Convergent sensing pathways mediate response to two extracellular competence factors in *Bacillus subtilis*

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Development of genetic competence in *Bacillus subtilis* is regulated by extracellular signaling molecules, including the ComX pheromone, a modified 9- or 10-amino-acid peptide. Here, we present characterization of a second extracellular competence stimulating factor (CSF). CSF appears to be, at least in part, a small peptide of between 520 and 720 daltons. Production of CSF requires several genes that are needed both for initiation of sporulation and development of competence (*spoOH, spoOA, spoOB,* and *spoOF*). Although both peptide factors regulate competence, two different sensing pathways mediate the response to the ComX pheromone and CSF. Analysis of double mutants indicated that ComX pheromone is on the same genetic pathway as the membrane-bound histidine protein kinase encoded by *comP* and that CSF is on the same genetic pathway as the oligopeptide permease encoded by *spoOK*. Furthermore, the cellular response to partly purified ComX pheromone requires the ComP histidine protein kinase, whereas the response to partly purified CSF requires the SpoOK oligopeptide permease. These two sensing pathways converge to activate transcription of *comS* (in the *srfA* operon), a key regulatory factor required for activation of additional competence genes. Both factors and their convergent sensing pathways are required for normal development of competence and might function to integrate different physiological signals.

[Key Words: *B. subtilis*; genetic competence; sporulation; signal transduction; cell–cell signaling]

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Cells communicate with each other to coordinate their activities. In bacteria, the secretion of and response to signaling molecules regulates many aspects of differentiation, development, pathogenesis, and symbiosis (Shapiro et al. 1993 and references therein). Characterizing the mechanisms by which bacterial cells produce, sense, and respond to extracellular signals is crucial to understanding these processes. The exchange of genetic material between bacteria is frequently regulated by cell–cell signaling. Transfer of conjugative plasmids in *Enterococcus faecalis* is induced by peptide pheromones (Clewell 1993). The development of genetic competence, the natural ability to take up DNA, is controlled by extracellular peptide factors in some species, including *Streptococcus pneumoniae* and *Bacillus subtilis* (Tomasz and Hotchkiss 1964; Tomasz and Mosser 1966; Joenje et al. 1972; Hui and Morrison 1991; Magnuson et al. 1994).

Development of competence in *B. subtilis* involves major changes in gene expression and metabolism. Under appropriate nutritional and cell density conditions a subpopulation of a culture of *B. subtilis* differentiates into a competent state (for review, see Dubnau 1991). Competent cells have a different buoyant density and are metabolically less active than noncompetent cells. During competence development, cells express specialized proteins that bind and take up DNA. Recombination is efficient between incoming DNA and homologous host sequences. The regulation of competence can be divided into two stages. The first stage leads to expression of the *srfA* operon. Gene products involved in producing and sensing extracellular factors all affect transcription of *srfA*, and expressing *srfA* from a heterologous promoter bypasses the need for genes upstream in the pathway and leads to constitutive levels of competence [Hahn and Dubnau 1991; Nakano and Zuber 1991]. Within the *srfA* operon is an open reading frame, *comS* (D'Souza et al. 1994; Hamoen et al. 1995), whose expression is required for the second stage of competence regulation, the activation of the ComK transcription factor [D'Souza et al. 1994; Kong and Dubnau 1994; Msadek et al. 1994; van Sinderen et al. 1994; Hamoen et al. 1995]. ComK activates transcription of the genes encoding components of the competence machinery, including the *comG* operon (Albano et al. 1987, 1989; Hahn et al. 1994; van Sinderen et al. 1994; van Sinderen and Venema 1994).

Expression of *srfA* increases as cells grow to high density as a result of the accumulation of extracellular peptide factors in the culture medium (Magnuson et al. 1994). One of these extracellular factors, the ComX phe-
omone, has been purified to homogeneity. It is a 9- to 10-amino-acid peptide with a modified tryptophan residue [Magnuson et al. 1994]. The peptide portion of ComX pheromone is encoded by the last 10 codons of comX. Production of the active pheromone also requires comQ, the gene immediately upstream of comX.

We describe the characterization and partial purification of a second extracellular competence factor that is distinct from the ComX pheromone. This competence-stimulating factor [called CSF] is, at least in part, a small peptide and is required for normal expression of srfA and the development of competence. Experiments described below also demonstrate that the two extracellular competence factors act upon two different sensing pathways that converge to stimulate expression of srfA to activate the next stage of competence regulation. The two pathways are summarized schematically in Figure 1.

