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THE EXTRACELLULAR PHR PEPTIDE-RAP PHOSPHATASE SIGNALING CIRCUIT OF BACILLUS SUBTILIS

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1. ABSTRACT

In the field of cell-cell communication, an emerging class of extracellular signaling peptides that function intracellularly has been identified in Gram-positive bacteria. One illustrative member of this group is the Phr family of extracellular signaling peptides of *Bacillus subtilis*. The Phr signaling peptides are secreted by the bacterium, and then, despite the presence of intracellular peptidases, they are actively transported into the cell where they interact with intracellular receptors to regulate gene expression. The intracellular receptors are members of a family of aspartyl-phosphate phosphatases, the Rap phosphatases. These phosphatases cause the dephosphorylation of response regulator proteins, ubiquitous regulatory proteins in bacteria. Immediately downstream of the genes for the Rap phosphatases are the genes for the Phr peptides, forming *rap phr* signaling cassettes. There are at least seven *rap phr* signaling cassettes in *B. subtilis*, and the genome sequence of other Gram-positive, endospore-forming bacteria suggests that similar cassettes may also function in these bacteria. In *B. subtilis*, the *rap phr* cassettes regulate sporulation, genetic competence, and genes comprising the quorum response (i.e. the response to high cell density). This review will address the mechanism of extracellular Phr signaling peptide production, transport, response, and their role in quorum sensing.

2. INTRODUCTION

Cell-cell signaling is a fundamental activity carried out by most cell types. Bacteria use cell-cell signaling for a variety of purposes, including detecting a different cell type (1) and determining spatial organization (2). However, the most common use of cell-cell signaling by bacteria is to monitor their population density, a process referred to as quorum sensing (3-6). Quorum sensing regulates a range of processes in bacteria, such as extracellular enzyme production (7), antibiotic production (4), biofilm formation (8), virulence (9), and development (10-12). Regulating these processes by quorum sensing allows bacteria to induce a process when there are a sufficient number of cells to efficiently carry out that process.

Bacteria sense a quorum through specific interactions between small, secreted signaling molecules and cellular proteins that comprise the quorum response mechanism. When the bacteria are at high density, the extracellular concentration of the signaling molecule is sufficiently high to bind to a cellular receptor and signal to the cells. In general, each bacterium produces its own set of unique signaling molecules. In Gram-negative bacteria, many of the signaling molecules are N-acylhomoserine lactones that vary in their acyl side chains (3). In Gram-positive bacteria, the signaling molecules are a diverse array of peptides (13). Many significant details about quorum sensing systems have been elucidated recently, making this one of the most active and exciting research areas in microbiology. This review will focus on the extracellular Phr signaling peptides of the Gram-positive bacterium, Bacillus subtilis.

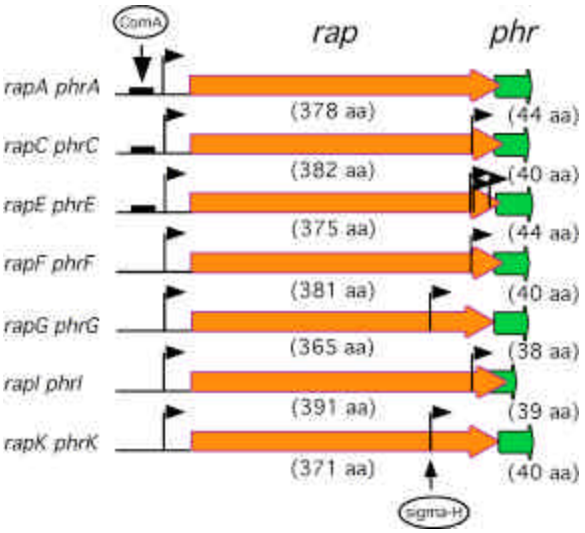

Figure 1. *rap phr* cassettes found on the chromosome of *Bacillus subtilis*. Orange arrows represent *rap*, and green arrows represent *phr*. The length of the Rap and Phr proteins is indicated below in parentheses (aa = amino acid). The arrows mark the start site of transcription. The transcription start sites indicated in front of *rapE*, *rapF*, *rapG*, *rapI*, and *rapK* are putative. Transcription start sites in front of *phr* are controlled by the minor sigma factor, sigma-H (27). The black boxes upstream of *rapA*, *rapC*, and *rapE* indicate potential sites recognized by the ComA transcription factor.

3. SIGNALING PEPTIDES OF BACILLUS SUBTILIS

B. subtilis serves as an ideal system to study extracellular signaling peptides as there are two developmental pathways that are stimulated by quorum sensing, sporulation (i.e. the formation of environmentally resistant spores) and genetic competence (i.e. the natural ability to take up exogenous DNA) (14). Studying how quorum sensing regulates these two diverse processes has led to the identification of multiple signaling peptides. Two extracellular signaling peptides have been identified that regulate genetic competence, ComX pheromone and the Competence and Sporulation Factor (CSF) (15, 16), and three extracellular signaling peptides have been identified that regulate sporulation, the PhrA peptide, CSF, and the PhrE peptide (16-18). These peptides can be divided into two groups based on differences in their mechanism of signaling; one functions extracellularly and one functions intracellularly to signal to the cells. The group that signals extracellularly is composed of ComX pheromone, which is functionally similar to extracellular signaling peptides produced by other Gram-positive bacteria (for a review see (19)). ComX pheromone appears to function extracellularly by interacting with a membrane receptor, a histidine-protein kinase, which signals to the inside of the cell by phosphorylating a response regulator protein (15, 20, 21). The group of peptides that signals intracellularly is composed of CSF, the PhrA, and the PhrE peptides. These signaling peptides have a novel mode of signaling for an extracellular peptide. They are secreted from the cell, but rather than interact with membrane receptors as many other extracellular signaling peptides do, these peptides are actively transported into the cell (17, 22, 23). Inside the cell, these peptides inhibit the activity of a member of the Rap family of protein aspartyl-phosphate phosphatases (18, 23).

CSF, the PhrA, and the PhrE peptides appear to be part of a larger family of peptides referred to as the Phr family of extracellular signaling peptides (24). This review will discuss the mechanisms of Phr production and the cell's response to extracellular Phr peptides. We will highlight what is known and what remains to be answered about the mechanism of signaling by the Phr peptides. Particular attention will be paid to the target of the Phr signaling peptides, the Rap phosphatases, as these dephosphorylate the ubiquitous response regulator type proteins found in bacteria. We discuss evidence that other bacteria may produce Phr signaling peptides and evidence both for and against these peptides playing a role in quorum sensing.

4. THE *BACILLUS SUBTILIS* FAMILY OF EXTRACELLULAR PHR SIGNALING PEPTIDES

The two defining members of the Phr family of extracellular signaling peptides are the PhrA peptide and CSF of *B. subtilis*. CSF was identified as a five amino acid peptide (sequence: ERGMT) present in the culture supernatants that stimulated the activity of a transcription factor ComA required for genetic competence development (16). The PhrA peptide (sequence: ARNQT) was identified as a peptide that when added exogenously to cells could rescue the sporulation defect of a mutant that was disrupted for the small gene, *phrA* (17). A combination of genetic and biochemical data, discussed below, indicate that CSF stimulates genetic competence development by inhibiting the RapC phosphatase and the PhrA peptide stimulates sporulation by inhibiting the RapA phosphatase.

