CSF, a Species-Specific Extracellular Signaling Peptide for Communication among Strains of *Bacillus subtilis* and *Bacillus mojavensis*[▽]

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ComX and CSF are *Bacillus subtilis* extracellular signaling peptides. Many different strains of *B. subtilis* do not communicate due to strain-specific variation of ComX. We demonstrate that CSF is a species-specific signaling molecule that partially compensates for the lack of ComX-mediated communication between different strains of *B. subtilis*.

Quorum sensing is the ability of cells to sense and respond to a high cell density. The gram-positive bacterium *Bacillus subtilis* may use as many as five secreted peptide signals to mediate quorum sensing and activate the transcription factor ComA (4, 8, 18). ComA is part of a two-component regulatory system and is activated by phosphorylation via the ComP histidine-protein kinase (11, 30). ComX pheromone is an extracellular signaling peptide that activates the phosphorylation of ComP (20, 27, 28). In addition to ComX, the CSF, PhrF, PhrK, and possibly PhrH peptides also stimulate ComA activity by first being transported into the cell by an oligopeptide permease (4, 19). In the cytoplasm, they bind to their cognate Rap protein to antagonize the inhibitory activity of the Rap proteins on ComA (8, 11). CSF is also able to inhibit ComA-controlled gene expression when present at high concentrations through an incompletely defined mechanism (19).

Knowing that five extracellular signaling peptides stimulate ComA activity raises the question of whether the different signaling peptides provide different information for *B. subtilis*. Genes activated by ComA include those involved in extracellular and membrane functions and regulators of development, including genetic competence and sporulation (10). At least two of these peptides, ComX and CSF, by virtue of their secretion into the growth medium, activate these processes in response to high cell density (5, 17, 20, 26). ComX also appears to signal whether there are other *B. subtilis* organisms of the same strain present in the environment. Genetic polymorphisms were found for different strains of *B. subtilis* and its close relative, *Bacillus mojavensis*, in genes involved in producing and responding to the ComX pheromone, *comQXP* (1, 2, 28, 29). Each strain produces a ComX that specifically activates ComA-controlled gene expression in that strain; however, in some cases, a limited set of pheromones from other strains partially activate or inhibit ComA-controlled gene

expression (2, 28). Since ComX is a strain-specific signaling molecule, this raised the question of whether other ComA-activating signal peptides exhibited strain-specific variation. Here, we show that CSF is a conserved signaling peptide produced by *B. subtilis* and other closely related species and that CSF can partially compensate for the lack of ComX pheromone communication between strains.

Conservation of *rapC-phrC* from *Bacillus* strains producing different ComX pheromones. To determine the CSF phenotypes of *B. subtilis* and *B. mojavensis* strains, we sequenced the *rapC-phrC* operon, encoding CSF and its cytoplasmic receptor RapC, from six strains previously shown to have different *comQXP* sequences (28) (Table 1). Chromosomal DNA was isolated from *B. subtilis* strains RO-OO-2, RO-FF-1, and RS-B-1 and *B. mojavensis* strains RO-B-2, RO-H-1, and RO-C-2 by using a standard protocol (9). The *rapC-phrC* locus was then PCR amplified using *Taq* polymerase (Qiagen) and primers that corresponded to the sequence of the *B. subtilis* 168 strain. For each strain, three independent PCR products were sequenced from two independent chromosomal DNA preparations.

Sequencing data showed that the *rapC-phrC* regions were greater than 95% and 97% identical between strains of *B. subtilis* and *B. mojavensis*, respectively. The *rapC-phrC* region showed the greatest difference between strains of *B. subtilis* and *B. mojavensis*, with a range of 89 to 90% identity. In contrast, the hypervariable region of *comQXP* from these strains showed ~70% identity (2, 28). None of the differences were predicted either to affect the function of *rapC-phrC* (Fig. 1) or to change the region corresponding to the ComA-binding box in the promoter region of *rapC*, which suggests that this promoter would be similarly regulated in these strains. Most of the amino acid differences in RapC and PhrC across the strains were conservative and would not be predicted to affect the function of these proteins. In particular, the sequence of the mature CSF peptide was conserved among all of the strains (Fig. 1B), and all of the PhrC protein variants were predicted by the SignalP 3.0 program to have a functional signal sequence and signal peptidase cleavage sites (7). These data suggest that for strains that produce different ComX pheromones, they all produce identical CSF peptides.