The histidine protein kinase encoded by comP [Weinrauch et al. 1990] was found to be required for response to ComX pheromone [Fig. 1]. The ComP histidine protein kinase has eight putative membrane-spanning domains [Weinrauch et al. 1990] and is a member of the large family of two component regulatory systems that sense and transduce a variety of signals in prokaryotes and eukaryotes [Bourret et al. 1991; Chang et al. 1993; Ota and Varshavsky 1993; Alex and Simon 1994]. These kinases autophosphorylate in response to a signal, often sensed by their amino-terminal domain, and the phosphate is transferred to the cognate response regulator, usually a transcription factor, which is activated by phosphorylation. The cognate response regulator for ComP is the comA gene product, a transcription factor that binds to the srfA promoter region [Roggiani and Dubnau 1993] and is required for transcription of srfA [Nakano and Zuber 1989; van Sinderen et al. 1990; Hahn and Dubnau 1991; Nakano and Zuber 1991; Nakano et al. 1991a,b].

The oligopeptide permease encoded by spoOK [Perego et al. 1991; Rudner et al. 1991] was found to be required for sensing CSF. SpoOK oligopeptide permease transports oligopeptides into B. subtilis and is a member of the ATP-binding cassette [ABC] family of transporters [Perego et al. 1991; Rudner et al. 1991] that link ATP hydrolysis to the import and export of a variety of compounds [Higgins 1992].

Results

The defect in expression of srfA caused by a null mutation in spo0H is rescued extracellularly

Expression of srfA is low at low cell densities, and when cells reach an optical density [at 600 nm] of 0.2–0.3 (~2 x 10^7 to cells/ml) extracellular factors accumulate to a critical level and expression of srfA increases [Fig. 2A; Magnuson et al. 1994]. Full expression of srfA [also known as csh293, comL] requires the spo0H gene product [Jaacks et al. 1989; van Sinderen et al. 1990; Nakano et al. 1991a], a σ factor [σ^H] of RNA polymerase that is required for the initiation of sporulation. Mutations in spo0H cause a defect in the development of competence [Sadaie and Kada 1983; Albano et al. 1987], at least in part because of a decrease in expression of srfA [Jaacks et al. 1989; Hahn and Dubnau 1991]. The defect in expression of srfA in the spo0H mutant was most severe before the culture entered stationary phase [Fig. 2A]. After entry into stationary phase, β-galactosidase specific activity from a srfA–lacZ fusion in the spo0H mutant reached ~30% of that in wild-type cells [data not shown], as described previously [Nakano et al. 1988; Hahn and Dubnau 1991]. The spo0H mutation also caused a defect in expression of comG [Fig. 2B; Albano et al. 1987], a late competence gene.

The defect in expression of srfA in the spo0H mutant was rescued by the addition of conditioned medium. Conditioned medium was made by growing spo0H^+ cells, [lacking any lacZ fusion] to high density, removing the cells by centrifugation, and filter sterilizing the medium [Materials and methods]. When added to a spo0H
mutant, conditioned medium restored expression of srfA to a level similar to that in wild-type cells (Fig. 2C). The addition of conditioned medium to the spoOH mutant also substantially restored expression of comG–lacZ (Fig. 2D).

Extracellular rescue of the spoOH mutant was also demonstrated in cell-mixing experiments. spoOH mutant cells containing the comG–lacZ fusion were grown in mixed culture with either wild-type or spoOH cells, without a lacZ fusion, at a ratio of ~1:1. Expression of comG–lacZ in the spoOH mutant was restored to near wild-type levels when mixed with wild-type cells (data not shown).

The decreased transformation frequency of the spoOH mutant was partially rescued extracellularly. Addition of conditioned medium increased the transformation frequency of the spoOH mutant by 5- to 20-fold. This rescue was never up to wild-type levels of transformation and was usually ~10% of wild-type. Thus, the spoOH mutant can be fully rescued for expression of srfA and partly rescued for expression of comG and competence, by the addition of conditioned medium.

Production of CSF is reduced in a spoOH mutant

Because expression of srfA is regulated by extracellular factors that accumulate in culture medium and the defect in expression of srfA caused by the spoOH mutation was rescued extracellularly, it seemed likely that the spoOH mutant (and possibly other spoO mutants) was defective in the production of an extracellular competence factor. While purifying the ComX pheromone from conditioned medium, we had noticed a second chromatographically distinct factor that stimulates expression of srfA–lacZ two- to threefold [Magnuson et al. 1994]. The spoOH mutant was defective in production of this second factor. We fractionated conditioned medium from spoOH and wild-type strains and tested the fractions for the ability to induce expression of srfA–lacZ. Conditioned medium was adjusted to pH 2 and applied to a C-18 Sep-pak column. The column was washed and step-eluted with increasing concentrations of acetonitrile, and fractions were dried in a Speedvac concentrator and resuspended in minimal medium (Materials and methods). The ComX pheromone elutes at ~50%–60% acetonitrile [Magnuson et al. 1994]. Fractions from the 10% acetonitrile eluate contained a second activity that also stimulated expression of srfA–lacZ in cells at low density [Magnuson et al. 1994]. Conditioned medium from the spoOH mutant had near normal levels of the ComX pheromone but had a reduced amount of the second factor (CSF), relative to conditioned medium from wild-type cells [Table 1]. It appears therefore, that even a partial defect in the production of CSF, such as is observed in a spoOH mutant, is sufficient to cause a defect or delay in expression of srfA and the development of competence.

