**An Exported Peptide Functions Intracellularly to Contribute to Cell Density Signaling in B. subtilis**

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Summary

Competence development and sporulation in B. subtilis are partly controlled by peptides that accumulate in culture medium as cells grow to high density. We constructed two genes that encode mature forms of two different signaling molecules, the PhrA peptide that stimulates sporulation, and CSF, the competence- and sporulation-stimulating factor. Both pentapeptides are normally produced by secretion and processing of precursor molecules. The mature pentapeptides were functional when expressed inside the cell, indicating that they normally need to be imported to function. Furthermore, at physiological concentrations (10 nM), CSF was transported into the cell by the oligopeptide permease encoded by spo0K (opp). CSF was shown to have at least three different targets corresponding to its three activities: stimulating competence gene expression at low concentrations, and inhibiting competence gene expression and stimulating sporulation at high concentrations.

Introduction

In bacteria, the ability to sense and respond to high population density, a trait common to many organisms, is often referred to as quorum sensing (Fuqua et al., 1996). In Bacillus subtilis, several peptide factors function in quorum sensing, and these factors contribute, in part, to the regulation of two different developmental pathways. The initiation of sporulation (Stragier and Losick, 1996) and the development of genetic competence (the natural ability to bind and take up exogenous DNA (Solomon and Grossman, 1996)) are most efficient in cells at high culture density (Grossman, 1995).

The competence and sporulation stimulating factor, CSF, is one of the extracellular peptide factors from B. subtilis that functions in quorum sensing. CSF is an unmodified pentapeptide that corresponds to the C-terminal 5 amino acids of the 40 amino acid polypeptide encoded by phrC (Solomon et al., 1996). CSF was purified from cell-free culture supernatant based on its ability to stimulate transcription from an early competence promoter (srfA) in cells at low cell density (Solomon et al., 1996).

Transcription of srfA is directly activated by the phosphorylated form of the transcription factor, ComA (Nakano and Zuber, 1989, 1991; Weinrauch et al., 1989; Nakano et al., 1991; Roggiani and Dubnau, 1993). ComA is phosphorylated on an aspartate residue in the N-terminal regulatory domain, typical of the response regulator family of proteins (Parkinson and Kofoid, 1992; Hoch and Silhavy, 1995). Phosphorylation of ComA is controlled primarily by ComP, a membrane-bound histidine protein kinase that autophosphorylates on a histidine residue and donates phosphate to ComA (Weinrauch et al., 1990). Transcription of the srfA operon increases as cells grow to high density due to accumulation of CSF and a second extracellular signaling factor, the ComX pheromone, a modified 10 amino acid peptide (Magnusson et al., 1994; Solomon et al., 1995, 1996).

Both CSF and the ComX pheromone stimulate transcription of srfA by increasing the concentration of phosphorylated ComA. The ComX pheromone stimulates activity of the kinase, ComP (Solomon et al., 1995), while CSF probably inhibits (either directly or indirectly) activity of RapC, a protein homologous to response regulator aspartyl phosphate phosphatases.

CSF has at least three activities. At low concentrations (1–10 nM), CSF stimulates expression of srfA, probably by inhibiting activity of the RapC phosphatase. At higher concentrations (20 nM–1 μM), CSF inhibits expression of srfA, by a mechanism that is independent of rapC. High concentrations of CSF also stimulate sporulation (Solomon et al., 1996). These responses to extracellular CSF depend on the spo0K (opp)-encoded oligopeptide permease (Solomon et al., 1995, 1996), a member of the ATP-binding cassette (ABC) transporter family (Perego et al., 1991; Rudner et al., 1991). Several ABC transporters in bacteria have dual roles, one in transport and a second as a receptor in signaling (Manson et al., 1986; Cox et al., 1988; Cangelosi et al., 1990; Wanner, 1993), raising the possibility that the oligopeptide permease may be functioning as a receptor for response to CSF.