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TABLE 1. Strains used in this study

Species and strain[a]	Relevant genotype[b]	Source or construction[c]
B. subtilis JH642 derivatives		
B. subtilis BAL188 (JRL293)	*amyE*::(*srfA-lacZ*Ω1974 *cat*)	27
B. subtilis BAL201 (AG1520)	Δ*comQ*::*spc*	20
B. subtilis BAL218 (JH642)	*trpC2 pheA1*	22
B. subtilis BAL941	Δ*phrC*::*tet*	16
B. subtilis BAL1141	*thrC*::(*xylAp-xylR erm*)	pRDC19→BAL218
B. subtilis BAL1385	*thrC*::(*comQXP*$_{JH642}$ *erm*)	pBL58→BAL218
B. subtilis BAL1386	*thrC*::(*comQXP*$_{RS-B-1}$ *erm*)	pBL220→BAL218
B. subtilis BAL1391	*thrC*::(*comQXP*$_{JH642}$ *erm*) Δ*comQ*::*spc*	BAL201→BAL1385
B. subtilis BAL1392	*thrC*::(*comQXP*$_{RS-B-1}$ *erm*) Δ*comQ*::*spc*	BAL201→BAL1386
B. subtilis BAL1397	*thrC*::(*xylAp-xylR erm*) Δ*comQ*::*spc*	BAL201→BAL1141
B. subtilis BAL1416	*amyE*::(*srfA-lacZ*Ω1974 *cat*) Δ*phrC*::*tet*	BAL941→BAL188
B. subtilis BAL1419	*thrC*::(*comQXP*$_{RS-B-1}$ *erm*) Δ*comQ*::*spc* Δ*phrC*::*tet*	BAL941→BAL1392
Non-*B. subtilis* JH642 derivatives		
B. mojavensis RO-B-2	Wild type	23
B. mojavensis RO-C-2	Wild type	23
B. subtilis RO-FF-1	Wild type	23
B. mojavensis RO-H-1	Wild type	23
B. subtilis RO-OO-2	Wild type	23
B. subtilis RS-B-1	Wild type	23
B. subtilis BAL1399	As RO-OO-2, except Δ*phrC*::*tet*	BAL941→RO-OO-2
B. subtilis BAL1400	As RS-B-1, except Δ*phrC*::*tet*	BAL941→RS-B-1
B. subtilis BAL1404	As RO-FF-1, except Δ*phrC*::*tet*	BAL941→RO-FF-1

[a] Names in parentheses are original laboratory strain names.
[b] All JH642 derivatives contain *trpC2* and *pheA1* mutations.
[c] The direction of the strain construction is indicated as DNA→recipient strain.

B. subtilis strains secrete a functional CSF peptide. To confirm that different *B. subtilis* strains secrete a functional CSF peptide, we asked whether three wild *B. subtilis* strains could secrete a factor that would induce ComA-controlled gene expression in the laboratory strain of *B. subtilis*. As the wild strains do not produce a form of ComX pheromone which the laboratory strain can sense and respond to, any ComA-activating factor secreted by the wild strains should be CSF or another Phr peptide. *B. subtilis* strains RO-OO-2, RO-FF-1, and

RS-B-1 were grown under standard conditions for the production of CSF by the laboratory strain (16, 17, 26), and then cell-free culture supernatants were harvested. These culture supernatants were tested for CSF activity using a standard CSF detection assay (16, 17, 26) in which dilutions of the culture supernatants were incubated with cells from the reporter strain BAL188 (*srfA-lacZ*) and then assayed for β-galactosidase specific activity (21). The *srfA-lacZ* fusion in the reporter strain is a fusion of the promoter region of the ComA-activated *srfA* gene to the gene for β-galactosidase (27). Culture supernatants from strains RO-OO-2, RO-FF-1, and RS-B-1 induced the expression of *srfA-lacZ* (Fig. 2). It is curious that the culture supernatant from strain RS-B-1 maximally induced *srfA-lacZ* expression 2.5-fold, whereas the other tested strains maximally induced *srfA-lacZ* expression 5.0-fold. The reason for this difference is most likely due to ComX of strain RS-B-1 (ComX$_{RS-B-1}$) inhibiting ComA-controlled gene expression by the laboratory strain, as ComX$_{RS-B-1}$ is very similar in sequence to ComX$_{RO-H-1}$, which has been shown to inhibit ComA-controlled gene expression by the laboratory strain (2). Furthermore, the slopes of the dose-response curves of the different strains are identical and differ only in the induction level at which they plateau, suggesting that that these strains produce similar levels of the stimulating factor, either CSF or another Phr peptide.