4.1. Structure of the *rap phr* Signaling Cassettes

CSF and the PhrA peptide are derived from small proteins encoded by the genes, *phrC* and *phrA*, respectively (16, 17). These small proteins are approximately 40 amino acids in length, have signal sequences for export, and have a sequence corresponding to the mature five amino acid signaling peptides at their C-terminus (Table 1). Interestingly, *phrA* and *phrC* are immediately downstream of and partly overlapping the genes, *rapA* and *rapC*, respectively (Figure 1). *rapA* and *rapC* encode the phosphatases that are required for the PhrA peptide and CSF, respectively, to stimulate development (16, 17). Thus, these *rap phr* genes appear to form a phosphatase/peptide-signaling cassette. Five other *rap phr* gene cassettes have been identified in *B. subtilis* based on amino acid sequence similarity to *rapA phrA* and *rapC phrC* (Figure 1). All the phosphatases are approximately 375 amino acids in length and share >25 % amino acid identity to each other. The Phr proteins do not share significant amino acid identity (Table 1); however, they are similar in size and possess putative signal sequences for export. One of these cassettes, *rapE phrE*, has been shown to function in a manner analogous to the RapA-PhrA- and

Table 1. Sequence of *Bacillus subtilis* Phr proteins

Phr Protein	Signal Sequence Domain[1]			Secreted Domain[2]	
	N	H	C	Pre-peptide	Mature Peptide[3]
PhrA	MKSK	WMSGLLLVAVGFSFTQVM	VHA	GETANTEGK**TFHIA**	A**R**NQT
PhrC	MKLKSK	LFVICLAAAIFTAAGVAS	ANA	EAL**DFHV**T	E**R**GMT
PhrE	MKSK	LFISLSAVLIGLAFFGS	MYN[4]	G**EMK**E**A**	S**R**NVT[5]
PhrF	MKLKSK	LLLSCLALSTVGVATTI	ANA	PTH**QIEVA**	Q**R**GMI
PhrG	MKR	FLIGAGVAAVILSGW	FIA[4]	DHQTHSQ**EMKVA**	E**K**MIG
PhrI	MKISR	ILLAAVILSSVFSI	TYL[4]	QSDHT**EIKVA**A	D**R**VGA
PhrK	MKK	LVLCVSILAVILSGVAL	TQL[4]	STDSPS**NIQVA**	E**R**PVG[5]
Phr-pLS20	MKKIN	GWIVVALLAVTTVGA	AAA	IQYTNNADSPG**QFQVA**	Q**K**GMY
Phr-pTA1040	MKFN	ALLLLIVCASLLIVSGSSF	VIQ[4]	QDS**NVSVA**	S**R**KAT
Phr-	MTFKK	IMAAVLILAVTVAPVY	GLA[4]	TQDN**SVSVA**	S**R**NAT
Phr-pTA1060	MKFK	GLFSAVLIVSLLVGAGY	SFV[4]	HHD**EVSVA**	S**R**NAT
Phr-pPL10	MSIKK	SFALAGLAITMLVGGSY	LNH[4]	TET**SMELA**	I**R**FVT
Consensus[6]	2-3 basic	14-19 hydrophobic		a̲h̲c̲VA p̲ p̲	b

[1] Putative signal sequences were identified using SignalP V1.1 program available at http://www.cbs.dtu.dk/services/SignalP/ (71, 72). [2] The amino acids in bold match the consensus sequence shown at the bottom. [3] Putative mature peptide sequences for PhrF, PhrG, PhrI, PhrK were identified based on amino acid sequence similarity to the mature signaling peptides derived from the PhrA, PhrC, and PhrE proteins. The amino acid in bold matches the consensus sequence shown at the bottom. [4] These Phr proteins were not predicted to have strong match to a signal peptidase cleavage site. [5] These Phr proteins have additional amino acids downstream of the C-terminal amino acid of the signaling peptide. [6] A potential consensus sequence for Phr proteins was derived from nine or more of the proteins have a particular type of amino acid at a position. Capital letters correspond to the single letter designations for amino acids. The letter "a" indicates an acidic amino acid; "p" indicates a polar amino acid; "h" indicates a hydrophobic amino acid, "c" indicates a charged amino acid; "b" indicates a basic amino acid.

RapC-PhrC-signaling circuits (18). These observations and others, discussed below, have led to the model where extracellular five amino acid peptides are derived from a Phr and then imported to inhibit the activity of their cognate Rap phosphatase.

The slightly overlapping location of *rap* with downstream *phr* suggests that a *rap* be co-transcribed with its cognate *phr*. The *rapA*, *rapC*, and *rapE* promoters are activated by the quorum response transcription factor, ComA, which is activated by the ComX pheromone and CSF signaling peptides (18, 25, 26) (Figure 1). Mutants defective for the transcription factor ComA are also defective in production of CSF, the product of *phrC*, suggesting that *rapC* and *phrC* are co-transcribed (26). While the *rap* appear to be co-transcribed with the *phr*, six of the seven *phr* are also transcribed independently of their cognate *rap*. Immediately upstream of *phrC*, *phrE*, *phrF*, *phrG*, *phrI*, and *phrK* is a promoter that is recognized by the alternative sigma factor, sigma-H (27) (Figure 1). Sigma-H is required for sporulation and is maximally active during the transition from exponential growth to stationary phase (28). This additional promoter may serve to ensure that the amount of the Phr signaling peptide relative to the Rap phosphatase is sufficiently high to inhibit the function of the phosphatase during the transition to stationary phase.

4.2. Phr Signaling Peptide Production

The sequence of the precursor proteins for the Phr signaling peptides suggests that this family of extracellular signaling peptides is produced through a similar mechanism. The precursor proteins range in size from 44 to 38 amino acids in length with the first ~24 amino acids comprising a putative signal sequence for the Sec-dependent export pathway (Table 1). These signal sequences all posses a positively charged "N" domain of 3-6 amino acids at their amino-terminus that is followed by a hydrophobic "H" domain of 14-19 amino acids to traverse the membrane. A signal peptidase cleavage site typically follows a "H" domain and includes an amino acid with a small side chain at the –3 and –1 positions relative to the cleavage site. Only the PhrA, PhrC, and PhrF precursor proteins are predicted to have a strong match to a signal peptidase cleavage site (Table 1). The remaining Phr precursor proteins could be cleaved by a signal peptidase at a non-canonical site. It may also be that these putative signal sequences could serve as membrane anchors. The polar nature of the 20-13 C-terminal amino acids of the precursor protein suggests that this portion of the protein be secreted from the cell.

