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 (*spo0H*, *spo0A*, *spo0B*, and *spo0F*). 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 *spo0K*. Furthermore, the cellular response to partly purified ComX pheromone requires the ComP histidine protein kinase, whereas the response to partly purified CSF requires the Spo0K 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 (Clewett 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 pher-

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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

Figure 1. Model for the production of and response to the two extracellular competence factors. The cell membrane is shown with the two competence pheromones, ComX pheromone and CSF, outside the cell. ComX is the precursor for the peptide portion of ComX pheromone, and ComQ is required for production (presumably processing or modification) of ComX pheromone (Magnuson et al. 1994). Normal production of CSF requires the alternate σ factor encoded by *spo0H*. Other *spo0* genes affect expression of CSF by inactivating a negative regulator of CSF production, *abrB* (Table 1). Both competence factors stimulate expression of *srfA*, which is part of a network of regulators that lead to competence development. The ComP histidine protein kinase, with eight putative membrane-spanning domains (Weinrauch et al. 1990), is required for detection of ComX pheromone but not for detection of CSF. Phosphate from ComP~P is probably transferred to and activates the ComA transcription factor, which acts directly at the *srfA* promoter (Roggiani and Dubnau 1993). The Spo0K oligopeptide permease is essential for detection of CSF but not for detection of ComX pheromone. (Spo0K is not required for production of either CSF or ComX pheromone). We hypothesize that Spo0K and CSF affect transcription of *srfA* by modulation of the levels of ComA~P, by activating another kinase, by inhibiting a phosphatase, or by interacting directly with ComA.

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 *spo0K* (Perego et al. 1991; Rudner et al. 1991) was found to be required for sensing CSF. Spo0K 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 ($\sim 2 \times 10^7$ 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 $\sim 30\%$ 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*

Figure 2. Expression of *srfA-lacZ* and *comG-lacZ* in wild type and a *spo0H* 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-lacZ* Δ 1974 wild-type); triangles indicate JMS139 (*srfA-lacZ* Δ 1974 *spo0H::cat*). (B,D) Circles indicate AG1046 (*comG-lacZ* wild type); triangles indicate JMS128 (*comG-lacZ spo0H::cat*). Note that the scales on the y-axis are different in each panel.

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 *spo0H* mutant also substantially restored expression of *comG-lacZ* (Fig. 2D).

Extracellular rescue of the *spo0H* mutant was also demonstrated in cell-mixing experiments. *spo0H* mutant cells containing the *comG-lacZ* fusion were grown in mixed culture with either wild-type or *spo0H* cells, without a *lacZ* fusion, at a ratio of $\sim 1:1$. Expression of *comG-lacZ* in the *spo0H* mutant was restored to near wild-type levels when mixed with wild-type cells (data not shown).

The decreased transformation frequency of the *spo0H* mutant was partially rescued extracellularly. Addition of conditioned medium increased the transformation frequency of the *spo0H* mutant by 5- to 20-fold. This rescue was never up to wild-type levels of transformation and was usually $\sim 10\%$ of wild-type. Thus, the *spo0H* 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 *spo0H* 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 *spo0H* mutation was rescued extracellularly, it seemed likely that the *spo0H* mutant (and possibly other *spo0* mutants) was defective in the production of an extracellular competence factor. While purifying the ComX pheromone from conditioned medium, we had noticed a second chro-

matographically distinct factor that stimulates expression of *srfA-lacZ* two- to threefold (Magnuson et al. 1994). The *spo0H* mutant was defective in production of this second factor. We fractionated conditioned medium from *spo0H* 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 $\sim 50\%$ – 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 *spo0H* 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 *spo0H* mutant, is sufficient to cause a defect or delay in expression of *srfA* and the development of competence.

In addition to *spo0H*, 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

Table 1. Production of CSF and the ComX pheromone in different mutants

Strain	Relevant genotype	Percent production ^a	
		CSF	ComX pheromone
JH642	wild type	100	100
AG665	<i>spo0H::cat</i>	9.5	68
AG503	Δ <i>spo0A475::cat</i>	3.2	129
AG141 (JH648)	<i>spo0B136</i>	19	110
AG144 (JH649)	<i>spo0F221</i>	23	110
AG132	<i>spo0A204 abrB703</i>	124	167

^aThe 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).

spo0A, *spo0B*, and *spo0F* were found to be required for normal production of CSF (Table 1). *spo0A* 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 *spo0F* and *spo0B* 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 *spo0A* 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 *spo0A*, *spo0F*, and *spo0B* 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 *spo0A*, *spo0B*, and *spo0F* was not fully relieved by the addition of conditioned medium (data not shown), in contrast to results with *spo0H* (above). These findings are consistent with the requirement for *spo0A* 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.

spo0K 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

Figure 3. HPLC reverse-phase chromatography of CSF. CSF was purified from conditioned medium and assayed as described in Materials and methods. A C-18 reverse phase column was eluted with a gradient of acetonitrile (0.04%/min), from 0% to 10% in 0.1% TFA. Milliabsorbance at 220 nm is indicated at left; CSF activity is indicated at right (●).