To demonstrate that the factor in the culture supernatants that was able to induce *srfA-lacZ* expression in the laboratory strain was CSF, a Δ*phrC* mutation was introduced into the RO-OO-2, RO-FF-1, and RS-B-1 strains. Genetically competent cells of these strains were prepared by growing the strains in a defined minimal medium (14) to an optical density at 600 nm (OD$_{600}$) of 2 to 3; these cells were then transformed with chromosomal DNA from BAL941 (Δ*phrC*::*tet*), using standard
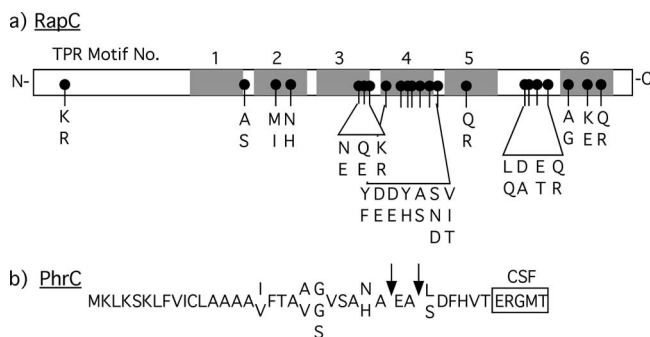

FIG. 1. Locations of amino acid differences in the RapC and PhrC proteins of *B. subtilis* and *B. mojavensis*. (a) Black dots show the locations of amino acid differences. Below these dots are the amino acids found at these positions. The positions of these substitutions are shown relative to the known structural feature of RapC. (b) Linear amino acid sequence of PhrC. Where there are amino acid differences, two or three amino acids are listed. The amino acid shown above the linear sequence is that found in *B. subtilis* strains. The amino acids shown below the linear sequence are those found in *B. mojavensis* strains. Arrows indicate putative signal peptidase cleavage sites as determined by SignalP 3.0 (7). The sequence of the mature CSF peptide is boxed.
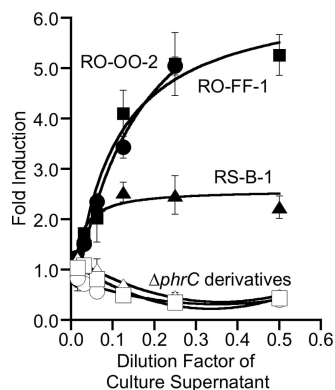
FIG. 2. Wild-type isolates of *B. subtilis* secrete CSF. Culture supernatants from *B. subtilis* wild isolates and their Δ*phrC* derivatives were tested for the ability to induce ComA-controlled gene expression in the BAL188 (JH642 *srfA-lacZ*) reporter strain. The increase in induction was calculated as the level of β-galactosidase specific activity for cells treated with culture supernatant divided by the level of activity for untreated cells. Plotted is the average increase in induction from three independent experiments with the standard error of the mean. Filled and open symbols correspond to results for culture supernatants from strains that either have the gene for CSF or do not (Δ*phrC*), respectively. Conditioned medium was from the following *B. subtilis* strains: RO-OO-2 (circles), RO-FF-1 (squares), and RS-B-1 (triangles).
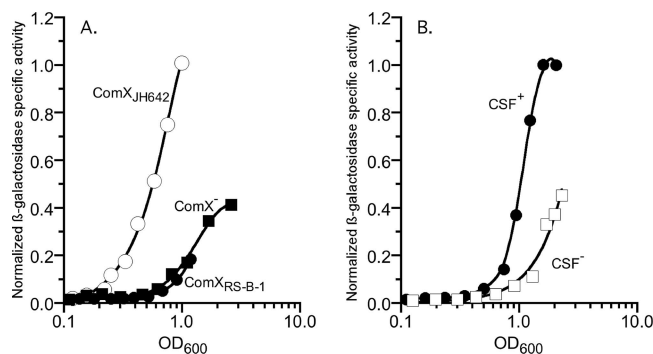