The extracellular C-terminal portions of the Phr proteins appear to be pro-peptides that are processed extracellularly to mature five amino acid signaling peptides (Figure 2). The processing of the pro-peptides is necessary for activity. Only a peptide corresponding to the C-terminal five amino acids of PhrA was able to inhibit RapA phosphatase activity (23). Peptides corresponding to the secreted C-terminal portion of PhrA longer than five amino acids were not capable of inhibiting the RapA phosphatase *in vitro*. It appears that the processing of the pro-peptide to the mature five amino acid peptide occurs extracellularly rather than intracellularly after transport. CSF was

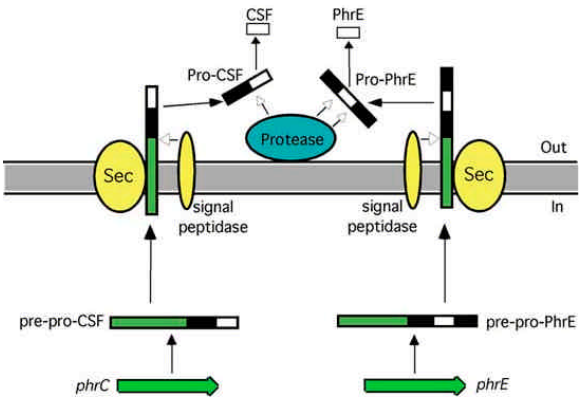

Figure 2. Model of the mechanism of production of the CSF and PhrE signaling peptides. Green arrows indicate genes. These genes encode pre-pro-peptides, the green portion of which represents signal sequence, the black portion of which represents secreted non-signaling peptide sequence, and the white portion of which represent the mature signaling peptide sequence. The gray bar represents the membrane. These pre-pro-peptides are secreted by the Sec-dependent export pathway, and the signal peptide is removed by the signal peptidase. The open-headed arrows indicate peptidase cleavage sites. The secreted pro-peptide is then cleaved by an unknown protease either once in the case of pro-CSF or twice in the case of pro-PhrE to release a mature five amino acid signaling peptide.

identified as a five amino acid peptide in culture supernatants of *B. subtilis* that stimulates the expression of ComA-controlled genes (16). That mature CSF is found extracellularly strongly supports the model that pro-peptides are processed extracellularly to mature signaling peptides.

Not all of the pro-Phr signaling peptides appear to be processed in an identical manner (Figure 2). The mature PhrA peptide and the CSF peptide appear to be derived from a single cleavage of their pro-peptide to release the C-terminal five amino acid signaling peptide. The mature PhrE signaling peptide, in contrast, appears to be derived from a double cleavage event that releases an internal five amino acid peptide. This peptide appears to be the mature signaling peptide, as only this peptide and not a peptide corresponding to the C-terminal five amino acids of pro-PhrE inhibited the activity of the RapE phosphatase (18). Thus, it is difficult to predict what the mature signaling peptide will be for the uncharacterized Phr proteins. Potential mature signaling peptides have been predicted on the basis of having a positively charged amino acid at the second position of the peptide, as is the case for the PhrA peptide, CSF, and the PhrE peptide (Table 1). If these predictions are correct, then pro-PhrF, pro-PhrG and pro-PhrI will be processed by a single cleavage step similar to pro-PhrA and pro-CSF, while pro-PhrK will be processed by a double cleavage step similar to PhrE.

The nature of the peptidase that processes the pro-Phr signaling peptides remains to be elucidated. This Phr processing enzyme is predicted to have an extracellular activity, and thus would be expected to be a membrane-bound, cell wall-associated, or secreted enzyme. Whether there is one Phr-processing enzyme or a unique enzyme for each Phr is unknown. That a single enzyme may be involved in processing is supported by the similarity among the Phr in the five amino acids preceding the mature signaling peptides (Table 1). For all the Phr proteins, the -5 position relative to the mature peptide is an acidic or polar amino acid, the -4 position is a hydrophobic amino acid, and the -3 position is a charged or polar amino acid. In all but one case, the -2 position is a valine, and the -1 position is an alanine. The importance of this putative motif is unknown; no mutational analysis of the amino acid sequence outside of the mature signaling peptide has been published.

4.3. Opp and Phr Signaling Peptide Transport

Once the mature five amino acid Phr signaling peptide has been produced extracellularly, the peptide needs to be transported into the cell to regulate gene expression. The Phr peptides are transported into the cell by an oligopeptide permease (Opp) (Figure 3). Opp is required for efficient genetic competence development and sporulation (29-31), processes regulated by the Phr signaling peptides, and mutants defective for Opp are defective in responding to exogenously added CSF or PhrA peptide (16, 17, 20). Indeed, Opp transported radiolabeled CSF at the low concentrations at which CSF signals cells, <1 nM (22). Furthermore, production of the mature PhrA peptide, which lacks the signal sequence and pro-PhrA sequence, inside the cell from a small gene, largely bypassed the requirement of Opp for sporulation ((22) and unpublished data). From these data, it seems likely that Opp transports the other Phr peptides into the cell to regulate gene expression.

Opp belongs to a large family of ATP-binding cassette (ABC) transporters, which hydrolyze ATP to drive transport (32), and Opp are widespread among bacteria. Opp consist of 5 subunits: one ligand-binding protein that is an extracellular lipoprotein, two transmembrane subunits that form a pore through the membrane, and two cytoplasmic ATPases (33, 34). Opp appear to transport peptides relatively non-specifically and ranging in size from 3-5 amino acids for the Opp from *Escherichia coli* and *Salmonella typhimurium* to 20 amino acids for the Opp from *Lactococcus lactis* (35-38). The major function of Opp for bacterial cells seems to be non-specific transport of peptides that will be used as carbon and nitrogen sources. This is thought to be the case for Opp of *B. subtilis* as well; it has been shown to transport one non-signaling peptide, the toxic tripeptide, bialophos (29, 31). Thus, it would seem that the Phr signaling peptides have co-opted the transport function of Opp for signaling. The putative lack of specificity in transport of peptides by Opp can explain how peptides with different sequences can all use the same transporter for signaling. This further suggests that the specificity in the Phr signaling peptide circuit occurs at the level of the intracellular receptor rather than at the transporter.

The use of Opp for transport of extracellularly signaling peptides is not unique to *B. subtilis*. Opp is also

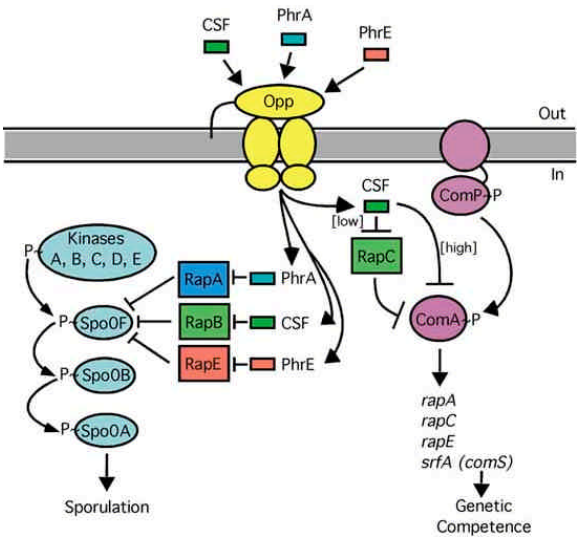

Figure 3. Model for the mechanism of response to the Phr signaling peptides. CSF (small green box), PhrA (small blue box), and PhrE (small red box) signaling peptides are transported into the cell via and oligopeptide permease (Opp). A gray bar represents the membrane. At low concentrations [low], CSF stimulates the activity of the response regulator ComA by inhibiting the phosphatase RapC. The kinase ComP activates ComA by phosphorylation. At high concentrations [high], CSF appears to inhibit the activity of ComA though an unknown mechanism. ComA activates several genes, one of which is required for genetic competence. The PhrA, CSF, and PhrE peptides stimulate sporulation by inhibiting the phosphatases RapA, RapB, and RapE, respectively. These phosphatases stimulate dephosphorylation of Spo0F. Spo0F is phosphorylated by one of five different kinases and then donates phosphate to Spo0A though the phospho-transfer protein Spo0B. Spo0A activates genes required for sporulation.

required for transport of mating pheromones into the cell where they interact with intracellular proteins to regulate plasmid transfer in *Enterococcus faecalis* (39). Opp have also been found to play a role in aerial hyphae formation in *Streptomyces coelicolor* (40), genetic competence development in *Streptococcus pneumoniae* (41, 42), extracellular virulence factor production in Group A streptococci (43) and *Bacillus thuringiensis* (44), adhesion production in *Streptococcus gordonii* (45), and hyaluronic acid production in *Streptococcus pyogenes* (46). It is enticing to speculate that Opp is transporting extracellular signaling peptides to regulate this diverse range of processes in these bacteria.

