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The intracellular function of extracellular signaling peptides

Beth A. Lazazzera*

Department of Microbiology, Immunology, & Molecular Genetics University of California, Los Angeles, Los Angeles, CA 90095, USA

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Abstract

A novel class of extracellular signaling peptides has been identified in Gram-positive bacteria that are actively transported into the cell to interact with intracellular receptors. The defining members of this novel class of signaling peptides are the Phr peptides of *Bacillus subtilis* and the mating pheromones of *Enterococcus faecalis.* These peptides are small and unmodified, gene encoded, and secreted by the bacterium. Most of these peptides diffuse into the extracellular medium, and when their concentration is sufficiently high, they are then actively transported into the cell by an oligopeptide permease (Opp). Once inside the cell, these peptides interact with an array of intracellular receptors. In *B. subtilis,* the Phr peptides regulate development of environmentally resistant spores and genetically competent cells (i.e. the natural ability to take up exogenous DNA). In *E. faecalis,* the mating pheromones regulate cell-cell transfer of plasmids, many of which encode antibiotic resistance or virulence factors. At least one component of the signaling pathway for these peptides is conserved in many bacteria, Opp. Opp is a non-specific transporter that transports peptides for use as carbon and nitrogen sources. The possibility that other bacteria could posses similar intracellularly functioning signaling peptides is discussed.

1. Introduction

1.1. Cell-cell signaling in bacteria

Bacteria regulate many important processes in response to cell-cell signaling. These processes include virulence, antibiotic production, biofilm formation, and development [5,13,16,18,20]. Bacteria often use cell-cell signaling to monitor their population density; a process referred to as quorum sensing. Quorum sensing is accomplished by the bacteria secreting small, extracellular signaling molecules whose concentration in an environment will be proportional to the concentration of cells in an environment. When the concentration of extracellular signaling molecule is sufficiently high, the same bacteria that secreted the signaling molecule will also sense and respond to the signaling molecule.

The extracellular signaling molecules used by bacteria can be divided into two general classes. Gram-positive bacteria use peptides as extracellular signaling molecules [16, 28]; Gram-negative bacteria appear to predominately use *N*-acyl homoserine lactones as extracellular signaling molecules [20,21,61]. In addition to this division, the types of signaling peptides that are used by Gram-positive bacteria can be further sub-divided into signaling peptides that function extracellularly and those that function intracellularly. This subdivision is based on the location of the receptor with which these peptides appear to interact, either in the membrane or in the cytoplasm. The mechanism of signaling by the intracellularly functioning signaling peptides represents a novel mechanism of signaling for peptides. It had been thought that signaling peptides would not function intracellularly due to the presence of peptidases. However, a subset of bacterial signaling peptides is actively transported into the cell where they interact with intracellular receptors. These intracellularly functioning signaling peptides are the subject of this review.

1.2. Intracellularly functioning signaling peptides

Intracellularly functioning signaling peptides have been identified in *Bacillus subtilis* and *Enterococcus faecalis.* The Phr peptides of *B. subtilis* and the mating pheromone of *E. faecalis* are the defining members of this class of signaling peptide. *B. subtilis* may produce as many as seven Phr peptides [50] (Table 1); three of these Phr peptides have been characterized, PhrA, PhrE, and CSF [27,52,58]. These three peptides inhibit the activity of a phosphatase that

* Tel.: +1-310-794-4804; fax: +1-310-206-5231.
E-mail address: bethl@microbio.ucla.edu (B.A. Lazazzera).

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Table 1
Structure of intracellularly-functioning signaling peptides

Bacillus subtilis		*Enterococcus faecalis*	
Name	Sequence	Name	Sequence
PhrA	ARNQT	cAD1	LFVVTLVG
CSF (PhrC)	ERGMT	cPD1	FLVMFLSG
PhrE	SRNVT	cCF10	LVTLVFV
PhrF*	QRGMI	cOB1	VAVLVLGA
PhrG*	EKMIG	cAM373	AIFILAS
PhrI*	DRVGA		
PhrK*	ERPVG		

* indicates putative signaling peptides. The production and function of these peptides has not been determined. The sequence of these peptides is inferred from the sequence of the corresponding gene product.

negatively regulates sporulation (i.e. the formation of environmentally resistant spores). CSF, which stands for *C*ompetence and *S*porulation *F*actor, has a second role, regulating the development of genetic competence (i.e. the natural ability to take up exogenous DNA).