PhrC, encoding the precursor of CSF, is a member of a family of at least four B. subtilis genes thought to encode precursors of signaling peptides (Perego et al., 1996). At least one other phr gene product has a role in sporulation. PhrA mutants have a sporulation frequency ~10% of that of wild-type cells, and sporulation can be restored to wild-type levels by addition to the medium of a peptide corresponding to the C-terminal portion of PhrA (Perego and Hoch, 1996). This rescue is dependent on the oligopeptide permease (Spo0K). PhrA is thought to inhibit activity of the phosphatase, RapA, a negative regulator of sporulation (Mueller et al., 1992; Mueller and Sonenshein, 1992; Perego et al., 1994; Perego and Hoch, 1996). It is likely that other Phr products work in a manner similar to that of CSF and PhrA.

Here, we document several aspects of Phr peptide function that are critical to understanding their mode of action. By changing, in turn, each residue of the CSF pentapeptide to alanine, we showed that different residues are required for each of the three activities of CSF, suggesting that CSF has at least three different targets inside the cell. In addition, we demonstrated that CSF can be transported into the cell when present extracellularly at physiologically relevant concentrations (10 nM), and that transport depends on the oligopeptide permease encoded by spo0K (opp). Furthermore, we showed

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that at least one form of CSF and the PhrA pentapeptide, when expressed from a small gene inside the cell, can function intracellularly. CSF and the PhrA peptide represent an emerging class of cell-cell signaling molecules that are actively imported and function intracellularly.

**Results**

**Effects of Alanine Substitutions on the Activity of CSF**

CSF has two distinct effects on expression of srfA (Solomon et al., 1996). In wild-type cells, low concentrations of CSF (2-10 nM) stimulate expression of srfA, while high concentrations of CSF (>20 nM) inhibit expression of srfA. To determine the residues of the CSF pentapeptide required for these activities, we used a series of peptides with each position of CSF changed (one at a time) to alanine. Each peptide was tested for the ability to stimulate or inhibit expression of srfA, as judged by accumulation of $\beta$-galactosidase from a srfA-lacZ fusion.

Each position of CSF was important for stimulation of expression of srfA. The alanine-mutant peptides were added to wild-type cells (containing a srfA-lacZ fusion) at low cell density, when srfA-lacZ expression is low. The wild-type CSF peptide (ERGMT) stimulated expression of srfA-lacZ such that $\beta$-galactosidase specific activity increased ~3-fold during the time (70 min) of the assay (Figure 1). The peak of activity was at a peptide concentration of ~5 nM, as seen previously (Solomon et al., 1996). In contrast, none of the alanine-mutant peptides stimulated expression of srfA-lacZ (Figures 1A-1E), indicating that each position of CSF is important for the inducing activity.

The alanine-mutant peptides were tested for the ability to inhibit srfA-lacZ in a rapC mutant. The rapC mutant was used because it has higher basal levels of expression of srfA-lacZ, making it easier to detect inhibition. In addition, srfA expression is not stimulated by CSF in a rapC mutant (Solomon et al., 1996), eliminating the overlapping regulation that causes stimulation. As expected, wild-type CSF (ERGMT) inhibited expression of srfA-lacZ (Figures 2A-2E). The effects of the alanine-mutant peptides were varied (Figure 2). Alanine in the first or third position resulted in little or no change in inhibitory activity, while alanine in the fifth position resulted in reduced, but still significant inhibitory activity. In contrast, the peptides with alanine in the second (arginine) or fourth (methionine) position had little or no inhibitory activity. These results indicate that only the second position, arginine, and the fourth position, methionine, are required for the inhibiting activity of CSF.

The results with the alanine-mutant peptides indicate that there are two different targets of CSF, one contributing to stimulation and a second contributing to inhibition of expression of srfA, and these targets recognize different residues in CSF. In wild-type cells, inhibition is observed at >20 nM CSF, whereas in the rapC mutant, inhibition is observed at ~2 nM CSF (compare Figure 1 and Figure 2). At low concentrations, CSF is probably causing inhibition in wild-type cells as well, but the inhibition is masked by the stimulatory activity. Furthermore, the stimulatory activity might be much greater in the absence of the inhibitory effect.

**Extracellular Control of Sporulation and CSF**

Multiple peptide factors contribute to the extracellular control of sporulation. To detect an effect of phrC on sporulation, and to test which positions of CSF are required for stimulation of sporulation, we made use of an allele of spo0H that causes a defect in the extracellular control of sporulation (see Experimental Procedures). spo0H encodes a sigma factor of RNA polymerase that is essential for sporulation (Stragier and Losick, 1996), and the ecs191 mutation of spo0H causes a defect in production of extracellular sporulation factors (Grossman and Losick, 1988). The sporulation frequency of the ecs191 mutant ranges from ~10^{-1} to 10^{-4} that of wild type, depending on the cell density at which sporulation is induced (Figure 3). Addition of other cells or conditioned medium rescues the sporulation frequency of the mutant up to ~1%-10% of that of wild type.