4.4. Rap Phosphatases and Phr Signaling Peptide Response

Once inside the cell, the Phr signaling peptides require intracellular proteins to regulate gene expression. The three characterized signaling peptides, CSF, PhrA, and PhrE, inhibit the activity of at least one protein aspartyl-phosphate phosphatase, a Rap phosphatase, to regulate gene expression (Figure 3). However, as Rap phosphatases can not directly regulate gene expression, additional proteins are required for the Phr signaling peptides to regulate gene expression. One is the response regulator protein that is dephosphorylated by the Rap phosphatases, and another is the histidine-protein kinase that phosphorylates the response regulator.

In vivo evidence for the mechanism of response to a Phr signaling peptide comes from studies of how CSF stimulates genetic competence development in *B. subtilis*. Exogenously added CSF stimulates expression of ComA-controlled genes, and mutations in the gene for CSF, *phrC*, result in decreased expression of ComA-controlled genes (16). ComA is a response regulator that binds to DNA and activates transcription of several genes, one of which is required for genetic competence development (10, 47-49). The RapC phosphatase of *B. subtilis* negatively regulates the expression of these same genes, suggesting that RapC dephosphorylates ComA (16, 26). RapC is required for CSF to stimulate ComA activity, as exogenous addition of CSF is unable to stimulate the expression of ComA-controlled genes in a *rapC* mutant (16, 26). Thus, the genetic data indicate that CSF stimulates ComA activity by inhibiting RapC, a negative regulator of ComA (Figure 3). That CSF may directly inhibit RapC dephosphorylation of ComA is suggested by the *in vitro* work done with the Phr signaling peptides that regulate sporulation.

In vitro evidence for the mechanism of response to Phr signaling peptides comes from work studying the regulation of the RapA, RapB, and RapE phosphatases. RapA, RapB, and RapE of *B. subtilis* have been shown in a purified system to dephosphorylate a response regulator, Spo0F, which is required for the initiation of sporulation (18, 50) (Figure 3). The ability of these Rap phosphatases to stimulate dephosphorylation was inhibited by a five amino acid Phr signaling peptide (18, 23). The inhibition by the Phr signaling peptide was specific; only the PhrA signaling peptide inhibited RapA, only CSF inhibited RapB, and only the PhrE peptide inhibited RapE (Figure 3). (Note: CSF inhibits both RapB and RapC phosphatases). While it is formally possible that the Phr signaling peptides bind to Spo0F to prevent Rap phosphatase activity, the specificity of the signaling peptides suggests that they interact directly with their cognate Rap phosphatase to inhibit its activity.

Many mechanistic details of how the Phr peptide signals regulate the activity of the Rap phosphatases remains to be elucidated. To understand this, it is necessary to understand how Rap phosphatases dephosphorylate response regulators. The current model proposes that the Rap phosphatase binds to the response regulator and causes a conformational change that increases the rate of autodephosphorylation of the response regulator. This conclusion is based on the observation that the Rap phosphatase does not appear to be phosphorylated upon dephosphorylation of a response regulator, as would be expected if Rap phosphatases acted as classical phosphatases (51).

A protein domain search of the Rap family members suggests that these phosphatases may be members

of the family of tetratricopeptide repeat (TPR) domain-containing proteins. TPR domains are comprised of 3-9 repeats of a degenerate 34 amino acid sequence (52-54). TPR domains are found in all cell types and are thought to function as protein-protein interaction domains (55). The Rap phosphatases appear to contain 6 TPR motifs spanning most of the length of the protein (24). Thus, it is tempting to speculate that the TPR motifs are involved in interacting with response regulators. A crystal structure of the TPR domain of the chaperone associated protein, Hop, was solved with the TPR domain bound to a 5 amino acid peptide derived from the protein with which it interacts, Hsp90 (56). This sets precedence for a short span of amino acids interacting with a TPR domain. Therefore, one can propose a model where the TPR domain of the Rap phosphatases binds the Phr signaling peptides. It may be that the signaling peptide and the response regulator bind to the same site on the TPR domain and competitively inhibit binding of each other. Alternatively, the Phr signaling peptides may bind to the TPR domain at a distant site from the response regulator and cause a conformational change in the TPR domain that prevents it from stimulating dephosphorylation of the response regulator. These are enticing models as they are possible to address experimentally. The possibility that the Phr signaling peptides may bind to TPR domains raises the question of whether other TPR domain-containing proteins are also subject to regulation by signaling peptides similar to the Phr signaling peptides.

4.5. Non-Rap phosphatase Receptors of Phr Signaling Peptides

The response to Phr signaling peptides may not be limited to interactions with Rap phosphatases. Studies with CSF indicate that it has at least one intracellular receptor that does not appear to be a Rap phosphatase. In addition to CSF's ability to stimulate ComA activity at low concentrations and stimulate sporulation at high concentrations, CSF can also inhibit ComA-controlled gene expression at high concentrations of the peptide (16, 26) (Figure 3). It inhibits all known genes regulated by ComA, suggesting that CSF inhibits ComA-controlled gene expression by inhibiting ComA activity ((16, 26) and unpublished data). The mechanism by which CSF may inhibit ComA activity is not understood, but several lines of evidence suggest that this mechanism does not involve inhibition of a Rap phosphatase.

Although CSF inhibits ComA-controlled gene expression at similar concentrations to which it stimulates sporulation, these two activities are separable. Changing the first, second, fourth or fifth position of CSF to an alanine dramatically reduced the ability of CSF to stimulate sporulation (22). In contrast, only the second and fourth positions of CSF were absolutely required to inhibit ComA-controlled gene expression (22). CSF inhibition of ComA-controlled gene expression is also independent of RapB, the phosphatase CSF appears to inhibit in order to stimulate sporulation, as this inhibition occurs in a *rapB* mutant (unpublished data).

The ability of CSF to inhibit ComA-controlled gene expression is also independent of RapC, the phosphatase that negatively regulates ComA activity. This inhibition is observed in a *rapC* mutant strain (16, 26), and different amino acids of CSF appear to be required for CSF to stimulate versus inhibit ComA-controlled gene expression (22). Furthermore, it is unlikely that CSF inhibits ComA activity by inhibiting a Rap phosphatase that stimulates dephosphorylation of a response regulator.

While the protein to which CSF binds to inhibit ComA activity is not known, it does appear that this protein has an intracellular domain. A mutant of CSF that has the first position changed from a glutamate to an alanine (ARGMT) is able to inhibit ComA activity, but it is unable to stimulate ComA activity (22). The ARGMT peptide was produced intracellularly from a small gene that encoded the five amino acid peptide and an initiator methionine, bypassing the export, processing, and import pathway for CSF. (Note: The initiator methionine is presumably proteolytically removed.) That this mutant CSF peptide was able to inhibit expression of ComA-controlled genes when expressed inside the cell strongly supports CSF binding to an intracellular protein to inhibit ComA activity (22). It will be interesting to determine the nature of this protein in order to understand the range of proteins to which these Phr signaling peptides can bind.

