-H is

involved in stationary phase and starvation response and is required for sporulation in *B. subtilis*. Sigma-H activity is regulated by many diverse factors at multiple levels. Transcription of *sigH* is negatively regulated by the repressor AbrB, which is in turn negatively regulated by the sporulation transcription factor Spo0A~P (Perego et al., 1988; Weir et al., 1991; Strauch, 1995). Sigma-H is also regulated at multiple levels by various conditions, including growth phase (Healy et al., 1991), pH (Cosby and Zuber, 1997; Cosby et al., 1998), amino acid availability (Eymann et al., 2001), and carbon sources (Palmer, 1999; Dixon et al., 2001). ClpX is required for the stimulation of sigma-H directed transcription in response to starvation conditions (Liu et al., 1999). In vitro studies of ClpX and sigma-H transcription suggest that ClpX may be involved in the dissociation of sigma-H from the core polymerase following transcription initiation (Liu and Zuber, 2000).

The *phr* genes are regulated by sigma-H in response to multiple stresses. An increase in production of Phr peptides during times of stress may serve to communicate the presence of adverse environmental conditions with other nearby bacteria. This could allow for the coordination and anticipation of appropriate responses to these stresses among the bacterial population.

Other cell-cell signaling systems are also interregulated with stress responses. Acid resistance in *Streptococcus mutans* is stimulated by cell-cell signaling through a secreted peptide also involved in competence development and biofilm formation (Li et al., 2001a; Li et al., 2001b). In *Pseudomonas aeruginosa* acylated homoserine lactones (AHL) signaling factors activate the transcription of genes involved in virulence (Brint and Ohman, 1995; Latifi et al., 1995). Induction of the stringent response artificially in

P. aeruginosa causes an increase in transcription of the genes responsible for production of AHLs with a corresponding increase in AHL levels and quorum sensing activity (van Delden et al., 1998; van Delden et al., 2001).

Links have also been observed between production and accumulation of AI-2 activity and stress conditions. In *S. typhimurium*, increased AI-2 signaling activity is observed when the cells are grown in glucose under conditions of high osmolarity (0.4 M NaCl) or low pH (pH 5.0) (Surette and Bassler, 1999). Low pH has also been shown to increase LuxS levels in *E. coli* (Stancik et al., 2002). Levels of AI-2 in *E. coli* are increased following addition of glucose, Fe(III), NaCl or dithiothreitol, whereas AI-2 levels decreased on amino acid starvation (DeLisa et al., 2001a). Overexpression of heterologous genes in *E. coli* was found to decrease accumulation of AI-2 activity proportional to the level of expression (DeLisa et al., 2001b).

It is not surprising that AI-2 levels are controlled by metabolic stresses, since the production of AI-2 activity is closely linked to cellular metabolism. The substrate for production of AI-2 by LuxS is produced from a byproduct of methylation by S-adenosylmethionine (SAM) (Schauder et al., 2001; Winzer et al., 2002). Many cellular components are methylated in SAM-dependent reactions, including proteins, DNA, and RNA. Methylation using SAM results in the toxic byproduct S-adenosylhomocysteine (SAH), which acts to inhibit methylation reactions. SAH is converted to adenine and S-ribosylhomocysteine (SRH) by the enzyme Pfs. SRH is the ultimate substrate of LuxS, which degrades SRH to homocysteine and 4,5-dihydroxy 2,3-pentanedione (DPD), which can then be converted to AI-2 activity by a mechanism that is poorly understood . Presumably, each methylation reaction yields one molecule of DPD and, potentially, AI-

2. In *S. typhimurium*, SRH substrate and not LuxS enzyme is the limiting factor for AI-2 production, and AI-2 production is roughly proportional to growth rate (Beeston and Surette, 2002).

SAM is also involved in the production of AHL signaling molecules. LuxI and homologous AHL synthases catalyze the condensation of fatty acids with SAM to produce the AHL and methylthioadenosine (MTA) (Parsek et al., 1999). Similarly to SAH, MTA is also a toxic intermediate that is degraded by Pfs to adenine and methylthioribose. Unlike AI-2 production, synthesis of AHLs is not directly related to growth rate and metabolism. AHL production is proportional to levels of LuxI homologs and not related to growth rate.

What is the primary function of LuxS?