**phrC and Sporulation**

Whereas a phrC single mutant has little or no defect in sporulation (Solomon et al., 1996), the phrC ecs191 double mutant had a sporulation frequency that was 5%-10% of that of the ecs191 single mutant (Figure 3).

![Figure 1. Effects of CSF Alanine-Mutant Peptides on Stimulation of Expression of srfA](image)

Cells containing a srfA-lacZ fusion (J RL476) were grown in defined minimal medium. At a low cell density, cells were mixed with the indicated amount of peptide and incubated for 70 min at 37°C, and $\beta$-galactosidase specific activity was assayed. Open circles indicate wild-type CSF (ERGMT); closed triangles indicate the alanine-mutant peptides. The alanine-mutant peptide is indicated in each panel. (A), ARGMT; (B), EAGMT; (C), ERAMT; (D), ERGAT; and (E), ERGMA.
Peptide Signaling in B. subtilis

Figure 3. phrC Affects Sporulation in the ecs191 Mutant Background
Cells were grown in defined minimal medium and induced to sporulate at the indicated density by addition of deoxycholine. Closed circles indicate wild type (AG174); open boxes indicate ecs191 (AG388); and closed triangles indicate ecs191 phrC (BAL81). The phrC mutation alone causes little or no defect in sporulation (not shown).

CSF Alanine Mutants and Sporulation
Each position of CSF, except the third position glycine, was needed for stimulation of sporulation. Each of the mutant peptides was added at a concentration of 1 μM to the ecs191 phrC double mutant, and the ability to stimulate sporulation was measured (Figure 4). Whereas wild-type CSF stimulated sporulation z~40-fold, the alanine-mutant peptides had greatly reduced activity, except for the third position glycine-to-alanine mutant. The glycine-to-alanine mutant peptide (ERAMT) consistently stimulated sporulation to higher levels than the wild-type peptide (ERGMT).

It is clear that the residues of CSF needed to stimulate wild-type CSF (ERGMT) and to stimulate sporulation are different from those needed to inhibit expression of srfA (Figure 2). The regulatory factors required to inhibit expression of srfA and to stimulate sporulation are not known, but our results indicate that the two processes are distinct. Taken together, the effects of the alanine-mutant peptides on stimulation and inhibition of expression of srfA, and on stimulation of sporulation, strongly suggest that CSF interacts with at least three different targets to affect gene expression and development.

CSF Is Transported into the Cell at the Low Concentrations Needed for Signaling
The oligopeptide permease encoded by spo0K (opp) is required for the cells to respond to CSF (Solomon et al., 1995, 1996). A simple model is that the oligopeptide permease transports CSF into the cell and the peptide interacts with intracellular targets. Alternatively, the oligopeptide permease could function as a receptor and uptake of CSF would not be important for signaling. There is precedent for ATP-dependent importers functioning as receptors to control signaling pathways (Cox et al., 1988; Wanner, 1993). There is no doubt that the oligopeptide permease is able to import CSF (ERGMT). Cells can utilize CSF (Solomon et al., 1996) and PhrA (our unpublished data), as well as many other oligopeptides (Mathiopoulos et al., 1991; Perego et al., 1991; Koide and Hoch, 1994) (our unpublished data), to satisfy an amino acid auxotrophy, and utilization depends on the...
Phr Peptides Can Function Intracellularly

Given that CSF is transported into the cell by the oligopeptide permease, we wished to determine if CSF actually has intracellular targets, and if CSF can function inside the cell. In addition, we wished to determine if a second Phr peptide, PhrA, also could function inside the cell. To test for intracellular function, we constructed two small genes (icp genes), each encoding an intracellular peptide (see Experimental Procedures). Each gene contains a ribosome binding site and an initiation codon, and is fused to the LacI-repressible, IPTG-inducible promoter Pspac. icpC encodes MARGMT, which corresponds to the CSF peptide with an alanine in place of the Gly-to-Ala mutant peptide (ERAMT) inside the cell.