4.6. Amino Acid Specificity in Phr Signaling Peptide Response

The informational content of a five amino acid peptide is small in comparison to a typical protein. Therefore, it was of interest to know the determinants of Phr peptide specificity. Two approaches were taken to address this question, replacing the amino acids of the peptide with an alanine (22) and changing amino acids in one signaling peptide to those found in another signaling peptide (23). These studies suggest that each position of the signaling peptide is important for specificity in regulating its cognate Rap phosphatase (i.e. the Rap phosphatase that is encoded in the same operon as the Phr signaling peptide). Changing each amino acid of CSF to an alanine independently and then assessing these peptides for the ability to stimulate ComA-controlled gene expression indicates that each position of CSF is required to inhibit RapC phosphatase activity (22). Similarly, changing the 1st position alanine of the PhrA peptide to glutamate or the 3rd position asparagine to a glycine abolished the ability of this peptide to inhibit RapA phosphatase activity (23).

The interaction of a Phr signaling peptide with a non-cognate Rap phosphatase or a putative non-Rap phosphatase receptor is less specific than the interaction of the signaling peptide with its cognate Rap phosphatase. Testing alanine substituted versions of CSF for the ability to stimulate sporulation, presumably by inhibiting the RapB phosphatase, revealed that four of the five amino acids of CSF were required to stimulate sporulation (22). Changing the 3rd position glycine of CSF to an alanine actually resulted in a peptide that stimulated sporulation more strongly than wild-type CSF (22). Consistent with this, changing this 3rd position glycine to an asparagine resulted in a peptide that more strongly inhibited the RapB phosphatase *in vitro* (23). The ability of CSF to inhibit

ComA-controlled gene expression appears to be even less specific than the ability to inhibit sporulation. The 1st position glutamate, 3rd position glycine, and 5th position threonine of CSF could be changed to an alanine and the peptide still retained the ability to inhibit ComA-controlled gene expression (22). The pattern of amino acid specificity for the three activities of CSF suggest that this peptide has co-evolved with the RapC phosphatase to inhibit it, and possibly as a consequence, CSF is not as specific in its interactions with its other receptors.

5. QUORUM SENSING AND THE PHR SIGNALING PEPTIDES

Having considered mechanistically how the Phr signaling peptides are produced and sensed, the question arises: for what purpose does the cell produce these Phr signaling peptides? It seems for at least one of these signaling peptides, CSF, that it is produced for the purpose of quorum sensing. Whether other Phr signaling peptides have a role in quorum sensing is still unknown, and for one Phr signaling peptide, PhrA, a role in modulating the timing of development, rather than quorum sensing, has been proposed. Below we consider the evidence both for and against these peptides playing a role in quorum sensing.

The idea of quorum sensing first came about in an effort to explain why the amount of light produced per cell by a culture of *Vibrio fischeri* was greater if the cells were at high cell density than at low density (3). Many elegant experiments led to the conclusion that *V. fischeri* was producing an extracellular signaling molecule, an *N*-acylhomoserine lactone, through the product of the *luxI* gene, to mediate quorum sensing (57, 58). One of the key features of the *N*-acylhomoserine lactone that allows it to function in quorum sensing is that it diffuses out of the cell and into the growth medium. When the *N*-acylhomoserine lactone reaches a sufficiently high concentration, the *N*-acylhomoserine lactone binds to an intracellular receptor protein, LuxR, to activate transcription of the genes for light production (58, 59). The *N*-acylhomoserine lactone can reach this high concentration for one of two reasons: the bacteria are in an enclosed space or the bacteria are at a high cell density.

In order to determine if Phr signaling peptides are agents that trigger a quorum response, it is necessary to show that quorum sensing regulates the Phr-regulated processes. As described below, there is evidence that competence development, which is regulated by CSF, and sporulation, which is regulated by the PhrA, CSF, and PhrE peptides, are regulated by quorum sensing. Whether the Phr signaling peptides mediate the quorum sensing control of these processes has been difficult to decipher, but at least for the CSF peptide, the preponderance of evidence supports this peptide having a role in quorum sensing.

5.1. Quorum Sensing Control of ComA and Genetic Competence

When *B. subtilis* is grown in the appropriate medium, genetic competence develops in a sub-population (1-10%) of the cells when the culture is near the end of exponential growth (60). It has been known for many years that there was a factor(s) present in culture supernatants that could induce genetic competence development (61). It is now known that genetic competence development requires activation of a cascade of transcription factors and that the extracellular factor(s) regulates the first transcription factor in this cascade, ComA.

Regulation of ComA-controlled genes shows a striking resemblance to regulation of light production genes in *V. fischeri.* Expression of ComA-controlled genes, like light production genes, is low at low cell density and high at high cell density (15). Furthermore, expression of both ComA-controlled genes and light-production genes at low cell density can be induced by the addition of culture supernatants from cells at high cell density (15). Like the *N*-acylhomoserine lactone that accumulates in culture supernatants to induce light production, there are extracellular signaling peptides that accumulate in culture supernatants to induce ComA-controlled gene expression. Thus, quorum sensing regulates expression of ComA-controlled genes and genetic competence development in *B. subtilis*, as it regulates light production in *V. fischeri.*

There are two peptides that are present in culture supernatants and induce expression of ComA-controlled genes, ComX pheromone and the Phr signaling peptide, CSF. Both of these peptides appear to function in an analogous manner to the *N*-acylhomoserine lactones of *V. fischeri* and other Gram-negative bacteria. Both CSF and ComX pheromone are produced by *B. subtilis* and accumulate in culture supernatants, as the *N*-acyl homoserine lactones do (15, 20, 26). In fact, the amount of CSF that accumulates in culture supernatants during growth of a wild-type culture of *B. subtilis* has been quantified and determined to increase throughout growth (26). That CSF and ComX pheromone accumulate in the extracellular environment indicates that these peptides are able to diffuse from the cell, a hallmark of agents that mediate a quorum response.

Further evidence of whether a signaling molecule has a role in quorum sensing is whether a mutant defective in the production of the signaling peptide is defective in quorum sensing. This is true of mutants defective in the production of ComX pheromone. These mutants have significantly decreased expression of ComA-controlled genes, and the fold-decrease in expression is higher at higher cell density than at low cell density, as would be expected of a mutant defective in quorum sensing (15).

Mutants defective in production of CSF also have decreased expression of ComA-controlled genes (16). However, the fold-decrease in expression is similar at all cell densities. This phenotype of a CSF-defective mutant would suggest that the peptide does not play a role in quorum sensing. However, it is necessary to consider that CSF has two activities on ComA-controlled gene expression: to stimulate at low concentrations of the peptide and to inhibit at high concentrations of the peptide (Figure 3). CSF accumulates to levels that should inhibit

expression of ComA-controlled genes at mid-to-late exponential growth. Thus, the phenotype of a CSF-defective mutant is the product of the loss of both activities of CSF. Isolation of mutants defective in the inhibition of ComA-controlled genes in response to CSF should aid in the determination of whether CSF mediates quorum-sensing control of ComA-controlled genes.