The mating pheromones of *E. faecalis* control plasmid transfer. There are five characterized mating pheromones, cCF10, cAD1, cPD1, cOB1, and cAM373 (Table 1), which control conjugal transfer of plasmids, pCF10, pAD1, pPD1, pOB1, and pAM373, respectively [37–40, 60]. These plasmids often encoded antibiotic resistance or virulence factors, such as hemolysin [11,17]. Plasmid-free recipient cells and plasmid-containing donor cells both produce the mating pheromones. However, as the plasmids encode proteins required for response to the pheromone, only the donor cells are able to sense the mating pheromone. To prevent autoinduction, the donor plasmids have at least two mechanisms to shut down response to the endogenously produced pheromone. One is to produce an inhibitor peptide, such that only when recipient cells are present does the concentration of pheromone exceed the concentration of inhibitor peptide. The second mechanism is to produce a membrane protein that reduces the amount of active pheromone in the culture supernatants in the case of cAD1 and cPD1 and in the cell wall in the case of cCF10 [2,10,43].

The mechanisms of signaling by the Phr peptides and the mating pheromones are similar (Fig. 1). Both types of peptides are small and unmodified; the Phr peptides are five amino acids in length and the mating pheromones are seven to eight amino acids in length (Table 1). Both types of peptides are transported into the cell by an oligopeptide permease (Opp). Once inside the cell, the peptides interact with intracellular receptors to regulate the functions described above. Below, I describe in detail each step in the production and sensing pathways for these peptides, and I discuss the possibility that this peptide-signaling pathway could be widespread in bacteria.

1.3. Production of intracellularly functioning signaling peptides

Some defining characteristics of these intracellularly functioning signaling peptides appear to be emerging. These peptides are small and unmodified. In addition, they are gene encoded, exported through the Sec-dependent export pathway, and undergo at least two processing events. Despite these similarities, there are differences in mechanism of production of Phr peptides and mating pheromones.

The seven Phr peptides appear to be produced through a similar mechanism [50], and the mechanism of production of CSF is an example (Fig. 1A). CSF is derived from the C-terminal 5 amino acids of a 40 amino acid pre-propeptide, PhrC. Mutants lacking *phrC* do not produce extracellular CSF, and *phrC* mutants can be rescued extracellularly by the CSF peptide [58]. PhrC has a signal sequence for Sec-dependent export and putative signal peptidase cleavage sites. This predicts that a 15–11 amino acid pro-CSF is secreted from the cell. How pro-CSF is processed extracellularly to mature 5 amino acid CSF is not yet known. It seems likely that there is a protease, which is either a membrane protein or secreted, that carries out this processing step. The exception to this mechanism of production of a Phr peptide is the mechanism of PhrE production. After removal of the signal sequence from the pre-propeptide, the secreted pro-PhrE needs to be processed twice in order to release mature PhrE from an internal portion of pro-PhrE [27].

All five characterized mating pheromones are produced in a similar manner (Fig. 1B). The five mating pheromones appear to be derived from the 21- to 22-amino acid signal sequences of five different lipoproteins [12]. A sequence corresponding to mating pheromones cAD1, cPD1, cOB1, and cAM373, was identified once in the genome database of *E. faecalis* in the signal sequences for lipoproteins; the sequence of cCF10 was identified twice, although the one corresponding to the signal sequence of a lipoprotein seems to be the more likely source of cCF10. This sequence analysis indicates that cAD1, cPD1, cOB1, and cAM373 are derived from the last seven or eight amino acids of the signal sequence. cCF10, in contrast, appears to be derived from the seven amino acids internal to the signal sequence. While it seems likely that these signal sequences are the source of the pheromones, the phenotype of mutants lacking the lipoproteins has not yet been reported.