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 $\sim 5\%$ and $\sim 2\%$, 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).

Figure 4. *comP* and *spo0K* are on different pathways for the activation of *srfA* transcription. Isogenic strains containing the *srfA-lacZ*Ω374 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-lacZ*Ω374 wild type); (○) JMS423 (*srfA-lacZ*Ω374 *comP::cat*); (Δ) JMS384 (*srfA-lacZ*Ω374 Δ*spo0K::erm*). (B) *srfA* expression in the *comP spo0K* double mutant is lower than in *spo0K* or *comP* single mutants. (○) JMS423 (*srfA-lacZ* *comP::cat*); (Δ) JMS384 (*srfA-lacZ*Ω374 Δ*spo0K::erm*); (□) JMS425 (*srfA-lacZ*Ω374 *comP::cat* Δ*spo0K::erm*); (▲) ROM306 (*srfA-lacZ*Ω374 *comA::cat*).

Expression of *srfA* in the *comP spo0K* double mutant was significantly lower than that in either single mutant, $\sim 0.1\%$ of wild type and barely above background, and was similar to that in a *comA* null mutant (Fig. 4B). These results indicate that the Spo0K 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 Spo0K 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 Spo0K 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, when production 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.

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

Figure 5. Effects of double mutant combinations on expression of *srfA*. Isogenic strains containing the *srfA-lacZ*374 fusion were grown as described in Fig. 4. (A) *comP* is on the same genetic pathway as *comQ* but on a different pathway than *spo0H*. (○) JMS423 (*comP::cat*); (□) ROM294 (*comP::cat comQ::spc*); (●) JMS478 (*spo0H::cat comP::spc*). (B) *spo0K* is on the same genetic pathway as *spo0H* but on a different pathway than *comQ*. (△) JMS384 (*spo0K::erm*); (●) JMS433 (*spo0K::erm spo0H::cat*); (■) JMS426 (*spo0K::erm comQ::spc*). (C) *comQ* and *spo0H* are on different genetic pathways for the activation of *srfA* expression. (●) JMS441 (*spo0H::cat*); (□) JMS424 (*comQ::spc*); (■) JMS477 (*comQ::spc spo0H::cat*).

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 Spo0K oligopeptide permease responded well to ComX pheromone, suggesting that Spo0K is not essential for detection of this factor (Fig. 6A). Spo0K, however, was needed for the sensing of CSF. Cells lacking the Spo0K oligopeptide permease were unable to in-

duce 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. *comP* (JRL177), *spo0K* (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 *spo0K 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 *comA*). These results are most consistent with the notion that a critical threshold level of expression of *srfA* must be

Figure 6. Response of *spo0K* and *comP* mutant cells to partly purified ComX pheromone and partly purified CSF. Strains JMS384 (*srfA-lacZ*374 Δ *spo0K::erm*) and JMS423 (*srfA-lacZ*374 *comP::cat*) were grown in defined minimal medium allowing at least three doublings before the start of the experiment. At an optical density (600 nm) of ~0.1, cells were diluted 1:1 into fresh medium, or fresh medium plus partly purified ComX pheromone or CSF. All samples contained 50 μ g/ml of bovine serum albumin to prevent the factors from adhering to the glass. The samples were incubated at 37°C in flasks in shaking water baths. Aliquots were taken for determination of β -galactosidase specific activity 45, 90, and 135 min after dilution. (A) Response to ComX pheromone requires ComP but not Spo0K. Data from the 45-min time point is shown. (– and +) The absence and presence of ComX pheromone. (B) Response to CSF requires Spo0K but not ComP. Data from the 90-min time point is shown. (– and +) The absence and presence of CSF.

reached to activate the next step in competence development.

In addition, the residual expression of *srfA* in the *comP* and *spo0K* 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 spo0K*) 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 *spo0H*, *spo0A*, *spo0B*, and *spo0F* mutants.

The oligopeptide permease encoded by *spo0K* 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 Spo0K 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 *spo0K* in competence development (Weinrauch et al. 1990; Dubnau 1993), indicating that ComA is downstream of both the ComP histidine protein kinase and the Spo0K 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).

Spo0K 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 Spo0K in competence (Koide 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. Spo0K 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). Spo0K 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 Spo0K 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 Spo0K 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 (Clewett 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 Kimmelmann 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 gonorrhoea*), 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

glutamine, which inhibits competence. In this way, 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* (Burbulys 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. Mu-

tant alleles used include *comP::cat* (Weinrauch et al. 1990), *comQ::spc* (Magnuson et al. 1994), *comA::cat* (D. Dubnau, Public Health Research Institute, NY), *spo0H::cat* (Jaacks et al. 1989), Δ *spo0K358::erm* (LeDeaux and Grossman 1995), and Δ *spo0A475::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-lacZ* Ω 1974 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-lacZ* Ω 374 *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 μ g/ml) as needed. SpII competence medium (Dubnau and Davidoff-Abelson 1971) was used in some experiments, except that CaCl_2 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 ($\Delta A_{420}/\text{min}$ per milliliter of culture per OD_{600}) $\times 1000$.