5.2. Quorum Sensing Control of Sporulation

For many years, it was thought that sporulation was a cell autonomous process that was induced solely by starvation. In 1988, Grossman and Losick showed that sporulation was regulated by cell density (62). Like light production in *V. fischeri*, sporulation was most efficient at high cell density, and cells at low density sporulated more efficiently in the presence of culture supernatants from cells at high density. These data indicate that there is an extracellular signaling molecule(s) that accumulates at high cell density to stimulate sporulation. Unlike light production in *V. fischeri*, quorum sensing alone does not induce sporulation. Starvation is absolutely required for sporulation, and quorum sensing appears to modulate the percent of cells in a culture that will sporulate.

5.2.1. Role of Spo0A in quorum sensing control of sporulation

Quorum sensing appears to stimulate sporulation by stimulating activity of the early sporulation transcription factor, Spo0A. Spo0A is activated by phosphorylation through a phosphorelay (63) (Figure 3). One of several histidine protein kinases donates phosphate to a response regulator, Spo0F, and the phosphate on Spo0F is subsequently transferred to Spo0A through a phospho-transfer protein, Spo0B (64, 65). Mutant cells with an altered Spo0A protein, which function in the absence of Spo0F and Spo0B, sporulate equally well at all cell densities (66). This indicates that quorum-sensing signals regulate sporulation at a point upstream of Spo0A activation.

Two lines of evidence implicate a Phr peptide in the quorum sensing control of Spo0A: One is that three Phr signaling peptides, CSF, the PhrA peptide, and the PhrE peptide, regulate phosphorylation of Spo0A (18, 23) (Figure 3). These peptides inhibit a Rap phosphatase that dephosphorylates the response regulator Spo0F that is required for phosphorylation of Spo0A. Second is that mutants defective in *opp*, the Phr signaling peptide transporter, are defective in sporulation at the level of activation of Spo0A (29, 31, 66). These data indicate that these Phr signaling peptides have the potential to mediate the quorum sensing control of sporulation; however, only CSF has been shown to be involved in quorum sensing.

5.2.2. Role of CSF in quorum sensing control of sporulation

One key piece of evidence that CSF is involved in the quorum sensing control of sporulation is the observation that the sporulation efficiency of mutants that do not make CSF are less sensitive to cell density than the parent control strain. This phenotype has only been observed when the parent strain is an *ecs191* mutant. The *ecs191* mutant has a missense mutation in *spo0H*, the gene encoding the early sporulation sigma factor, sigma-H (22). The sporulation defect of an *ecs191* mutant is largely rescued by culture supernatants from wild-type cells at high cell density, indicating that the *ecs191* mutant is at least partially defective in the production of an extracellular signaling molecule(s). This mutant is still subject to quorum sensing control of sporulation as the sporulation efficiency of the *ecs191* mutant drops 5000-fold when comparing cells at high density (OD_{600} 1.0) to cells at low density (OD_{600} 0.2) (22). In contrast, the *ecs191 phrC* mutant, which does not make CSF, drops only 250-fold under the same conditions (22).

A *phrC* mutation in an otherwise wild-type strain does not result in a sporulation defect (22). The reason for the synergistic effects of the *phrC* and *ecs191* mutations is not known. One model is that sporulation is controlled by at least two extracellular signaling molecules, CSF and another unknown signaling molecule, that are redundant. Removing either one of these signaling molecules does not result in a drop in sporulation efficiency. However, when the levels of both signaling molecules are decreased, as may be the case in the regulatory mutant *ecs191*, then preventing the production of one (i.e. a *phrC* mutation) will now result in a drop in sporulation. *phrC* and 5 other *phr* are transcribed from sigma-H promoters (26, 27) (Figure 1), suggesting that one or all of these Phr signaling peptides may have a role in the quorum sensing control of sporulation.

An additional piece of evidence that CSF is involved in the quorum sensing control of sporulation is that exogenously added CSF can partially rescue the sporulation defect of wild-type cells at low density. Consistent with CSF working synergistically with a second signaling molecule, CSF was only able to stimulate sporulation when in the presence of conditioned medium from *spo0A* mutant cells (16). Spo0A, the key early sporulation transcription factor, appears to be partially defective for the production of an extracellular signaling molecule(s), as conditioned medium from a *spo0A* mutant strain is unable to stimulate sporulation of cells at low density to the same extent as conditioned medium from a wild-type strain (16). However, the *spo0A* mutant appears to produce a low level of an extracellular signaling molecule(s), as it was able to stimulate sporulation of cells at low density in the presence of CSF (16). Like sigma-H, Spo0A is required for transcription of *phrC* and five other *phr* genes (26, 27), further suggesting that a Phr signaling peptide family member in addition to CSF is involved in the quorum sensing control of sporulation.

From the evidence presented, it appears that the physiological function of CSF for *B. subtilis* is to participate in quorum sensing. That CSF only functions in quorum sensing in conjunction with other signaling molecules (e.g. ComX pheromone) has made it more difficult to study the physiological function of this signaling peptide. To have a more general understanding of the physiological signal(s), be it quorum sensing or something else, that Phr signaling peptides integrate onto