Following the lipoprotein signal sequence for Sec-dependent export are putative signal peptidase cleavage sites that indicate that the signal sequence from which the mating pheromones are derive is proteolytically removed from the lipoprotein (Fig. 1B). The mechanism by which the mating pheromones are released from the signal se-

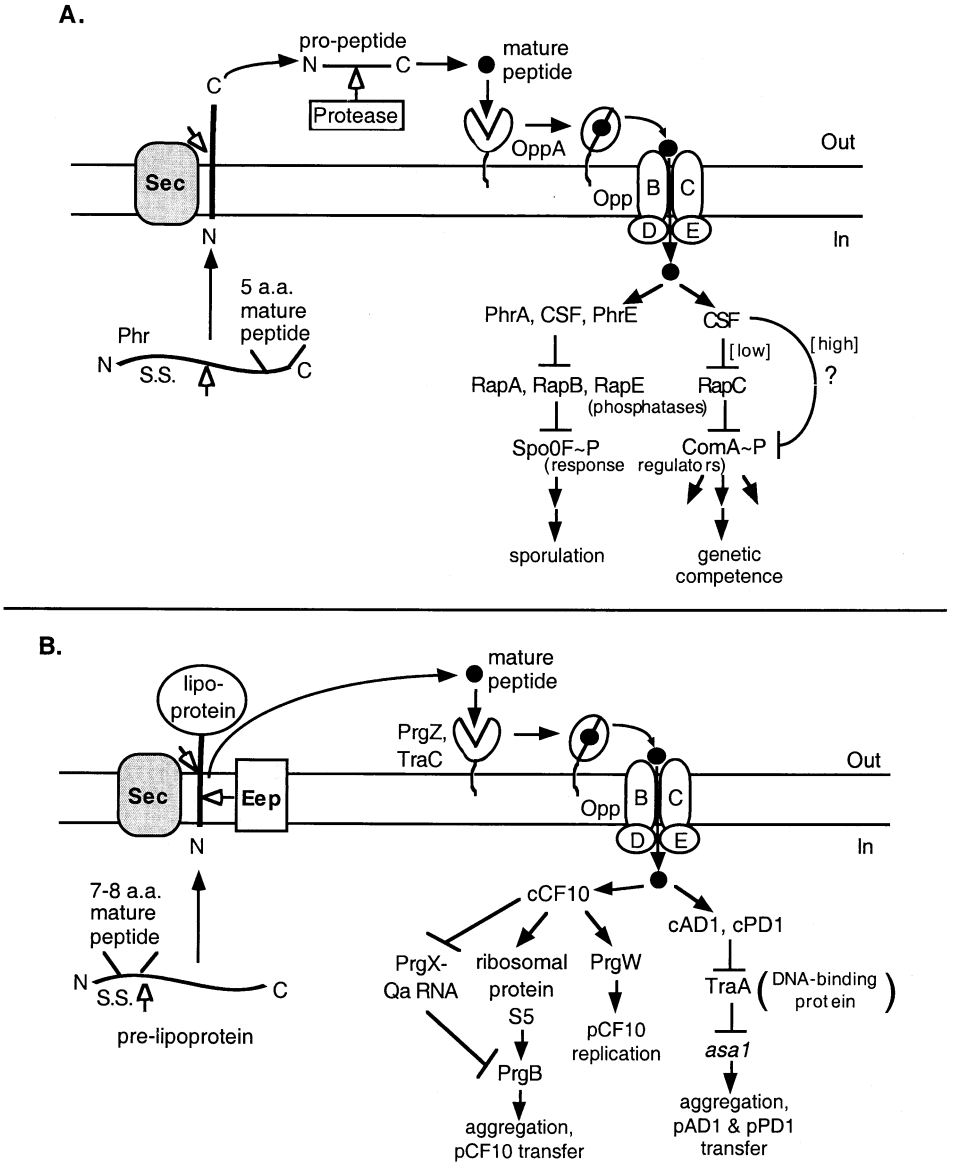

Fig. 1. Model for the production, transport and response to intracellularly functioning signaling peptides. The two straight lines represent the membrane, and the inside and outside of the cell is indicated. Dark lines indicate unfolded polypeptide precursors for the peptides. S.S. is an abbreviation for signal sequence. The box labeled Sec represents the Sec export machinery. Small arrows with unfilled heads indicate the location of putative peptidase cleavage sites. The small black circle represents the mature peptide. The five subunits of Opp transport complex are indicate by unfilled oblong circles. The OppA/PrgZ/TraC subunit is shown in the peptide bound and unbound forms. (A). Model for the mechanism of production transport and response to the Phr peptide of *B. subtilis*. (B) Model of the mechanism of production, transport, and response to the *E. faecalis* mating pheromones.

quence is not clear. An additional protein, Eep, has been identified that is required for production of all but one of the signal peptides, cAM373 [3]. Eep has similarity to membrane metalloproteases [9] and is presumed to play a role in processing mating pheromones from the signal sequence. The precise step in the production pathway that an *eep* mutant is blocked is not yet known. It is interesting to note that unknown proteins similar to Eep are found in other bacteria [9]. Could these proteins play a role in the production of extracellular signaling peptides that function intracellularly in other bacteria?

1.4. Transport of intracellularly functioning signaling peptides by oligopeptide permease

Both the Phr peptides and the mating pheromones appear to be transported into the cell by Opp (Fig. 1). *B. subtilis* cells lacking Opp are unable to respond to the CSF peptide [59], and *E. faecalis* cells lacking Opp are unable to respond to cCF10 [34]. In addition, cAD1 and cPD1 have been shown to bind Opp [40,63]. This raised the question, was the function of Opp in response to these signaling peptides to transport these peptides into the cell or simply to bind the