FIG. 3. CSF partially compensates for the lack of ComX pheromone communication and leads to the induction of ComA-controlled gene expression at a lower cell density. Mixed cultures in which 10% of the cells were of a reporter strain that had a *srfA-lacZ* fusion and 90% were of a neighbor strain producing a different complement of extracellular signaling peptides were grown. β-Galactosidase specific activity measurements were normalized by dividing the level of β-galactosidase specific activity for each point by the highest level of β-galactosidase specific activity obtained for the group of strains assayed in one experiment. The graphs shown are representative of three independent experiments. (A) For all three cultures, all cells produced CSF, and BAL188 (*srfA-lacZ* ComX$_{JH642}$) was the reporter strain. The neighbor strain either produced ComX$_{JH642}$ (BAL1391) (open circles), ComX$_{RS-B-1}$ (BAL139) (filled circles), or no ComX pheromone (ComX$^-$; BAL1397) (filled squares). (B) BAL1416 (*srfA-lacZ* Δ*phrC* ComX$_{JH642}$) does not produce CSF and was the reporter strain. The neighbor strain produced ComX$_{RS-B-1}$ and was either phenotypically CSF$^+$ (BAL1392) (filled circles) or CSF$^-$ (BAL1419) (open squares).

protocols (12). Culture supernatants from these Δ*phrC* derivatives were assayed, as described above, and were found to be unable to induce *srfA-lacZ* expression (Fig. 2). These data indicate that *phrC*, and by extension CSF, was required for the inducing activity produced by the wild *B. subtilis* strains.

Effect of CSF on the quorum response of cultures of mixed-ComX-pherotype cells. To determine the role of CSF in the context of a mixed community producing different ComX pheromones, we created cultures in which 10% of the cells were of a "reporter strain" that produced and responded to ComX$_{JH642}$ and had a *srfA-lacZ* fusion. The remaining 90% of cells were of a "neighbor strain" that produced ComX$_{JH642}$, ComX$_{RS-B-1}$, or no ComX pheromone. To create isogenic neighbor strains, either the *comQXP* region from JH642 or RS-B-1 or a heterologous gene was introduced into the chromosome at the *thrC* locus. The *comQXP* locus was PCR amplified, from 200 bp upstream of *comQ* to 11 bp downstream of *comP*, and then cloned into the BamHI and EcoRI sites of the *thrC* integration vector pRDC19 (3). The resulting plasmids, pBL58 [*thrC*::(*comQXP*$_{JH642}$ *erm*)], pBL220 [*thrC*::(*comQXP*$_{RS-B-1}$ *erm*)], and pRDC19 [*thrC*::(*xylAp-xylR erm*)], were each transformed into BAL218, and transformants in which *thrC* was replaced with the indicated alleles were selected. To prevent ComX pheromone production from the native locus, the Δ*comQ*::*spc* allele was backcrossed into these strains.

The reporter and neighbor strains were grown separately in defined minimal medium with shaking at 37°C to an OD$_{600}$ of ~0.1, at which point the reporter strain and one of the neighbor strains were mixed. β-Galactosidase specific activity was measured during growth of the mixed culture, and the percentage of the culture that was comprised by each of the two strains was measured at the end of growth as the number of CFU per ml on the appropriate selective agar plates.

When the reporter strain was grown in the presence of a

neighbor strain producing ComX$_{JH642}$, the expression of *srfA-lacZ* was induced at an OD$_{600}$ of ~0.2 (Fig. 3A). In contrast, *srfA-lacZ* expression was induced two cell doublings later when the reporter strain was grown in the presence of a strain that either did not produce ComX (*n* = 3; *P* < 0.01) or produced ComX$_{RS-B-1}$ (*n* = 3; *P* < 0.05) (Fig. 3A). These data confirm that the laboratory strain, JH642, does not respond to the ComX pheromone from the RS-B-1 strain. They further indicate that the inability to sense the presence of cells from other strains of *B. subtilis* via the ComX pheromone delays the expression of ComA-controlled genes when cells are grown in a mixed community.