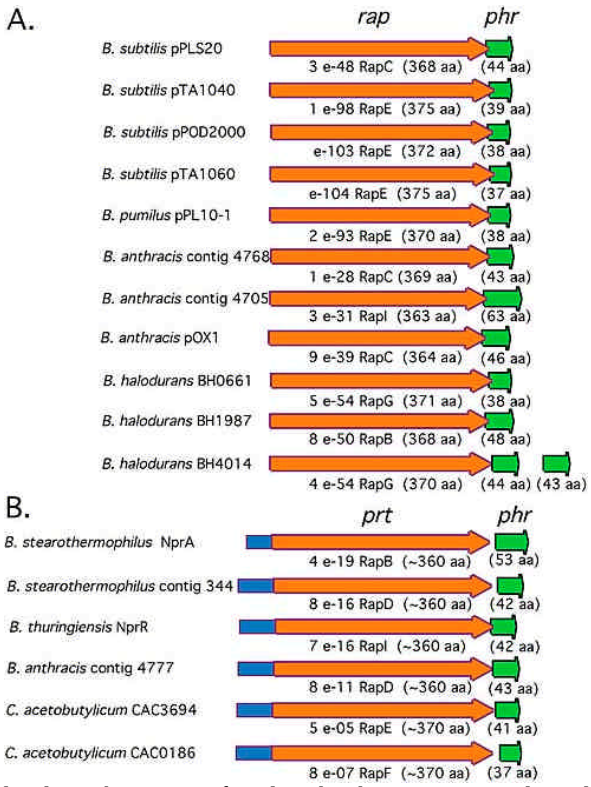

Figure 4. *rap phr* cassettes and related *prt phr* cassettes found on the chromosomes or plasmids of spore-forming, Gram-positive bacteria. Arrows indicate open reading frames with the head of the arrow representing the 3-prime-end of the gene. The orange arrows represent genes whose proteins have significant similarity to the Rap phosphatases of *B. subtilis*. Below these genes is the E-value obtained from a Blast of these proteins against the *B. subtilis* Rap phosphatases (see http://genolist.pasteur.fr/SubtiList). Next to this is the name of the Rap phosphatase to which the protein has the highest degree of similarity. The green arrows represent putative *phr* genes, and beneath these, in parenthesis, is the number of amino acids (aa) encoded by these *phr*. (**A.**) Represented are putative *rap phr* cassettes. The number in parentheses below the putative *rap* is the length of the protein encoded by these proteins. To the left of the cassettes is the name of the bacterium in which this cassettes is found. For those that are found on plasmids, the name of the plasmid is listed (e.g. pLS20). BH06601, BH1987, and BH4014 are the currently annotated names of the *B. halodurans* genes. Contig 4768 and 4705 are the sequenced contig of the *B. anthracis* genome on which the putative *rap* were identified (see http://tigrblast.tigr.org/ufmg). Please note that the putative *rap* on contig 4705 has a stop codon in the middle of the gene. This could either be a sequencing error or an inactivated gene. (**B.**) Represented are putative *prt phr* cassettes. The region in blue corresponds to the portion of the *prt* that encodes a putative helix-turn-helix motif. The approximate length of this Rap domain is indicated below in parentheses. To the left is the name of the bacterium in which these cassettes are found. NprA, NprR, CAC3694, and CAC0186 indicate the annotated names of the proteins encoded by the *prt.* The *B. stearothermophilus* NprA protein was sequenced from strain TELNE (70), and the NprA homologue encoded on contig 344 was sequenced from *B. stearothermophilus* strain 10 (see http://www.genome.ou.edu/bstearo.html). Contig 4777 is the *B. anthracis* genome sequence contig on which the putative *prt* is found. Please note that the putative *prt* CAC3694 is a fusion of open-reading frames encoding CAC3694 and CAC3695. The stop codon that separates these two genes could either be a sequencing error or an inactivated gene.

the processes that they regulate will require understanding the function of the other members of this family of signaling peptides. Below, we discuss how widespread this family of Phr signaling peptides appears to be.

6. *BACILLUS SUBTILIS* PLASMID-ENCODED PHR SIGNALING PEPTIDE FAMILY MEMBERS

Putative *rap phr* cassettes appear to be found on plasmids that replicate in *B. subtilis*. Five putative *rap phr* cassettes are found on five different *B. subtilis* plasmids, and one putative *rap phr* cassette is found on a plasmid that replicates in *Bacillus pumilus*, a close relative of *B. subtilis* (Figure 4A). These plasmid-encoded Rap proteins are similar to those encoded on the *B. subtilis* chromosome. Likewise, similarities can be discerned between the plasmid- and chromosome-encoded Phr proteins (Table 1). These plasmid-encoded Phr all have a basic N-terminus, followed by a hydrophobic region, typical of a signal sequence. The C-terminal portion of these Phr proteins is

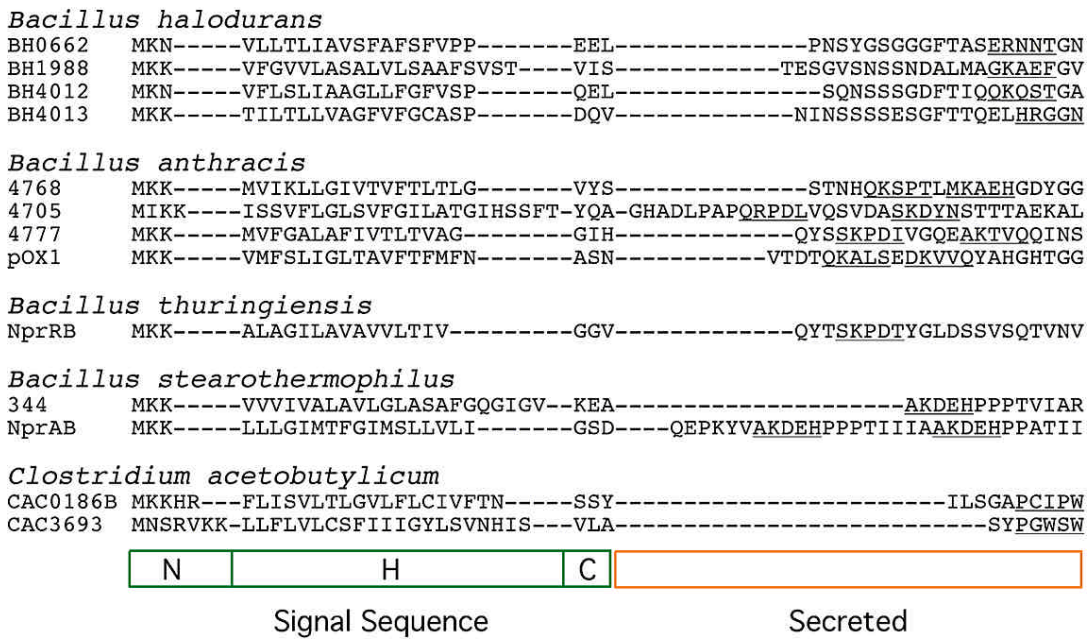

Figure 5. Alignment of putative *phr* found in *B. halodurans*, *B. anthracis*, *B. thuringiensis*, *B. stearothermophilus*, and *C. acetobutylicum*. The sequence of the proteins encoded by the putative *phr* is shown using the single letter amino acid code. The different domains of the Phr proteins are separated by dashes. Below the sequence is a schematic of the domain structure of the Phr proteins. The signal sequence is subdivided into a basic N-terminal region (N), a hydrophobic region (H), and the putative three amino acids that occurs prior to the cleavage site (C). The signal sequences were identified using SignalP V1.1 program (http://www.cbs.dtu.dk/services/SignalP/#submission) (71, 72). The underlined amino acids are those of the putative mature extracellular signaling peptide. To the left of the sequence of the *B. halodurans* Phr is the annotated name of the *phr*. To the left of the sequence of the *B. anthracis* Phr is the number of the contig or the name of the plasmid (pOX1) on which the *phr* is found. Also for a *B. stearothermophilus* Phr, the number represents the contig on which this Phr is encoded. *B. thuringiensis* NprRB and *B. stearothermophilus* NprAB are the Phr associated with NprR and NprA, respectively. CAC3693 is the annotated name of the putative Phr shown. CAC0186B is the *phr* associated with CAC0186.

polar, consistent with this portion of the protein being secreted. The C-terminal five amino acids of four of these Phr proteins differ from the PhrE signaling peptide in only 1 or 2 positions. The C-terminal five amino acids of the fifth plasmid Phr protein differs at only 2 positions from the putative PhrF signaling peptide. These plasmid Phr proteins also share amino acid similarity to the chromosomal Phr proteins in the five amino acids preceding the sequence of the predicted mature signaling peptide (Table 1). From these similarities, it is reasonable to assume that these plasmid *rap phr* cassettes function similarly to the chromosomal *rap phr* cassettes, with the *phr* encoding an extracellular signaling peptide that is transported into the cell to inhibit the activity of its cognate Rap phosphatase that dephosphorylates a response regulator. The similarity of the plasmid-encoded Rap phosphatases to RapE raises the possibility that they negatively control sporulation by dephosphorylating Spo0F, a reaction catalyzed by RapE. The selective advantage for the plasmid of having a *rap phr* cassette, which inhibits sporulation or some other processes, is not clear.