In addition to spoOH, several other early sporulation genes were found to be required for normal production of CSF. spo0A encodes a transcription factor, the activity of which is regulated by phosphorylation, and the spo0F and spo0B gene products are required to transfer phosphate from histidine protein kinases to Spo0A [Burbulys et al. 1991]. One function of Spo0A–P is to repress transcription of abrB [Perego et al. 1988; Strauch et al. 1990], the product of which is a repressor of many functions.

Sensing pathways for two competence factors

Figure 2. Expression of srfA–lacZ and comG–lacZ in wild type and a spoOH mutant in the presence and absence of conditioned medium. Cells were grown in defined minimal medium for at least three doublings before the start of the experiment. When cells reached an optical density (600 nm) of ~0.1, an equal volume of either fresh medium (A,B) or conditioned medium from wild-type cells (C,D) was added. Samples were then taken at the indicated densities for determination of β-galactosidase specific activity. (A, C) Circles indicate JRL293 [srfA–lacZΔ1974 wild-type], triangles indicate JMS139 [srfA–lacZΔ1974 spoOH::cat]. (B, D) Circles indicate AG1046 [comG–lacZ wild type], triangles indicate JMS128 [comG–lacZ spoOH::cat]. Note that the scales on the y-axis are different in each panel.
Table 1. Production of CSF and the ComX pheromone in different mutants

| Strain   | Relevant genotype | CSF percent production | ComX pheromone percent production |
|----------|-------------------|------------------------|-----------------------------------|
| JH642    | wild type         | 100                    | 100                               |
| AG665    | spo0H::cat        | 9.5                    | 68                                |
| AG503    | spoOA475::cat     | 3.2                    | 129                               |
| AG141    | spoOB136          | 19                     | 110                               |
| AG144    | spoOF221          | 23                     | 110                               |
| AG132    | spoOA204 abrB703  | 124                    | 167                               |

*The amount of CSF and ComX pheromone per milliliter of conditioned medium was determined for each strain indicated. Data are normalized to the amount of CSF and ComX pheromone determined from wild-type conditioned medium prepared and treated similarly to a given mutant. For the experiments shown, the amount of CSF from conditioned medium from wild-type cells ranged from 1170 to 1460 U/ml. The amount of ComX pheromone in conditioned medium from wild-type cells ranged from 72 to 210 U/ml. Some of the variability in the measurements of ComX pheromone probably results from its tendency to stick to glass surfaces (Magnuson et al. 1994). Similar results were obtained in multiple experiments from several different preparations of conditioned medium.

that are expressed during the transition from growth to stationary phase (Strauch and Hoch 1993), including competence development (Albano et al. 1987). spoOA, spoOB, and spoOF were found to be required for normal production of CSF (Table 1). spoOA mutants are defective in expression of srfA (Nakano et al. 1988; Hahn and Dubnau 1991) and the development of competence (Sadaie and Kada 1983; Albano et al. 1987) as well as sporulation (Hoch 1993). Mutations in spoOF and spoOB caused a defect in expression of srfA-lacZ and comG-lacZ (data not shown), and transformation frequencies were ~0.2%-1% of wild-type in both minimal and complex (SpII) competence medium. The defect in CSF production caused by a null mutation in spoOA was relieved by a null mutation in abrB (Table 1), indicating that production of CSF is controlled by AbrB, a regulator of stationary-phase gene expression (Strauch and Hoch 1993).

Although spoOA, spoOF, and spoOB are needed for production of CSF, they also appear to be required for cells to respond to CSF. The defect in srfA-lacZ and comG-lacZ expression caused by mutations in spoOA, spoOB, and spoOF was not fully relieved by the addition of conditioned medium (data not shown), in contrast to results with spoOH (above). These findings are consistent with the requirement for spoOA and abrB in the expression of the late competence transcription factor encoded by comK (Hahn et al. 1994; van Sinderen and Venema 1994).