peptides and then signal to an intracellular protein? It had been shown that PhrA accumulated to higher levels in culture supernatants of an *opp* mutant strain [52], suggesting Opp transports the PhrA peptide into the cell. This was confirmed when it was shown that ^{3}H-labelled version of CSF and cPD1 were transported into the cell by Opp at the low concentrations at which they signal, $\leq$1 nM [32,42].

Simply knowing that these peptides are transported into the cell does not indicate whether these peptides function inside the cell. The following experiments suggest that the sole function of Opp appears to be to transport these peptides into the cell. The need for export and import of the Phr peptides through Opp could be bypassed by producing, from a synthetic gene, a mature form of the PhrA peptide inside the cell. Intracellularly produced mature PhrA peptide fully rescued the sporulation defect of a *phrA* mutant [32]. In addition, it was shown with the mating pheromone that simply binding to Opp was not sufficient to induce a response. cCF10 was conjugated to Sephadex beads, which prevented cCF10 from being transported into the cell but still allowed cCF10 to bind to Opp at the cell surface [34]. cCF10 bound to Opp at the cell surface was not sufficient to stimulate a cellular response. Further evidence that the role of Opp was to transport peptides into the cell came when the Phr peptides and the mating pheromones were shown to regulate the activity of their intracellular receptor proteins in a purified system.

Opp belongs to the large family of ATP-binding cassette (ABC) transporters, which hydrolyze ATP to drive transport [23]. Opp are comprised of 5 subunits: one ligand-binding protein that is either a periplasmic protein or an extracellular lipoprotein, two transmembrane subunits that form the pore through the membrane, and two cytoplasmic ATPases [24, 48]. Opp have been shown to transport peptides relatively non-specifically in *Escherichia coli*, *Salmonella typhimurium*, and *Lactococcus lactis* [22,30,57,62]. The Gram-negative bacteria, *E. coli* and *S. typhimurium*, appear to transport peptides of 3–5 amino acids in length [22,57,62]; whereas, the Gram-positive bacterium, *L. lactis*, appears to transport peptides of up to 20 amino acids in length [30]. This size difference could be due to differences in the nature of the Opp in these bacteria or to the outermembrane of Gram-negative bacteria limiting the size of peptides that are available for transport by Opp.