Figure 5. Expression of the CSF Ala1 Peptide, (M)ARGMT, inside the Cell Inhibits Expression of srfA rapC phrC mutant strains (BAL68, BAL69, and BAL59) containing a srfA-lacZ fusion and one of three different multicopy plasmids (pDG148, pBL3, and pBL4, respectively) were grown in minimal medium. IPTG (1 mM) was added at an optical density (600 nm) of ~0.05 to induce transcription from the Pspac promoter on each plasmid. Samples were taken for determination of β-galactosidase-specific activity at the indicated densities. Closed circles indicate pDG148 (cloning vector); open triangles indicate pBL3 [icpC, encoding the CSF Ala1 peptide (M)ARGMT]; and open boxes indicate pBL4 [icpA, encoding the PhrA peptide (MARNQT)].
were wild type with the cloning vector, pDG148 (KI2006), the phrA mutants are defective in sporulation and can be known, but may happen during secretion in the membrane. The PhrA peptide is also able to function inside the cell. Secreted precursors to the mature pentapeptide is not also a pentapeptide. The mechanism for processing the PhrA pentapeptide expression, and that target is not RapC. We propose that the mature extracellular form of PhrA is a CSF interacts with an intracellular target to inhibit srfA expression, and that target is not RapC.

**PhrA Pentapeptide**

The PhrA peptide is also able to function inside the cell. phrA mutants are defective in sporulation and can be rescued by adding extracellular peptides that correspond to the C-terminal portion of the PhrA precursor protein (Perego and Hoch, 1996). A peptide that corresponds to the last 5 amino acids of PhrA, ARNQT, functions most efficiently for rescue of the sporulation defect of a phrA mutant (data not shown). The 5 amino acid peptide is active at approximately one-tenth of the concentration of a peptide that corresponds to the C-terminal 6 amino acids of the PhrA precursor (data not shown). Therefore, we asked if the defect in sporulation of a phrA mutant could be rescued intracellularly by the IcpA peptide, (M)ARNQT. When expression of IcpA was induced with IPTG, the level of sporulation in the phrA mutant was restored to that of the congeneric wild-type strain (Figure 6).

To test if the icp products were getting out of the cell and functioning extracellularly, we tested culture supernatant from strains expressing icpC and icpA for peptide activity. The culture supernatant from the icpC strain was not able to inhibit expression of srfA, nor was the supernatant from the icpA strain able to rescue the sporulation defect of a phrA mutant (data not shown). As an additional control, we verified that we would have detected activity had the peptides been in the culture supernatant. We added ARGMT (CSF Ala1) to the icpC culture supernatant and ARNQT (PhrA pentapeptide) to the icpA culture supernatant and tested for activity under the appropriate conditions. Each peptide was able to function when mixed with the culture supernatant from the icp strain (data not shown). Based on these results, we conclude that CSF and the PhrA pentapeptide can function inside the cell. It seems likely that other Phr peptides also function inside the cell.

**Discussion**

Our results indicate that the extracellular signaling peptides, CSF and PhrA, are actively transported into the cell and interact with intracellular receptors. CSF interacts with multiple intracellular receptors, resulting in different physiological responses at different concentrations of the peptide. Because it is secreted, CSF serves as a signal for high population density, one of the conditions that regulates initiation of competence development and sporulation. CSF accumulates extracellularly to concentrations necessary to elicit a physiological response as cells in culture grow to high density. Response to extracellular peptides that are transported back into the cell represents an emerging mechanism for cell-cell signaling, involving active import of a signaling molecule and subsequent interaction with an intracellular receptor.

CSF and the PhrA peptide are produced from secreted precursors, the phrC and phrA gene products, that are 40 and 44 amino acid peptides, respectively, each with a signal sequence (Perego and Hoch, 1996; Solomon et al., 1996). The mature form of CSF is a pentapeptide that accumulates in the culture medium (Solomon et al., 1996). The mature form of PhrA that accumulates in the culture medium has not been identified directly, but we propose that the mature extracellular form of PhrA is a also a pentapeptide. The mechanism for processing the secreted precursors to the mature pentapeptides is not known, but may happen during secretion in the membrane or on the cell surface.