**CSF is distinct from ComX pheromone and appears to be a small peptide**

Preliminary characterization of CSF from conditioned medium indicated that it is at least in part a small peptide. CSF activity passed through filters with a nominal molecular weight cutoff of 10,000 daltons and was sensitive to treatment with trypsin or pronase (data not shown). CSF was partially purified from conditioned medium from spo0H+ cells (Materials and methods). Briefly, conditioned medium was adjusted to pH 2 by addition of trifluoroacetic acid (TFA) and passed over a Sep-pak C-18 cartridge. CSF was eluted with 10% acetonitrile, dried in a Speedvac concentrator and resuspended. Material was then applied to a sulfopropyl Sephadex (SP) column, and CSF activity eluted at ~60 mM NaCl. Active fractions were pooled and rechromatographed over a C-18 Sep-pak cartridge and then applied to an HPLC C-18 column. The column was eluted with a gradient of acetonitrile from 0 to 10%, and active fractions were pooled and rerun under similar conditions. Active fractions were again pooled and rerun, this time eluting with a very shallow gradient (Fig. 3).

To date, the most pure preparations of CSF have a complex elution profile from reverse-phase chromatography (Fig. 3) and contain multiple components as indicated by mass spectrometry. Mass spectrometry analysis of the active fractions from this purification (Fig. 3) revealed seven components ranging in mass from ~520 to 720 daltons. If CSF is an unmodified peptide and its mass is identical or similar to any of the components detected, then CSF is probably a peptide of 4–7 amino acids.

**spoOK and comP appear to be on different, but convergent, response pathways**

To determine whether there is more than one pathway activating expression of srfA in response to the two extracellular competence factors, a variety of double mutant strains were constructed and analyzed. If two genes are on the same pathway, then a double (null) mutant should have the same phenotype that is observed in the strongest single mutant. If two genes are on different...
pathways that affect the same process, then the double mutant should have a more severe phenotype than either of the single mutants.

Expression of srfA was greatly reduced in comP [histidine protein kinase] and spo0K (oligopeptide permease) null mutants (Fig. 4A), as seen previously (Hahn and Dubnau 1991; Magnuson et al. 1994). At an optical density of ~2 [at 600 nm], accumulation of β-galactosidase specific activity from srfA-lacZ in the comP mutant and the spo0K mutant was ~5% and ~2%, respectively, of that in the wild type (Fig. 4). Despite the large effects (Fig. 4A), there was still detectable expression of srfA in these mutants (Fig. 4B). Expression of srfA was more readily detectable in these experiments than in previous work (Hahn and Dubnau 1991; Magnuson et al. 1994) because of the use of a more active srfA-lacZ fusion (Materials and methods).

Expression of srfA in the comP spo0K double mutant was significantly lower than that in either single mutant, ~0.1% of wild type and barely above background, and was similar to that in a comA null mutant [Fig. 4B]. These results indicate that the SpoOK oligopeptide permease and the ComP histidine protein kinase are on different pathways for activation of srfA transcription. The residual expression of srfA in cells lacking the SpoOK oligopeptide permease depends on the presence of ComP, and the residual expression of srfA in cells lacking the ComP histidine protein kinase depends on the presence of Spo0K. Normal expression of srfA requires both ComP and Spo0K, and eliminating both of these components is similar to eliminating the ComA transcription factor that directly regulates expression of srfA.

We also used double mutant analysis to determine which of the extracellular competence factors, ComX pheromone or CSF, is on the same pathway as the ComP histidine protein kinase and/or the SpoOK oligopeptide permease. Cells lacking ComX pheromone [because of a nonpolar mutation in comQ] and ComP [a comQ comP double mutant] were no more impaired in srfA transcription than cells lacking only ComP (Fig. 5A), indicating that the ComX pheromone is on the same pathway as the ComP histidine protein kinase. In contrast, expression of srfA was lower in the spo0H comP double mutant than in either single mutant [Fig. 5A,C]. The spo0H mutation causes reduced production of CSF [above], and this combined effect on srfA expression suggests that CSF and ComP are on different pathways for activation of srfA transcription [Fig. 1].

The Spo0K oligopeptide permease and CSF were found to be on the same pathway. Cells lacking Spo0K and producing reduced amounts of CSF [a spo0K spo0H double mutant] were no more impaired for srfA expression than cells lacking only Spo0K [Fig. 5B]. In contrast, movement of the ComX pheromone was eliminated in cells lacking Spo0K [a comQ spo0K double mutant], expression of srfA was lower than in either single mutant [Fig. 5B]. These results indicate that Spo0K and ComX pheromone are on different pathways [Fig. 1].

We also measured expression of srfA in cells that are fully capable of responding to both factors but that do not produce ComX pheromone and produce reduced amounts of CSF, a comQ spo0H double mutant. As expected, this double mutant was more defective in expression of srfA than either single mutant [Fig. 5C].