The major function of Opp for bacterial cells is probably to transport peptides to be used as carbon and nitrogen sources. It is not known how "food" peptides affect the activity of signaling peptides. Do these peptides compete for binding to Opp? Or, does Opp bind signaling peptides preferentially? *E. faecalis* seems to have addressed this problem by using an alternate ligand-binding protein, PrgZ or TraC, to recognize mating pheromones and deliver them to the transmembrane proteins of Opp for transport [34,42, 56,63]. PrgZ and TraC are both homologous to OppA, the ligand-binding subunit of Opp, and the gene encoding these proteins are found on the mating plasmids, pCF10 and pAD1/pPD1, respectively. The use of an alternate ligand-binding protein presumably reflects the need of cells to sense the mating pheromones at very low concentrations, 1 pM. An alternate ligand-binding protein does not appear to be required for *B. subtilis* to respond to CSF. Deletion of OppA has the same phenotype as deleting OppB, a membrane spanning subunit [33,51,54].

1.5. Intracellular receptors of intracellularly functioning signaling peptides

Intracellular proteins are involved in responding to the Phr peptides of *B. subtilis* and the mating pheromones of *E. faecalis*. However, this is the point at which the similarities between these intracellularly functioning peptides diverge. The types of intracellular proteins with which these peptides interact with are diverse. These proteins are described below, and they range from transcriptional regulators to phosphatases to ribosomal proteins (Fig. 1).

The Phr peptides of *B. subtilis*, PhrA, PhrE, and CSF, all regulate the activity of a protein aspartyl-phosphate phosphatase, a Rap phosphatase. The PhrA, PhrE, and CSF peptides have been shown in a purified system to inhibit RapA-, RapE-, and RapB-mediated dephosphorylation of the response regulator protein, Spo0F, respectively [27,49]. There appears to be specificity in this peptide regulation, as each peptide was only able to inhibit the activity of their cognate phosphatase. While these experiments clearly demonstrate that these peptides directly regulate the activity of an intracellular protein, these experiments do not tell us whether the peptide binds to the Rap phosphatase or the response regulator, Spo0F.

CSF is a particularly interesting peptide as genetic data indicates that this peptide interacts with at least two other intracellular receptor proteins. One of the putative intracellular receptors is the RapC phosphatase, which is homologous to the other Rap phosphatases. RapC negatively regulates the activity of a response regulator, ComA, and is required for CSF to stimulate the activity of ComA, in vivo [58]. The second putative intracellular receptor of CSF has not been identified. While CSF, at low concentrations (1–5 nM), stimulates the activity of ComA through inhibition of RapC, CSF, at high concentrations ($>$20 nM), inhibits the expression of ComA-controlled genes through an unknown mechanism [32,58]. CSF may accomplish this by stimulating the activity of a transcriptional repressor, or it may inhibit the activity of ComA itself.

The mating pheromones of *E. faecalis* induce conjugal transfer of plasmids from a donor cell to a plasmid free recipient cell. The mating pheromones appear to accomplish this in part by stimulating the production of a cell surface aggregation protein, PrgB, in the case of pCF10, and Asa1, in the case of pAD1 [6,7,65]. The aggregation protein causes plasmid donor and recipient cells to clump, which facilitates plasmid transfer. The mating pheromones stimulate the production of the aggregation protein through dif-

ferent mechanisms. The cAD1 and cPD1 peptides bind to a DNA-binding protein TraA and inhibit its activity [19,42]. TraA binds to DNA and prevents transcriptional read-though to *traE1;* TraE1 is a positive regulator of the gene encoding Asa1 [19,64,65].