At least two other putative phr genes have been identified based on their size and location adjacent to and downstream from a rap gene (Perego et al., 1996). Completion of the B. subtilis genome sequencing project may allow identification of additional phr genes. A function has not been ascribed to these phr gene products, but they probably encode secreted peptides that mediate regulation of cellular processes by cell-cell signaling. The production of multiple Phr peptides by B. subtilis indicates that multiple processes are probably regulated by cell-cell signaling, that different peptides might be produced under different growth conditions, and/or that there is considerable redundancy in signaling.

**Intracellular Targets of the Phr Peptides**

At least one target of CSF and the PhrA peptide appears to be the cognate Rap phosphatase encoded upstream of phrC and phrA, respectively (Perego et al., 1996; Solomon et al., 1996). These peptides may bind directly to the Rap phosphatase inhibiting its activity, or to an unidentified target that inhibits the activity of the Rap phosphatase.

CSF has at least two additional intracellular targets. The proteins required for CSF to inhibit srfA expression or stimulate sporulation have not been identified, but our results with the alanine-mutant peptides indicate that the targets required for stimulating sporulation and inhibiting expression of srfA in response to CSF are not the same. We speculate that the inhibition of srfA expression by CSF might be due to inhibition of the autokinase activity (or stimulation of a potential phosphatase activity) of ComP, the histidine protein kinase.
required for phosphorylation of ComA and subsequent activation of transcription of srfA. CSF probably affects sporulation by affecting the level of Spo0A−P, the key transcription factor required for initiation of sporulation (Hoch, 1993; Grossman, 1995). Constitutively active forms of Spo0A bypass the requirement for high cell density and extracellular spore factors for efficient sporulation (Ireton et al., 1993). CSF could increase the level of Spo0A−P by inhibiting the activity of a Rap phosphatase that dephosphorylates Spo0A−P or a component of the phosphorelay that activates Spo0A.

**The Role of the Oligopeptide Permease in B. subtilis**

Our results show that CSF is transported into the cell at concentrations that are physiological relevant for cell-cell signaling, and that transport depends on the oligopeptide permease encoded by spo0K (opp). The oligopeptide permease is able to import CSF at the lowest concentrations we tested, 2 nM. It seems likely that the PhrA peptide is also imported via the oligopeptide permease, as spo0K is required for cells to respond to the PhrA pentapeptide (our unpublished data). Thus, one role of the oligopeptide permease for competence and sporulation is to import the CSF and PhrA signaling peptides.

The spo0K null mutants cause competence and sporulation defects that are more severe than simply the absence of CSF and/or PhrA, indicating an additional role for the oligopeptide permease. This additional role could be in controlling other Rap phosphatases that normally do not affect competence or sporulation, but that might be hyperactive in a spo0K null mutant (the “phosphatase run amok” model). In addition, the oligopeptide permease might interact directly with one or more of the Rap phosphatases, or other intracellular receptors, to facilitate inhibition of phosphatase activity by the Phr peptides. Consistent with the notion that the developmental defect of a spo0K mutant is more than simply inability to transport CSF and the PhrA peptides, we found that expression of the IcpA (PhrA pentapeptide) inside the cell did not fully suppress the sporulation defect of a spo0K (opp) null mutant (our unpublished data). It may be possible to obtain altered function mutations in spo0K that affect response to some peptides but not others, and to begin to dissect the different roles of the oligopeptide permease in development.

**Mechanisms for Cell-Cell Signaling**

Mechanisms for responding to cell-cell signaling molecules generally have been divided into two types: (1) interaction of the signaling molecule with a membrane-associated receptor and transmembrane signaling, and (2) passive diffusion of the signaling molecule across the membrane to interact with an intracellular receptor. Examples of signaling molecules that interact (or are thought to interact) with a membrane receptor include mating pheromones of Saccharomyces cerevisiae (Kurjan, 1992; Herskowitz, 1995), the ComX pheromone of B. subtilis (Magnuson et al., 1994; Solomon et al., 1995), the competence pheromone of Streptococcus pneumoniae (Hävarstein et al., 1995; Hävarstein et al., 1996; Pestova et al., 1996), and the AgrD peptide pheromone of Staphylococcus aureus (Ij et al., 1995). All of these signaling molecules activate a membrane-bound receptor, and in the bacterial examples, the receptor is a histidine kinase that autophosphorylates and donates phosphate to a cognate transcription factor. Cell-cell signaling molecules that passively diffuse across the membrane and interact with an intracellular receptor exist in both eukaryotes and prokaryotes. The steroid hormones interact with intracellular transcription factors to regulate gene expression (Tsai and O’Malley, 1994). Quorum sensing in many gram-negative bacteria involves diffusion of the signaling molecule, an acylhomoserine lactone, into the cell and interaction with an intracellular receptor, usually a transcription factor of the LuxR family (Fuqua et al., 1996).