The genetic evidence clearly shows that two pathways stimulate expression of srfA. ComX pheromone and the ComP histidine protein kinase are on one pathway, and CSF and Spo0K are on the other pathway. Double mutants affecting both pathways had a combined effect on srfA expression, and double mutants affecting only a single pathway had the same effect as the single mutants.

**Figure 4.** comP and spo0K are on different pathways for the activation of srfA transcription. Isogenic strains containing the srfA-lacZ374 fusion were grown in defined minimal medium for at least three generations before the start of the experiment. Samples were taken for determination of β-galactosidase specific activity at the indicated cell densities. [A] Expression of srfA is reduced in comP and spo0K mutants. [□] JMS374 [srfA-lacZ374 wild type]; [□] JMS423 [srfA-lacZ374 comP::cat]; [□] JMS384 [srfA-lacZ374 Δspo0K::erm]. [B] srfA expression in the comP spo0K double mutant is lower than in spo0K or comP single mutants. [□] JMS423 [srfA-lacZ comP::cat]; [□] JMS384 [srfA-lacZ374 Δspo0K::erm]; [□] JMS425 [srfA-lacZ374 comP::cat Δspo0K::erm]; [□] ROM306 [srfA-lacZ374 comA::cat].

The ComP is required for response to ComX pheromone and Spo0K is required for response to CSF

We determined directly whether the Spo0K oligopeptide permease or the ComP histidine protein kinase is needed
for response to either ComX pheromone or CSF. We measured induction of \( srfA-lacZ \) in cells at low density in response to addition of either partly purified ComX pheromone or partly purified CSF. ComP was required for sensing of ComX pheromone. Cells lacking the ComP histidine protein kinase were unable to induce transcription of \( srfA-lacZ \) in response to the addition of partly purified ComX pheromone [Fig. 6A]. In contrast, cells lacking the SpooK oligopeptide permease responded well to ComX pheromone, suggesting that SpooK is not essential for detection of this factor [Fig. 6A]. SpooK, however, was needed for the sensing of CSF. Cells lacking the SpooK oligopeptide permease were unable to induce transcription of \( srfA-lacZ \) in response to the addition of partly purified CSF [Fig. 6B]. Cells lacking ComP had the normal two- to threefold response to CSF [Fig. 6B], indicating that ComP was not essential for detection of CSF.

**Effect of double mutants on transformation efficiency**

Analysis of the competence defects in double mutants indicates that a threshold level of \( srfA \) expression may be needed to activate the next step in competence development. Double mutations that had combined effects on expression of \( srfA \) did not have combined effects on competence development, determined by measuring transformation frequency. \( \text{comP} \) [JRL177], \( \text{spoOK} \) [JRL358], and \( srfA \) [ROM77] single mutants all had transformation efficiencies ~1–2% of that of otherwise isogenic wild-type, as reported previously [Jaacks et al. 1989; van Sinderen et al. 1990; Weinrauch et al. 1990; Rudner et al. 1991; van Sinderen and Venema 1994]. We found that a \( \text{spoOK} \) \( \text{comP} \) double mutant [JMS425] also had a transformation frequency ~1–2% of wild-type, indistinguishable from that of either single mutant. In essence, mutations in either branch of the response pathway that cause reduced expression of \( srfA \) cause a defect in competence development similar to that caused by no expression of \( srfA \) [a null mutation in \( srfA \), or \( \text{comA} \)]. These results are most consistent with the notion that a critical threshold level of expression of \( srfA \) must be
reached to activate the next step in competence development.

In addition, the residual expression of srfA in the comP and spoOK mutants probably does not represent a subpopulation of cells that are fully induced for expression of srfA. If a small fraction of the single mutants had full levels of expression of srfA, then it would be expected that some of the fully expressing cells would go on to develop competence. In the double mutant (comP spoOK) there is less expression of srfA than in either single mutant, and if this represented a further reduction in the size of the subpopulation that was expressing srfA, it should also have caused a further reduction in the transformation frequency.

Discussion

This work demonstrates that two different extracellular signaling factors and two different response pathways, one for each of the extracellular factors, are necessary for the initiation of competence development in B. subtilis [Fig. 1]. CSF is biochemically distinct from ComX pheromone, and different genes are required for production of each of these factors. Production of ComX pheromone [but not CSF] absolutely depends on comX and comQ, the gene immediately upstream of comX [Magnuson et al. 1994]. Production of CSF [but not ComX pheromone] is reduced significantly in spoOH, spoA, spoB, and spoOF mutants.