We next determined what role CSF had in activating ComA-controlled gene expression in a mixed culture that does not communicate by ComX pheromone. To accomplish this, we determined the effect of the presence and absence of CSF in the mixed-culture assay described above. Strains lacking CSF were created by backcrossing the Δ*phrC* mutation into the reporter strain (BAL188) and the neighbor strain expressing ComX$_{RS-B-1}$ (BAL1419) (Table 1). Two mixed cultures were then set up, in which the reporter strain, which cannot produce CSF (BAL1416), was grown with either a neighbor strain that expressed *comQXP*$_{RS-B-1}$ and CSF (BAL1392) or a neighbor strain that expressed *comQXP*$_{RS-B-1}$ but not CSF (BAL1419) (Fig. 3B). For the culture in which CSF was not produced, *srfA-lacZ* expression was induced at 0.5 cell doublings later than it was induced in the culture that produced CSF (*n* = 3; *P* < 0.02). These data indicate that CSF mediates communication between different ComX pherotype strains and leads to the induction of ComA-controlled gene expression at a lower cell density than can be achieved without CSF. CSF only par-

tially compensated for the lack of ComX pheromone communication, as ComA-controlled gene expression was delayed when strains of different ComX phenotypes were grown together, even in the presence of CSF.

Conclusions and implications. The reason that *B. subtilis* produces multiple signaling molecules that converge to regulate the quorum response is unclear. In light of the strain specificity exhibited by ComX pheromone, we set out to determine whether the CSF signaling molecule was also strain specific. Our data indicate that CSF is not; it is a conserved signaling molecule produced by strains of *B. subtilis* and the closely related species *B. mojavensis*. Furthermore, CSF can mediate communication between strains that are unable to communicate via ComX. This is the first evidence that a bacterium that exhibits strain level specificity for a secreted signaling molecule produces an additional signaling molecule to allow communication between strains. Strain-specific variation has also been observed for the extracellular signaling peptides that induce genetic competence in *Streptococcus pneumoniae* (31) and virulence gene expression in *Staphylococcus aureus* (15) and *Bacillus cereus* (24), and it will be interesting to learn whether these bacteria also produce a species-specific signaling molecule, as does *B. subtilis*.

It is unknown why bacteria secrete multiple signaling molecules that differ in the ability to communicate the relatedness of the surrounding bacteria. In addition to the fact that *B. subtilis* produces strain- and species-specific signals, *Vibrio harveyi* produces species- and genus-specific signals, as well as a universal signal (6, 13, 32). The fact that bacteria as distantly related as *V. harveyi* and *B. subtilis* exhibit this phenomenon suggests that this may be a common property of signaling molecules produced by many bacterial species.

To understand why *B. subtilis* produces both strain- and species-specific signaling molecules, it will be necessary to discern the selective pressure that has led to the strain level of variation in ComX pheromone. Since ComX pheromone is required for the development of genetic competence (i.e., the natural ability to take up DNA), one consequence of ComX pheromone variation is sexual isolation from different strains (25). Therefore, it has been proposed that by *B. subtilis* cells responding only to ComX pheromone from genetically identical clones, these cells avoid the uptake of DNA from closely related, but not genetically identical, strains that could carry harmful genetic elements (1). If sexual isolation is the fitness-enhancing factor that has selected for the diversification of the ComX pheromone, then what advantage does communication via CSF provide for strains of different ComX phenotypes? Perhaps *B. subtilis* has to strike a balance between sexual isolation and cooperation for other processes activated by ComX and CSF through the ComA transcription factor (e.g., surfactin or secreted pectate lyase production) (10). And so, although *B. subtilis* possibly excludes harmful genetic variation via the lack of ComX communication, it can still gain the benefits of cooperation by signaling via the conserved molecule CSF. If CSF accumulates to sufficient levels to be sensed by *B. subtilis* cells, which is dependent on the accumulation of ComX itself (17), the likelihood of the existence of a high density of cooperative cells may be sufficiently great to make induction of the quorum response possibly beneficial. Clearly, there are many experiments yet to be done to understand the potential benefits

offered to a bacterium that can communicate at both the strain and species levels.

Nucleotide sequence accession numbers. The GenBank accession numbers for the obtained sequences are as follows: AY664811 (*B. subtilis* RO-OO-2), AY667584 (*B. subtilis* RO-FF-1), AY667585 (*B. subtilis* RS-B-1), AY667586 (*B. mojavensis* RO-B-2), AY672642 (*B. mojavensis* RO-H-1), and AY672643 (*B. mojavensis* RO-C-2).

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