A third mechanism for responding to cell-cell signaling molecules is emerging in prokaryotes, as illustrated by the mechanism of responding to the Phr peptides of B. subtilis. In contrast to the two mechanisms described above, the extracellular Phr peptides of B. subtilis must interact with two proteins: an importer to actively transport the signaling molecule into the cell, and a second

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**Table 1. B. subtilis Strains**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant Genotype [comments]</th>
</tr>
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<tbody>
<tr>
<td>AG174 (J H642)</td>
<td>trpC2, pheA1</td>
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<tr>
<td>AG388</td>
<td>spo0HT106 [ecs191]</td>
</tr>
<tr>
<td>BAL59</td>
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</tr>
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</tr>
<tr>
<td>J RL476</td>
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</table>

* Magnuson et al., 1994.
* Solomon et al., 1996.
intracellular receptor to cause a change in gene expression. Several other signaling molecules in prokaryotes also appear to be transported into the cell by an active mechanism. These often include peptides that are taken up by an oligopeptide permease. In addition to Phr peptides of B. subtilis, the mating pheromones of Enterococcus faecalis appear to be taken up by an oligopeptide permease (Leonard et al., 1996). Competence regulation in S. pneumoniae is modulated by several oligopeptide binding proteins and an oligopeptide permease. While these are not required for response to the major competence pheromone, they do play a role in modulating competence and suggest a possible role for additional peptide signals during competence development in S. pneumoniae (Pearce et al., 1994; Alloing et al., 1996). Development of aerial mycelium in Streptomyces coelicolor also requires an oligopeptide permease, suggesting that an extracellular signaling peptide(s) is required for development (Nodwell et al., 1996). It seems likely that these two signaling molecules function similarly to the Phr peptides of B. subtilis, with import required for interaction of the signaling molecule with an intracellular receptor. Expressing mature forms of the signaling molecules inside the cell could be generally useful in dissecting signaling mechanisms and signal transduction pathways. Considering the ubiquitous nature of ABC transporters (Higgins, 1992), the combined use of such a transporter and an intracellular receptor for response to cell-cell signaling molecules may prove to be widespread.

**Experimental Procedures**

**Construction of Plasmids Containing icpC and icpA**

Standard cloning and sequencing techniques were used (Ausubel et al., 1990). For each icp gene, two oligonucleotides were synthesized (Integrated DNA Technologies) that were complimentary except for 5′ HindIII and 3′ SphI sticky ends. The oligonucleotides for icpC contained 18 bp upstream of the start of phrC and encoded the peptide MARGMT and a stop codon. The two icpA oligonucleotides, with bold and underlined bases, were: 5′-GCAGCTAAAGAGGATGGTGTTGCTGTCCAAACATACCGTTCTCCTTACTGCTT-3′ and the complimentary 3′-ATTTCCTCCTCTCCAAATCACCTGGTCCTCTCCCTTCACTGGTCTCGCCGTCTC-5′. Similarly, the oligonucleotides for icpC contained 18 bp upstream of the start of phrC and encoded the peptide MARGMT and a stop codon. The icpA oligonucleotides, with bold and underlined bases as above, were: 5′-GCAGCTAAAGAGGATGGTGTTGCTGTCCAAACATACCGTTCTCCTTACTGCTT-3′ and the complimentary 3′-ATTCCTCCTCTCCAAATCACCTGGTCCTCTCCCTTCACTGGTCTCGCCGTCTC-5′.