cCF10 appears to stimulate the production of the aggregation protein, PrgB, by enhancing translation and transcription of *prgB.* cCF10 has been shown to interact with the ribosomal protein S5 [6]. This is proposed to lead to increased translation of a positive regulator, PrgS, which enhances translation of *prgB* [41]. Although, cCF10 has been shown to bind to a ribosomal protein, cCF10 has not yet been shown to affect the function of ribosomes in vitro. cCF10 has also been proposed to inhibit a PrgX protein-Qa RNA complex that negatively regulates transcription of *prgB* [4]. A direct interaction between cCF10 and PrgX-Qa RNA has not been shown.

cCF10 also appears to have another effect on the mating plasmid pCF10. Data indicate that cCF10 is required for pCF10 maintenance, possible by affecting plasmid replication. cCF10 appears to interact with PrgW [34], a putative replication initiator protein of pCF10 [55,66]. In addition, the host range of pCF10 could be extended to *Lactococcus lactis* by producing cCF10 peptide from a synthetic gene expressed in *L. lactis* [16]. While it is clear that cCF10 affect plasmid maintenance, how this is accomplished is unclear. The role that PrgW plays in replication of pCF10 is unknown, and it is not know what affect cCF10 binding has on PrgW activity.

A detailed mechanistic understanding of how these peptides bind to their intracellular receptors and regulate their activity is not yet known. Peptides may act like many other small molecules, binding to their targets and causing a conformational change. It appears that cAD1 acts as an allosteric inhibitor to regulate TraA DNA binding. Deletions of the N-terminus of TraA eliminate cAD1 binding, but do not affect DNA binding [19]. Peptides may also act as competitive inhibitors disrupting protein-protein interactions, an activity for which peptides are ideally suited. It is possible that the Phr peptides of *B. subtilis* regulate the activity of Rap phosphatases by binding to the interaction surface of Rap phosphatases and response regulators. However, it is unlikely that cCF10 enhances translation by disrupting a protein interaction with ribosomal protein S5. Knowing how peptides interact with their intracellular targets will tell us the range of proteins with which these intracellularly functioning peptides may interact. Is there an allosteric regulatory domain to which these peptides interact? Is this domain found on other proteins? If peptides mimic and disrupt protein/protein interaction sites, then the types of proteins that a peptide can regulate are only limited by the need for a protein to interact with another protein. In addition to an intracellularly functioning peptide affecting the activity of a protein, these peptides could also regulate the activity of large multi-protein complexes.

1.6. Do other bacteria utilize intracellularly functioning peptides?

How widespread is this class of intracellularly functioning signaling peptides? Many bacteria possess components necessary for the production and the sensing of these signaling peptides. The peptides are secreted through the Sec-dependent export machinery, which is possessed by all cell types. The precursors for the peptides undergo at least two processing events. The first is by a signal peptidase as part of export. The enzyme that carries out the subsequent processing steps has not been clearly identified yet for all the signaling peptides. Identification of this processing enzyme will reveal how widespread is the mechanism for production of these peptides.

The putative protease, Eep, involved in production of the some of the mating pheromones of *E. faecalis*, appears to be widely conserved from bacteria to mammalian cells. This indicates that many bacteria may have the capacity to produce signaling peptides from signal sequences. In fact, *Staphylococcus aureus* and *Streptococcus gordonii* produces a peptide identical to the cAM373 mating pheromone of *E. faecalis* [8,15]. This *S. aureus* peptide also appears to be derived from the signal sequence of a lipoprotein.

The key component that all these signaling peptides share is transport into the cell via an oligopeptide permease. Opp are widespread; 13 of the 20 microbial genomes listed at the TIGR web site (*http://www.tigr.org/tigr-scripts/CMR2/CMRHomePage.spl*) appear to have at least one putative peptide permease. As Opp are thought to transport a range of peptides relatively non-specifically, this indicates that any number of different peptides could serve as signaling peptides. That a bacterium possesses an Opp does not necessarily indicate that this bacterium uses intracellularly functioning peptides as signaling peptides. The main function of Opp for the cell is to transport peptides found in the environment for food (i.e. carbon and nitrogen sources). The signaling peptides appear to be co-opting the transport function of Opp for signaling.