The oligopeptide permease encoded by spoOK is required for response to CSF, and the membrane-bound histidine protein kinase encoded by comP is required for response to the ComX pheromone [Fig. 1]. Double mutant analysis indicated that CSF is on the same signaling pathway as SpoOK oligopeptide permease, and the ComX pheromone is on the same signaling pathway as ComP histidine protein kinase. Both pathways converge to activate expression of srfA, and we suspect that they converge to regulate production and accumulation of ComA−P. Consistent with this notion is the finding that overexpression of comA on a multicopy plasmid bypasses the need for comP and spoOK in competence development [Weinrauch et al. 1990; Dubnau 1993], indicating that ComA is downstream of both the ComP histidine protein kinase and the SpoOK oligopeptide permease.

ComX pheromone probably interacts directly with ComP histidine protein kinase to stimulate autophosphorylation activity of ComP. ComP has eight putative membrane-spanning domains, and it is likely that several regions of the protein are exposed on the cell surface [Weinrauch et al. 1990]. Many other members of the family of histidine protein kinases are membrane proteins involved in signal transduction [Bourret et al. 1991; Parkinson and Kofoid 1992].

SpoOK oligopeptide permease probably transports CSF into the cell where CSF then interacts with a downstream target. CSF is in the size range of peptides transported by oligopeptide permeases [Tynkkynen et al. 1993], and a functional transporter seems to be required for the response. In addition, mutations that activate a cryptic oligopeptide permease bypass the need for SpoOK in competence [Koida and Hoch 1994] [J.M. Solomon, N. Gunther, S. Shyn, and A.D. Grossman, unpubl.], consistent with the notion that CSF is transported into the cell. SpoOK oligopeptide permease belongs to a large family of transporters that couple ATP hydrolysis to the import or export of specific compounds. The cystic fibrosis transmembrane regulator (CFTR), multidrug resistance protein (MDR), and many bacterial importers, including those for maltose, phosphate, and histidine, are members of this family [Higgins 1992]. SpoOK is able to transport a variety of peptides into B. subtilis, and these peptides can be used as a nutrient source. However, such peptides do not stimulate competence development.

Another possibility that cannot yet be ruled out is that SpoOK functions as a receptor, and in the presence of CSF, is able to send a transmembrane signal to stimulate srfA expression. The Pst ABC transporter imports phosphate ions into Escherichia coli and also functions as a receptor to regulate the activity of the PhoR/PhoB two component regulatory system [Cox et al. 1988; Wanner 1993]. In either model, transporter or receptor, we suspect that SpoOK and CSF act to stimulate accumulation of ComA−P, perhaps by stimulating the activity of a kinase, inhibiting the activity of a phosphatase, or interacting with ComA directly.

It seems that the sensing of extracellular signaling molecules by components of ABC transporters might be widespread. ABC transporters are involved in the response to opines in Agrobacterium tumefaciens [Valdivia et al. 1991; Zanker et al. 1992]. Some of the proteins required for the response to mating pheromones in E. faecalis are similar to the oligopeptide-binding proteins OppA and Spo0KA [Clewell 1993; Ruhfel et al. 1993; Tanimoto et al. 1993]. In addition, oligopeptide-binding proteins are involved in the response to extracellular competence factors in S. pneumoniae [Pearce et al. 1994].

While pathways with multiple intercellular signals occur frequently in higher organisms [Cornell and Kimelman 1994], it is not clear why the development of genetic competence in B. subtilis requires two extracellular signals. One possibility is that two signals contribute to the species specificity of competence and DNA uptake. In contrast to some naturally competent organisms [e.g., Haemophilus influenzae and Neisseria gonorrhoeae], which prefer to take up DNA containing a species-specific sequence [Smith and Danner 1981; Stewart 1989], B. subtilis will bind and take up DNA of any sequence. Two signals could help to ensure that competence is induced only in the presence of other B. subtilis cells and not simply in the presence of a species that might produce one homologous signal.

It is also possible that the two signals each provide different information to the cells, perhaps indicating the density of the culture as well as some aspect of the nutritional state of the cells. For example, production of one or the other of the factors might be stimulated by glucose, which stimulates competence, or inhibited by...
Expression of srfA could serve to integrate two different signals that affect competence development. Signal integration during competence development also seems to occur at the step of activation of the ComK transcription factor, which depends on srfA (comS) and several other genes [Hahn et al. 1994; Kong and Dubnau 1994; Msadek et al. 1994; van Sinderen and Venema 1994; van Sinderen et al. 1994]. Signal integration affecting the activity of a single transcription factor also occurs during the initiation of sporulation, the other developmental process associated with B. subtilis. Multiple diverse signals regulate the initiation of sporulation by affecting the phosphorylation of the transcription factor encoded by spo0A [Burbulis et al. 1991; Ireton and Grossman 1992, 1994; Ireton et al. 1993, 1994].