Equimolar amounts of the complimentary Icp oligonucleotides were mixed, heated to 70°C, and annealed during slow cooling to room temperature. The annealed oligonucleotides were ligated into the HindIII-SphI vector fragment of pDG148 (Stragier et al., 1988), resulting in the icp genes under control of the LacI-repressible, IPTG-inducible promoter, Pspac (Yansura and Henner, 1984). pBL4 is pDG148 with icpC, and pBL3 is pDG148 with icpA. The inserts were confirmed by DNA sequencing.

**B. subtilis Strains**

B. subtilis strains are derivatives of AG174 (J H642, trpC, pha) unless indicated otherwise. Strains and relevant genotypes are listed in Table 1. Standard procedures were used for transformations and strain constructions (Harwood and Cutting, 1990). The srfA-lacZ fusion (Hahn et al., 1994; Magnuson et al., 1994) was integrated into the chromosome at the amyE locus. Integration of pBL1 into the B. subtilis chromosome by single crossover resulted in disruption of phaA, as described (Perego and Hoch, 1996). pBL1 contains the 614 bp EcoRI-BsiEI fragment from pJ PM74 (Mueller et al., 1992), that is internal to the rapA pha operon cloned into pJ H101 (Ferrari et al., 1983). Multicopy plasmids pDG148, pBL3, and pBL4 were transformed into B. subtilis selecting for resistance to neomycin. Antibiotics were used at the following concentrations: chloramphenicol, 5 μg/ml; neomycin, 5 μg/ml; phleomycin, 0.5 μg/ml; erythromycin and lincomycin together at 0.5 μg/ml and 12.5 μg/ml, respectively.

**β-Galactosidase Assays and the Effect of CSF on srfA Expression**

Cells grown in defined minimal medium with glucose (1%) and glutamate (0.1%) and required amino acids (40 μg/ml) as previously described (Jaacks et al., 1989; Magnuson et al., 1994; Solomon et al., 1995, 1996). When necessary, antibiotics were included to maintain selection for plasmids. β-galactosidase-specific activity is presented as (ΔA420 per min per ml of culture per OD600 x 1000 (Miller, 1972; Jaacks et al., 1989; Magnuson et al., 1994). To measure the effect of exogenously added CSF on srfA expression, β-galactosidase-specific activity was measured after incubating 0.25 ml of cells at low cell density (OD600 0.1) with 0.25 ml of CSF peptide (at indicated concentrations) for 70 min at 37°C as previously described (Solomon et al., 1995, 1996).

**Sporulation Assays**

Cells grown in defined minimal medium with glucose and glutamate and induced to sporulate by the addition of decomycin (U-7984; Upjohn Co.) to 500 μg/ml. Decomine is an inhibitor of GMP synthetase and induces sporulation by causing a drop in intracellular GTP (Mitani et al., 1977). Alternatively, cells were grown in nutrient sporulation medium (Harwood and Cutting, 1990) and sporulation was induced upon entry into stationary phase. Sporulation was measured 18-20 hr after induction as the number of colony forming units (CFU) per ml after heat treatment (80°C for 20 min) as a fraction of the total number of CFU per ml before heat treatment (Harwood and Cutting, 1990).

**Isolation and Characterization of ecs191, A Mutant Defective in the Extracellular Control of Sporulation**

The rationale for the mutant hunt was based on that used for the isolation of mutants defective in extracellular signaling in Myxococcus xanthus (Hagen et al., 1978). Briefly, mutants were identified that were defective in sporulation, but that were stimulated to sporulate in the presence of other cells or in the presence of conditioned medium (culture supernatant) from other cells.

B. subtilis strains K5306 (prototroph, cotA::Tn917) (Donovan et al., 1987; Sandman et al., 1987) was mutagenized with nitrosoguanidine (Mitani et al., 1972) and allowed to sporulate in nutrient sporulation medium. Any mutant defective in the extracellular control of sporulation should be stimulated to sporulate due to the presence of mostly Spo+ cells in the mutagenized culture. After enough time for sporulation, the culture was subjected to heat killing (80°C for 15 min), lysozyme treatment (1 mg/ml for 60 min at 37°C), and then another heat treatment (80°C for 15 min). The surviving spores were then diluted and spread on sporulation plates containing the indicator XP, and excess phosphate to prevent induction of the vegetative alkaline phosphatase. Colonies that appeared to be defective in production of the sporulation-associated alkaline phosphatase, as indicated by lack of blue color, were chosen for further study. The cotA mutant was used to facilitate visualization of the presence of alkaline phosphatase, cotA encodes a spore coat protein that is responsible for the dark brown color of sporulating colonies, and the cotA mutant produces colonies lacking the brown spore pigment (Donovan et al., 1987).