The involvement of an Opp in sensing a signaling peptide can be inferred from the requirement of an Opp for a process that is regulated by cell-cell signaling. This is the case for a number of bacteria (Table 2). One example is *Streptomyces coelicolor.* Arial hyphae development appears to be regulated by cell-cell signaling, as developmental mutants of *S. coelicolor* exist that can be rescued by growing in the presence of wild-type cells [44]. Although the nature of the extracellular signaling molecules has not been identified, an Opp mutant has been identified as being blocked in sensing one of the early extracellular signaling events [44]. Thus, it seems very likely that the role of Opp in this system will be to transport an extracellular signaling peptide into the cell where this peptide then regulates the activity of intracellular proteins. Table 2 lists bacterial processes that are regulated by cell-cell signaling for which an Opp regulates the processes. These represent potential sys-

Table 2
Potential bacterial systems with intracellularly functioning signaling peptides

Bacterium	Regulated process*	Reference
Group A streptococci	Cysteine protease (SpeB) production, Virulence	(53)
	Export/processing of secreted proteins	
Streptococcus gordonii	CshA adhesion production	(36)
	Genetic competence	(26)
	Growth on complex nitrogen sources	
Streptococcus pneumoniae	Genetic competence	(1)
Streptococcus pyogenes	Hyaluronic acid capsule production	(14)
Streptomyces coelicolor	Arial hyphae development	(44)

* An Opp has been identified as regulating these processes.

tems for which an intracellularly functioning peptide may be present.

The identified intracellular receptors of intracellularly functioning signaling peptides do not help us predict whether other bacteria will have members of this class of signaling peptides. Homologues of the Rap phosphatases involved in sensing Phr peptides of *B. subtilis* have not been identified in other genus of bacteria. Furthermore, homologues of the TraA DNA-binding protein and the PrgW protein, which bind mating pheromones of *E. faecalis*, have not been identified in other bacteria. However, the mating pheromone, cCF10, binds to a protein conserved in all bacteria, ribosomal protein S5. If cCF10 does indeed regulate the function of ribosomes, this raises the possibility that intracellularly functioning peptides could regulate translation through binding to ribosomes in other bacteria. Additional intracellularly functioning signaling peptides will need to be identified and studied before we have an appreciation of all the types of proteins these peptide can regulate.

1.7. Do Gram-negative bacteria utilize intracellularly functioning peptides for cell-cell signaling?

Thus far, intracellularly functioning signaling peptides have only been identified in Gram-positive bacteria. Why have similar intracellularly functioning peptides not been identified in Gram-negative bacteria? The outermembrane certainly provides a barrier to the types of peptide signals that could be sensed by Gram-negative bacteria. Gram-negative bacteria would need a way for the peptide to pass through the outermembrane. The porins could provide this role [29]. It is clear that peptides are able to pass through the outermembrane, as Gram-negative bacteria are able to import peptides up to five amino acids through Opp [46,47]. This size limit indicates that Gram-negative bacteria could sense peptides similar to the 5 amino acid Phr peptides.

One example of a type of signaling peptide that does function intracellularly in Gram-negative bacteria is the sensing of cell wall murein breakdown products (muropeptides) by the intracellular transcription factor AmpR [25, 45]. In Gram-negative bacteria such as *Escherichia coli*, the cell wall is rapidly turned-over and the muropeptides are recycled. The muropeptides are transported from the periplasm to the cytoplasm by the AmpG transporter. If these muropeptides accumulate in the cytoplasm, as appears to be the case in the presence of β-lactam antibiotics, then muropeptides bind to AmpR, which in turns activates transcription of a gene encoding β-lactamase.

1.8. Physiological function of intracellularly functioning signaling peptides

What purpose do these intracellularly functioning signaling peptides serve for the bacterium? For any secreted peptide that can diffuse into the extracellular environment, the extracellular concentration of the peptide will be proportional to the density of cells secreting this peptide. Under these conditions, this peptide can serve to monitor cell density; a process also referred to as quorum sensing. The main function of the CSF peptide of *B. subtilis* appears to be in quorum sensing. CSF is secreted and diffuses into the extracellular medium, and the extracellular concentration of CSF accumulates as cells grow to high cell density [31]. This allows CSF to regulate gene expression in response to high cell density.