Competence is not the only developmental process in B. subtilis that is regulated by extracellular peptide factors. The initiation of sporulation is also regulated, in part, by cell crowding or high cell density. Cultures at low cell density do not sporulate efficiently, whereas similarly treated cultures at high density sporulate efficiently [Vasantha and Freese 1979; Grossman and Losick 1988; Waldburger et al. 1993]. The decreased sporulation frequency of cells at low density is partly rescued by the addition of conditioned medium made from cells grown to high density [Grossman and Losick 1988; Waldburger et al. 1993]. The conditioned medium appears to contain peptide factors that accumulate as cells grow to high density. The production of at least one extracellular sporulation factor is regulated by spo0A, spo0B, spo0F, and abrB [Grossman and Losick 1988], similar to production of CSF.

It is tempting to speculate that CSF might be involved in the initiation of sporulation as well as the initiation of competence development. However, we have not found conditions in which partly purified CSF stimulates cells at low density to sporulate. It is possible that CSF is not involved in sporulation and that another density factor with some properties similar to CSF is required for efficient sporulation. Alternatively, if CSF is involved in the initiation of sporulation, we might not have found the proper conditions in which to measure its activity. The ComX pheromone plays a role in sporulation, at least under some conditions [Magnuson et al. 1994]. However, it is not responsible for the entire effect of cell density on sporulation, and we have not found conditions in which CSF and ComX pheromone together have significantly greater effects on sporulation than ComX pheromone alone. Currently, we favor the hypothesis that there is probably a third extracellular factor, distinct from CSF and the ComX pheromone, that is involved in sporulation.

Materials and methods

Strains

Strains used are listed in Table 2. All are derived from B. subtilis strain JH642 and contain the trpC2 and pheA1 mutations. Mutant alleles used include comP::cat [Weinrauch et al. 1990], comQ::spc [Magnuson et al. 1994], comA::cat [D. Dubnau, Public Health Research Institute, NY], spoOH::cat [Jaacks et al. 1989], Δspo0K358::erm [LeDeaux and Grossman 1995], and Δspo0A475::cat [Grossman et al. 1992]. The comG–lacZ fusion is a transcriptional fusion located at amyE [Magnuson et al. 1994].

srfA–lacZ fusions

Two different srfA–lacZ fusions were used. The srfA–lacZ01974 fusion is a translational fusion located in single copy at the amyE locus and was provided by J. Hahn and D. Dubnau [Hahn et al. 1994]. It was used in most of the experiments involving the purification of CSF and had been used previously in the characterization of the ComX pheromone [Magnuson et al. 1994].

In addition, we constructed a new srfA–lacZ transcriptional fusion at the amyE locus, amyE::[srfA–lacZ01374 neo]. The srfA promoter fragment, from −291 to +140 nucleotides relative to the srfA transcription start site, with flanking EcoRI and BamHI restriction sites, was first isolated by PCR amplification of chromosomal DNA and cloned into the vector pGEM-cat [Youngman et al. 1989]. DNA sequence was determined to verify that there were no changes compared with the published sequence of the srfA promoter region [Nakano et al. 1991b]. The promoter fragment was then cloned into the lacZ fusion vector pKS2 [Magnuson et al. 1994] to generate pJS34, and the fusion was recombined into the chromosome by single crossover, selecting for neomycin resistance. β-Galactosidase specific activity from this transcriptional fusion is three- to fourfold higher than specific activity from the srfA–lacZ translational fusion described previously [Hahn et al. 1994; Magnuson et al. 1994]. Expression of this fusion was similar to that of fusions described previously [Nakano et al. 1988; Jaacks et al. 1989; van Sinderen et al. 1990; Hahn and Dubnau 1991, Nakano and Zuber 1991, Hahn et al. 1994; Magnuson et al. 1994] in that it was dependent on the same regulatory genes and was controlled similarly by cell density and nutritional conditions (data not shown).

Media

Defined minimal medium was used for most experiments and contained S7 salts [Vasantha and Freese 1980] except that MOPS buffer was used at 50 rather than 100 mM [Jaacks et al. 1989]. Medium contained glucose (1%) and glutamate (0.1%) and required amino acids (40 or 50 μg/ml) as needed. SpI competence medium [Dubnau and Davidoff-Abelson 1971] was used in some experiments, except that CaCl2 was left out [Albano et al. 1987]. The important difference between SpII medium and the defined minimal medium is the presence of yeast extract and casamino acids in the SpII medium. These components cause competence to develop after the end of exponential growth [Dubnau et al. 1991].

β-Galactosidase assays

β-Galactosidase specific activity was measured essentially as described [Miller 1972; Jaacks et al. 1989; Magnuson et al. 1994] and is presented as ΔA420/min per milliliter of culture per OD600×1000.