Competence assays

Cells were grown in defined minimal medium (or SpII), and the transformation frequency was determined by mixing cells with

Table 2. *Strains used*

Strain	Genotype or description
JH642 (AG174)	<i>trpC2 pheA1</i>
AG132	<i>spo0A204 abrB703</i> (Grossman and Losick 1988)
AG141 (JH648)	<i>spo0B136</i> (Piggot and Coote 1976)
AG144 (JH649)	<i>spo0F221</i> (Piggot and Coote 1976)
AG503	$\Delta spo0A475::cat$ (Grossman et al. 1992)
AG665	<i>spo0H::cat</i> (Jaacks et al. 1989)
AG1046 (JMS107)	<i>amyE::(comG-lacZ neo)</i> (Magnuson et al. 1994; Siranosian and Grossman 1994)
JMS128	<i>amyE::(comG-lacZ neo) spo0H::cat</i>
JMS139	<i>amyE::(srfA-lacZΩ1974 cat::spc) spo0H::cat</i>
JMS374	<i>amyE::(srfA-lacZΩ374 neo) fusion 374</i>
JMS384	$\Delta spo0K::erm$ <i>amyE::(srfA-lacZΩ374 neo)</i>
JMS423	<i>comP::cat amyE::(srfA-lacZΩ374 neo)</i>
JMS424	<i>comQ::spc amyE::(srfA-lacZΩ374 neo)</i>
JMS425	$\Delta spo0K::erm$ <i>comP::cat amyE::(srfA-lacZΩ374 neo)</i>
JMS426	<i>comQ::spc $\Delta spo0K::erm$ amyE::(srfA-lacZΩ374 neo)</i>
JMS433	<i>spo0H::cat $\Delta spo0K::erm$ amyE::(srfA-lacZΩ374 neo)</i>
JMS441	<i>spo0H::cat amyE::(srfA-lacZΩ374 neo)</i>
JMS477	<i>spo0H::cat comQ::spc amyE::(srfA-lacZΩ374 neo)</i>
JMS478	<i>spo0H::cat comP::spc amyE::(srfA-lacZΩ374 neo)</i>
JRL177	<i>comP::cat</i>
JRL293 (JMS108)	<i>amyE::(srfA-lacZΩ1974 cat)</i>
JRL358 (JMS122)	$\Delta spo0K358::erm$ (LeDeaux and Grossman 1995)
ROM77 (JMS117)	<i>srfA::pRO106 (cat)</i> plasmid pRO106 integrated, disrupting <i>srfA</i>
ROM294	<i>comP::cat comQ::spc amyE::(srfA-lacZΩ374 neo)</i>
ROM297	<i>comQ::spc</i>
ROM302	<i>comQ::spc spo0H::cat</i>
ROM306	<i>comA::cat amyE::(srfA-lacZΩ374 neo)</i>

chromosomal DNA ($\sim 1\mu\text{g/ml}$) 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 $\sim 5 \times 10^{-5}$ 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 10–20 ml of conditioned medium was adjusted to pH 2 with TFA and applied to a 0.3-gram Sep-pak cartridge. CSF was eluted in 10% acetonitrile (0.1% TFA), whereas the ComX pheromone was eluted in 60% acetonitrile (0.1% TFA). Before elution of the ComX pheromone, the column was washed with 20% and 30% acetonitrile. Samples were typically stored frozen at -20°C . Before use, samples were thawed at room temperature and dried down completely in a Speedvac concentrator, resuspended in fresh minimal medium for physiological assays, and stored at -20°C .

Purification of CSF

CSF was purified from conditioned medium made from strain ROM186 (prototroph, $\Delta spo0K357::neo$ *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 10-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 ($\sim 0.2\%$ /min) in 0.1% TFA. Active fractions were pooled and rerun under similar conditions. Finally, active fractions were pooled and rerun, this time eluting with a very shallow gradient of acetonitrile (0.04%/min). Under these conditions, CSF elutes at $<5\%$ acetonitrile. After each step in the purification, active fractions were concentrated by roto-vap and/or Speedvac as necessary. Concentrated fractions were resuspended in buffers appropriate for the next step in the purification, and the pH was adjusted as necessary.

Assay of CSF activity

CSF activity was measured essentially as described for the

ComX pheromone (Magnuson et al. 1994). Cells containing the *srfA-lacZ*Ω1974 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-lacZ*Ω1974 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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