7. NON-*BACILLUS SUBTILIS* PHR SIGNALING PEPTIDE FAMILY MEMBERS

Putative *rap phr* cassettes are found in two species of *Bacillus*: two cassettes on the chromosome of *Bacillus anthracis*, three cassettes on the chromosome of *Bacillus halodurans*, and one cassette is found on *B. anthracis* virulence plasmid, pOX1. The putative Rap proteins from these cassettes are similar in size and have significant amino acid similarity to the chromosomal *B. subtilis* Rap proteins (Figure 4A). For five of the cassettes, there is one putative *phr* that is partially overlapping the putative *rap*, similar to the cassettes of *B. subtilis*. For the cassette that encodes *B. halodurans* protein BH4014, there is one putative *phr* immediately downstream of the putative *rap* and a second putative *phr* 54 base pairs downstream from the first *phr* (Figure 4A). It is possible that this second putative Phr regulates the adjacent Rap phosphatase, but it is also possible that it regulates another Rap phosphatase.

The *B. halodurans* and *B. anthracis* Phr are similar in size to the Phr of *B. subtilis*, except for the *phr* on *B. anthracis* contig 4705, which is ~20 amino acids longer than the other Phr proteins (Figure 5). That these putative Phr are the precursors for extracellular signaling peptides is supported by the similarity of their domain structure to the Phr proteins of *B. subtilis*. The N-terminal portion of these proteins is similar to signal sequences, and the C-terminal portion is polar, consistent with this portion being secreted. It is reasonable to predict that the secreted portions of the proteins are processed to five amino acid signaling

peptides. Predicting the mature signaling peptide is difficult as most of these Phr do not have a strong match to a mature Phr signaling peptide from *B. subtilis*. The one conserved residue among the known and putative mature Phr signaling peptides of *B. subtilis* is a basic amino acid at the second position of the pentapeptide (Table 1). Among the *B. halodurans* Phr, there is only one basic amino acid in the predicted secreted portion of the protein. This would predict that the mature signaling peptides are derived from either the C-terminal five amino acids or two amino acids away from the C-terminus (Figure 5). One of these putative mature Phr signaling peptides (ERNNT) shares significant similarity to the CSF peptide of *B. subtilis* (ERGMT), supporting the prediction of this peptide as the mature peptide. For the three *B. anthracis* Phr, there are two potential pentapeptides that can be derived from the predicted secreted domain that would have a basic amino acid at the second position (Figure 5). At this time, we do not know whether a basic amino acid at the second position of the mature signaling peptide is necessary to regulate the activity of the putative Rap phosphatases in these bacteria.

There are at least five genes on the chromosomes of other Gram-positive, spore-forming bacteria whose encoded proteins share weak similarity to the Rap phosphatases of *B. subtilis* and have a putative *phr* immediately downstream (Figure 4B). Two of these genes are found in the genome of *Clostridium acetobutylicum*, one in the genome of each *B. anthracis*, *Bacillus thuringiensis*, and *Bacillus stearothermophilus* (the two shown for *B. stearothermophilus* are the same gene from two different strains). The similarity of these proteins to the Rap phosphatases spans the ~360 C-terminal amino acids of the proteins, the size of the Rap phosphatases of *B. subtilis*. Although the similarity to the Rap phosphatases is weak, these proteins appear to have multiple TPR motifs when compared to the Pfam multiple protein database (67-69), similar to the Rap phosphatases (24). However, these proteins all differ from the Rap phosphatase in that, at the N-terminus of these proteins, there is an ~65 amino acid region that is identified as being a helix-turn-helix motif using the Pfam multiple protein database. Thus, it seems that these proteins may not encode phosphatases, but DNA-binding proteins that regulate transcription directly. Indeed, the *B. stearothermophilus* protein, NprA, was identified as a protein required for maximal expression of the neutral protease gene, which is located adjacent to the NprA gene (70). The *B. thuringiensis* NprR protein and the *B. anthracis* protein appear to be homologues of NprA, as they have strong similarity to NprA and are located adjacent to the gene for neutral protease.

The putative Phr downstream of these Rap phosphatase-helix-turn-helix motif proteins have a similar structure to the *B. subtilis* Phr (Figure 5). These Phr proteins appear to have a signal sequence for export and a polar C-terminus. If we apply the rule that the signaling peptide have a basic amino acid at the second position of the pentapeptide, the *B. anthracis*, *B. thuringiensis*, and *B. stearothermophilus* Phr proteins have the ability to encode one or two pentapeptide signaling molecules. However, the *C. acetobutylicum* Phr do not have any basic or charged amino acids in their putative secreted domain, suggesting that the rules for the sequence of a functional Phr signaling peptide are different for this bacterium. It is intriguing to speculate that these Rap phosphatase-helix-turn-helix motif proteins are regulated by extracellular signaling peptides derived from these Phr proteins. Extracellular signaling peptides can be used as agents that are monitored during quorum sensing, and a gene regulated by NprA shows a pattern of expression consistent with regulation by quorum sensing (i.e. low expression at low cell density and high expression at high cell density) (70). Furthermore, quorum sensing in other bacteria often regulates production of secreted enzymes, like the neutral protease (7). Thus, peptides derived from these Phr may be secreted into the growth medium for quorum sensing. When the cell density is high, these peptides may be transported back into the cell by an oligopeptide permease where they may activate the Rap phosphatase-helix-turn-helix motif proteins to bind DNA and stimulate gene expression. We propose that these Rap phosphatase-helix-turn-helix motif proteins be called Peptide-responsive transcription Factors (Prt).

8. SUMMARY AND PERSPECTIVE

The characterized *rap phr* cassettes of *B. subtilis* encode phosphatase-peptide-signaling circuits. The Rap phosphatases appear to be TPR domain proteins that bind to response regulators stimulating the dephosphorylation of these transcription factors. The activity of these Rap phosphatases is inhibited by five amino acid signaling peptides derived from these Phr proteins. The mature signaling peptides are derived by secretion of the Phr proteins through the Sec-dependent export pathway, which may or may not include signal peptidase cleavage of the signal sequence. The secreted domain of the Phr protein is then cleaved by an unknown protease at least once to release the mature five amino acid peptide. Some of these Phr signaling peptides have the ability to diffuse from the cell into the growth medium, enabling them to participate in quorum sensing. When the concentration of the mature signaling peptide becomes sufficiently high, it is then transported into the cell by an oligopeptide permease where it inhibits the activity of the Rap phosphatases. This mode of signaling by the Phr signaling peptides is relatively novel, only the peptide pheromones of *Enterococcus faecalis* that regulate plasmid transfer are also transported into the cell by an oligopeptide permease and interact with intracellular receptors (1). It seems likely that, as more examples of peptide signaling are studied in other bacteria, more examples of intracellularly functioning, extracellular signaling peptides will be identified.

rap phr cassettes appear on the genomes and plasmids of several *Bacillus* species. It is likely that these cassettes will function as phosphatase-peptide signaling circuits. However, as Rap phosphatases do not appear to be enzymes, but a TPR protein-protein interaction domain, it is possible that Rap proteins could interact with proteins that are not phosphorylated to regulate their activity. Consistent with Rap domains not necessarily acting as a phosphatase domain is the finding of putative Rap domains associated with DNA-binding domains in several species of

Bacillus and *C. acetobutylicum*. Clearly, more *rap phr* cassettes need to be studied to determine the range of functions that these cassettes may have. It seems unlikely that all the cassettes with putative *phr* have been identified among the genomes that are currently sequenced. We have applied a fairly strict definition of a *rap phr* cassette to identify additional members of this family. TPR domains are conserved from bacteria to man, most of which will not be revealed by a blast search with a Rap phosphatase. How many of these TPR domain proteins have small Phr like proteins associated with them?

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