The mating pheromones of *E. faecalis* are also secreted and diffuse into the extracellular medium. However, in this case, the cells that produce the pheromone are different from the cells that sense the pheromone. This allows plasmid-donor cells to sense the concentration of plasmid-free recipient cells. This function is different than the function of CSF, which senses the density of the entire population.

The PhrA peptide is not proposed to play a role in quorum sensing. Although this peptide is secreted, it is not clear that this peptide diffuses into the extracellular medium. Opp may immediately import this peptide into the same cell that secreted the peptide. In this case, the signaling peptide does not play a role in sensing the cell density. The role of this signaling peptide is less obvious. It has been suggested to play a role in modulating the timing of sporulation. PhrA peptide synthesis is induced along with synthesis of the phosphatase, RapA, that PhrA inhibits. This provides a window of time between the secretion of the peptide, when the phosphatase will be active to inhibit

sporulation, until the peptide is imported by Opp to inhibit the phosphatase. An alternative role for the PhrA peptide could be as a checkpoint for either a functional secretion pathway or a functional Opp. This peptide could also serve to monitor cell-cell contact, if the peptide were taken up into another cell only when that cell came into direct contact with the PhrA-producing cell. This cell-cell contact mechanism of PhrA peptide transfer has been suggested as an explanation, for how the sporulation defect of PhrA mutants can be rescued by the presence of wild-type cells in liquid culture [52]. Monitoring cell-cell contact could be used as an indirect measure of cell density, as more cell contact should occur at high cell density.

Another function that intracellularly functioning peptides could serve is to monitor the nutritional status of the environment. In *Lactococcus lactis*, small di- and tri-peptides, which are released by proteolysis of larger peptides and proteins present in the growth environment, inhibit the production of proteinases [35]. This regulation di- and tri-peptides was dependent on uptake of these peptides into the cell, indicating that these peptides are likely to interact with an intracellular receptor to regulate production of these proteinases.

It is clear that the role that intracellularly functioning signaling peptides can play is varied. In the case of environmental peptides not encoded for by the bacterium, they can function to sense the nutritional status of the environment. For those peptides that are gene encoded, the function will in large part be dictated by whether the peptide can diffuse into the environment. If the peptide can diffuse, then the peptide will have a role in sensing cell density. Whether the type of cell that produces the peptide is the same type of cell that senses the peptide will determine whether the signaling molecule is involved in sensing an entire population or sensing a particular cell type.

2. Future Directions

This class of intracellularly functioning signaling peptides is still rather small. It seems probably that other bacteria, particularly Gram-positive bacteria, will exhibit this type of peptide signaling. By identifying more of these signaling molecules, we will develop a better understanding of the range of ways that cells can produce and sense these peptides.

Will a theme emerge in terms of the types of proteins that these peptides regulate? Knowledge of these intracellularly functioning signaling peptides may enable us to design new antibiotics that are peptide based, transported into the cells and inhibit particular protein targets. To do this, it will be important to understand what role intracellular peptidases play in degrading these signaling peptides once they are transported into the cell.

How will additional intracellularly functioning signaling peptides be identified in other bacteria? Knowledge that Opp is required for the regulation of a process is a starting point. Identifying the nature of the signaling peptides will likely require purification of these peptides from the culture supernatants. These signaling peptides are not likely to be identified as part of the sequencing of microbial genomes. If the peptides are encoded for by small genes, as the Phr peptides, these genes are not likely to be annotated as part of genome sequencing. Those that are encoded as part of signal sequences may be easier to identify. If a gene encoding a lipoprotein is identified as regulated a process, it will be important to remember that the lipoprotein may play not role in regulating the process, only the peptide derived from the signal sequence may have a role.

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