Competence assays

Cells were grown in defined minimal medium (or SpII), and the transformation frequency was determined by mixing cells with
chromosomal DNA (~1 μg/ml) containing a selectable marker (e.g., spc, spectinomycin resistance) for 20 or 40 min (depending on the experiment) at 37°C and plating on selective plates. Transformation frequency is the total number of transformants per viable cell. Typical frequencies for our wild-type strains (JH642 and derivatives) ranged from 5 × 10^{-5} to 8 × 10^{-4} transformants per viable cell.

Conditioned medium and separation of CSF from ComX pheromone
Conditioned medium was prepared by growing cultures in S7 minimal medium with glucose and glutamate, essentially as described (Grossman and Losick 1988; Magnuson et al. 1994) to an optical density of 2.5–3.5 at 600 nm. Cells were removed by centrifugation, and supernatant medium was sterilized by filtration. CSF and the ComX pheromone were partially purified and separated from each other on a Sep-pak C-18 cartridge (Waters). Approximately 2 hr after the onset of stationary phase, cells were removed by centrifugation and the supernatant was filter sterilized to produce cell-free conditioned medium. Nine hundred milliliters of this conditioned medium was adjusted to pH 2.0 with TFA and applied to a 0.3-gram Sep-pak C-18 cartridge (Waters). Active CSF was recovered after step elution with 10% acetonitrile and 0.1% TFA (pH 2.0). This material was applied to a SP column and eluted with a linear gradient of NaCl in 25 mM sodium acetate (pH 4.0). CSF was concentrated by roto-vap and/or Speedvac as necessary. After each step in the purification, active fractions were pooled and rerun under similar conditions. Finally, active fractions were pooled and rerun under similar conditions. Frequently for our wild-type strains (JH642 and derivatives) ranged from ~5 × 10^{-5} to 8 × 10^{-4} transformants per viable cell.

Purification of CSF
CSF was purified from conditioned medium made from strain ROM186 [prototroph, ΔspoOK357::neo spoIVC::Tn917]. The spoIVC mutation was used to block spore formation completely. The spo0K allele is a deletion insertion (LeDeaux and Grossman 1995) and was used because preliminary results indicated that spo0K null mutations caused increased production of CSF, especially after entry into stationary phase (data not shown). The cells were grown in defined minimal medium with S7 salts (Vasantha and Freese 1980), trace metals, glucose (1%), and glutamate (0.1%), at 37°C, essentially as described previously (Magnuson et al. 1994). Approximately 2 hr after the onset of stationary phase, cells were removed by centrifugation and the supernatant was filter sterilized to produce cell-free conditioned medium. Nine hundred milliliters of this conditioned medium was adjusted to pH 2.0 with TFA and applied to a 0.3-gram Sep-pak C-18 cartridge (Waters). Active CSF was recovered after step elution with 10% acetonitrile and 0.1% TFA (pH 2.0). This material was applied to a SP column and eluted with a linear gradient of NaCl in 25 mM sodium acetate (pH 4.0). CSF eluted at ~60 mM NaCl, and active fractions were pooled and applied to a Vydac C-18 column for HPLC purification. Material was eluted using a linear gradient of acetonitrile (~0.2%/min) in 0.1% TFA. Active fractions were pooled and rerun under similar conditions. Finally, active fractions were pooled and rerun under similar conditions. Frequently for our wild-type strains (JH642 and derivatives) ranged from ~5 × 10^{-5} to 8 × 10^{-4} transformants per viable cell.

Assay of CSF activity
CSF activity was measured essentially as described for the
ComX pheromone [Magnuson et al. 1994]. Cells containing the srfA–lacZ1974 fusion [JRL293] were grown for at least three doublings to an OD600 of ~0.1. Cells [0.25 ml] were mixed with 0.25 ml of the sample to be assayed, with 50 μg/ml of BSA to prevent nonspecific loss of activity [Magnuson et al. 1994], in a 2.2-ml plastic tube (Marsh Biomedical), incubated at 37°C for 70 min, and assayed for β-galactosidase specific activity. Samples to be assayed typically included conditioned medium, fresh medium, and column fractions diluted into fresh medium. Induced β-galactosidase specific activity is that induced by a given sample, above the background specific activity from cells incubated with fresh medium. The response to CSF was linear only over a small concentration range and assays were typically done on a series of twofold dilutions. The greatest dilution that gave an induced specific activity approximately twofold above background (fresh medium) was used to calculate the units of CSF per milliliter of conditioned medium. One unit of CSF activity is defined as the amount needed to induce expression of the srfA–lacZ1974 fusion in strain JRL293 to an activity of one β-galactosidase specific activity unit above the background of untreated cells [fresh medium] in 70 min.

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