Candidate mutants that appeared to be defective in sporulation as isolated colonies on solid sporulation medium were chosen for further analysis. Candidates were tested in liquid medium for the ability to sporulate in pure culture, or in the presence of other cells, typically a spoIIA mutant that produces normal amounts of extracellular sporulation factors, but is blocked at stage II of sporulation and produces <10 spores/ml. The mutant with the strongest phenotype, ecs191, was stimulated to sporulate ~100-fold in the presence of the spoIIA mutant cells, or in the presence of conditioned medium.
The mutation causing the phenotype was backcrossed into unmutagenized cells by unlinked cotransformation (congression). Strain AG232 (sigB-cat.dal) was transformed to dal by nutrient sporation plates in the absence of D-alanine. Transformants that also became Spo were chosen and retested in liquid culture for the ability to be rescued extracellularly. Strain AG398 is an isolate that contained the ecs191 mutation. In nutrient sporation medium, the ecs191 mutant has a sporeulation frequency (~0.1%) of wild type, and this defect is rescued to 10- to 100-fold in the presence of other cells. ecs191 was mapped by transduction and transformation using standard procedures (Harwood and Cutting, 1990) and found to be an allele of spoOH. Cloning and DNA sequence analysis indicated that ecs191 caused a change from threonine to isoleucine at amino acid 106 of sigma-H, the spoOH gene product, a sigma factor of RNA polymerase (Stragier and Losick, 1996).

**Peptide Synthesis**

Nonradioactive peptides were purchased from Genmed Biotechnologies (So. San Francisco, CA). Radioactive CSF (ERGMT) was synthesized with 'H-glycine (DuPont NEN). Met-Thr dipeptide (10 μmol) was synthesized on an Advanced Chemtech MPS-350 automated peptide synthesizer. 'H-glycine (5 mCi; 98 nmol) was N-terminally blocked with β-fluoromethyl chloroformate (fmoc) by the micropreparation protocol essentially as described (Moyle and Boning, 1979), except that the fmoc-'H-Gly was used directly in the peptide synthesis, without purification. fmoc-'H-Gly was coupled to the Met-Thr peptide. The remaining (excess) Met-Thr was coupled to fmoc-Lys, creating a mixture of tripeptides 'H-GMT and KMT. After addition of the Arg and Glu residues, the radioactive ERGMT pentapeptide was separated from ERKMT by HPLC chromatography in the MIT Biopolymers Laboratory.

**Peptide Transport**

Strains JM753 (pHC) and JM754 (pHC, spo0K) were used to measure the rate of uptake of radiolabeled CSF (ERGMT). Cells were grown in defined minimal media to an optical density (600 nm) of ~1. Cells were removed from medium by centrifugation, washed twice, and resuspended in minimal salts (medium lacking a carbon source and amino acids) in one fifth of their original volume. Radiolabeled peptide was added to cells at room temperature to the desired final concentration. 2 or 10 nM Aliquots (0.1 ml) were removed every 10 sec after the addition of the peptide, filtered over a 0.45 μm HA filter (Millipore), and washed with 10 ml of medium lacking carbon or amino acids. Filters were counted in a Beckman LS 6000 scintillation counter.

Using the specific activity of the 'H-glycine (51.1 mCi/mmol), the amount of ERGMT peptide taken up per min per mg of cellular protein or per cell was determined. The concentration of cellular protein from lysed cells was determined using the Bio-Rad Protein Assay Kit and BSA as a standard. The cells were diluted and plated on LB to determine the number of cells used in the experiment. In a typical experiment, the amount of ERGMT taken up versus time was linear during the first two minutes, and the rate of uptake was determined from these early time points.

Import by the ABC transporters is sensitive to azide due to ATP depletion. To show that uptake of ERGMT was ATP dependent, cells were washed as above and treated with the respiratory chain inhibitor sodium azide (10 mM) for 1 hr at 37°C, prior to adding the radioactive peptide (Parnes and Boos, 1973; Berger and Heppel, 1974).

**References**


Peptide Signaling in B. subtilis