Because of the intimate link between AI-2 production, LuxS and metabolism, there has been much discussion about the primary function of LuxS. Is LuxS primarily an enzyme involved in production of an interspecies signaling activity or is LuxS principally involved in cellular metabolism? The work presented in this thesis does not support the model that LuxS and AI-2 act as a signaling system in *B. subtilis* under the conditions tested, as no effect on transcription was observed in a *luxS* null mutant under conditions where AI-2 accumulation is high. However, *luxS* appears to be completely dispensable in *B. subtilis* under laboratory conditions as the *luxS* mutant has no discernable phenotype. LuxS may be important metabolically for growth and survival under conditions not studied here. Alternately, *B. subtilis* may use LuxS for production of signals for interaction with other bacterial species in its natural environment.

It is clear that LuxS produces a cell-cell signaling activity in *Vibrio* spp. A specific receptor protein has been shown to respond to the AI-2 molecule to induce luminescence, a response unrelated to AI-2 processing or utilization. This is distinct from the response to AI-2 identified in *S. typhimurium*. In this species, AI-2 induces expression of the *lsr* operon encoding an ABC transporter required for AI-2 uptake as well as other proteins involved in AI-2 processing (Taga et al., 2001; Taga et al., 2003). In this model, a transcriptional regulator represses transcription of the *lsr* operon in the absence of AI-2. When AI-2 is present, this repression is relieved and the operon is expressed. The only identified genes in *S. typhimurium* regulated by AI-2 are involved in the processing of AI-2 itself and its removal from the medium. However, the purpose of this uptake is unclear and not necessarily nutritional, as *S. typhimurium* has not been shown to be able to utilize AI-2 as a sole carbon source.

So what is the primary function of LuxS? A subset of bacteria that contain *pfs* homologs do not contain *luxS* homologs as well, indicating that *luxS* is dispensable for basic metabolism, although it may provide a growth advantage. However, *luxS* does not appear important for growth and survival in some bacteria tested, including *B. subtilis*. Also, *luxS* does not appear to affect gene expression in some bacteria, again including *B. subtilis*. It is clear that a subset of bacteria can respond to LuxS-produced signals, whereas a perhaps larger subset of bacteria can produce signals. This may be responsible for interspecies interactions of bacteria in the environment. For example, LuxS is dispensable for formation of biofilms in pure cultures of *Streptococcus gordonii,* but required for formation of mixed biofilms with *Porphyromonas gingivalis* (McNab et al., 2003).

Inhibition of quorum sensing

Often, quorum sensing controls processes that are disadvantageous to other organisms. Many bacteria use quorum sensing to regulate such processes as virulence and biofilm formation. To combat this, some organisms have developed methods to interfere with the signaling mechanisms involved in this quorum sensing to defend themselves or to compete more efficiently.

Some bacteria produce enzymes that can break down AHL signals. Some *Bacillus* species produce an AHL lactonase (AiiA) that can cleave the lactone ring to form acylhomoserine, which is incompetent for cell-cell signaling (Fig. 4-1) (Dong et al., 2000; Dong et al., 2002). Expression of AiiA by the plant pathogen *Erwinia carotovora* or its plant host greatly reduced AHL production and the ability of the bacteria to form a productive infection (Dong et al., 2000; Dong et al., 2001). It is not known what role AiiA plays in *Bacillus* species, but it may aid in competition for resources with AHL-producing bacteria in the environment. Another AHL-degrading enzyme, AiiD, is produced by a *Ralstonia* species (Lin et al., 2003). Instead of cleaving the lactone ring like AiiA from *Bacillus* species, AiiD is an acylhomoserine lactone acylase that separates the acyl chain from the intact homoserine lactone ring (Fig. 4-1). This also serves to eliminate the signaling function of the AHL molecule. Expression of *aiiD* in *Pseudomonas aeruginosa* greatly reduces AHL accumulation and inhibits production of virulence factors and paralysis of *Caenorhabditis elegans* (Lin et al., 2003). These findings suggest that inhibition of AHL signaling may be a common strategy for competition among bacterial species.

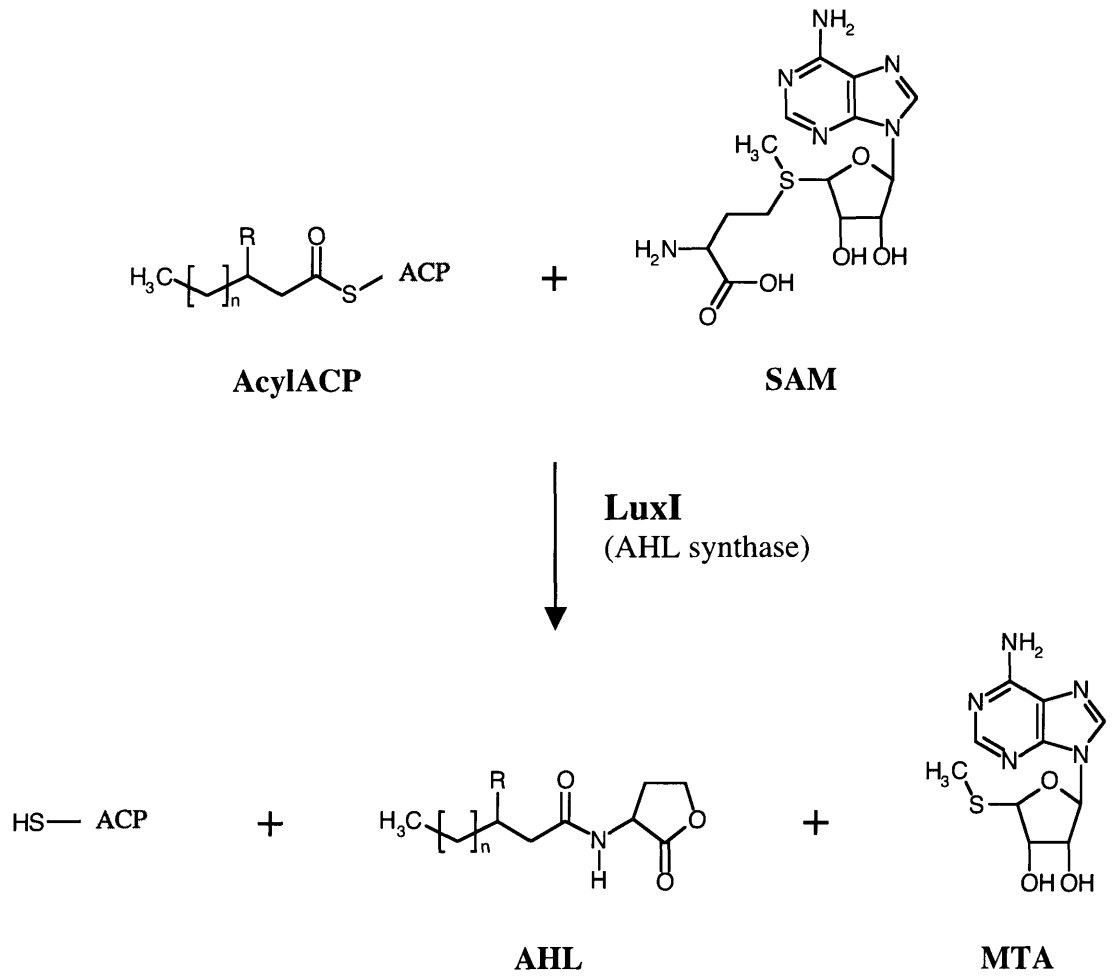

Figure 4-1. Synthesis of acyl homoserine lactones from S-adenosylmethionine. LuxI homologs catalyze the condensation of acylated acyl carrier protein (ACP) with S-adenosylmethionie (SAM). The reaction produces an acylated homoserine lactone (AHL) along with free ACP and methyltyioadenosine (MTA). Like S-adenosylhomocysteine, MTA is a substrate of the Pfs enzyme, which cleaves MTA to adenine and methylthioribose (MTR). The fate of MTR is unknown.

Inhibition of peptide signaling has been observed in the Gram-positive bacterium *Staphylococcus aureus*. *S. aureus* also regulates the production of virulence factors through quorum sensing using a thiolactone peptide, AgrD. Each *S. aureus* strain group produces a peptide signal that activates its own expression of virulence factors and inhibits the expression of virulence factors in other groups (Ji et al., 1997). Presumably, this system will serve to reward the *S. aureus* strains that can reach a higher density first in competition with other strains.

Conclusions

Through cell-cell signaling, bacteria exchange important information with their neighbors. Species-specific signals that indicate population density tell related bacteria that partners or present for genetic exchange or a that a "quorum" exists of sufficient numbers for production of efficient concentrations of exoproducts such as degradative enzymes, virulence factors and antibiotics. Bacteria may actively produce interspecies signals for the coordination of bacterial communities. In other bacteria, these interspecies "signals" may simply be secreted metabolites that other bacteria use to monitor their competitors. Signals produced in times of stress can coordinate an appropriate response among the population.

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