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Translation of the Open Reading Frame Encoded by *comS*, a Gene of the *srf* Operon, Is Necessary for the Development of Genetic Competence, but Not Surfactin Biosynthesis, in *Bacillus subtilis*

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A small open reading frame, *comS* of the *srf* operon, is the site of mutations that impair competence development in *Bacillus subtilis*. *comS* open reading frame translation was required for competence, as was confirmed by the suppression of a *comS* amber mutation [*comS*(Am)] by the nonsense suppressor *sup-3*. *comS*(Am), when introduced into the *srf* operon, eliminated late competence gene expression but had no significant effect on surfactin production.

The development of genetic competence in the spore-forming bacterium *Bacillus subtilis* is promoted in glucose-ammonium-phosphate medium when cultures reach a high cell density. The isolation of *com* mutants having reduced transformation efficiency has resulted in the identification of several genes that function to regulate competence establishment or function more directly in DNA internalization (6). Studies of the *com* gene products have uncovered a complex signal transduction network controlling the development of competence (6). In the initiation of competence, the pheromone ComX accumulates as culture cell density increases (21) and is sensed by the ComP-ComA signal transduction system (8). ComP, a membrane protein of the sensor class of the two-component regulatory proteins (42), is thought to undergo autophosphorylation in response to ComX and then to facilitate the phosphorylation of ComA, a transcriptional activator (31, 41). Phosphorylation of ComA is also stimulated by a mechanism that depends upon another extracellular factor, Csf, the production of which is sensitive to the nutritional environment and is regulated by the Spo0 phosphorelay (35). The function of the Spo0 system is to overcome the negative control of Csf production imparted by the transition state regulator AbrB (36). Csf-dependent activation of ComA is ComP independent and involves the peptide transport complex coded for by the *spo0K* operon (30, 33). ComA, thus phosphorylated, is a transcriptional activator of the *srf* operon (formerly *srfA*; also called *comL*) (3, 13, 25–28, 31, 39).

The *srf* operon is required for the nonribosomal biosynthesis of the lipopeptide antibiotic surfactin (26) but also serves a regulatory role in the development of competence (8, 25, 37, 39). The *srf* operon (Fig. 1), composed of the four genes *srfA*, *srfB*, *srfC*, and *srfD* (formerly *srfAA*, *srfAB*, *srfAC*, and *srfAD*, respectively) (3, 9), encodes the surfactin synthetase subunits, each containing the amino acid-activating domains necessary for the activation of the surfactin substrate amino acids (3, 9, 10, 22, 37, 40). The peptide synthetic capacity of *srf* is dispensable with respect to competence development (4). Instead, a small gene called *comS*, a 46-codon open reading frame (ORF) nested within and out of frame with *srfB*, is the *srf*-associated competence gene (5, 15). In this paper, we present evidence that the *comS* ORF is translated in *B. subtilis* and that the translation is necessary for competence development but not for surfactin production.

Strains used for this study are listed in Table 1.

To determine if the *comS* ORF is expressed in *B. subtilis*, translational *comS′*::*′lacZ* fusions were constructed. Plasmid pZΔ329 (Fig. 2A) bearing a promoterless *lacZ* gene was first digested with *Hin*dIII, the ends were rendered flush with mung bean nuclease, and this was followed by *Eco*RI digestion. A 2.5-kb pCD95 *Eco*RI-*Hpa*I fragment bearing the *cat* gene, the *srf* promoter, and the putative *cis*-acting translational sequence elements of *comS*, namely, the Shine-Dalgarno sequence and the TTG codon, was inserted into pZΔ329 to yield the plasmid pCD96 (Cm^r) (Fig. 2A). This resulted in the fusion of the TTG start codon of *comS* to the 16th codon of *lacZ*, with 8 codons (LACLQVDR) between *comS* and *lacZ*, as was verified by the sequencing of the fusion junction. pCD96 was introduced into *B. subtilis* JH642 by transformation (7, 29) with selection for Cm^r, resulting in integration by Campbell recombination into the P*srf* sequence and the *srfB* gene to create LAB1771 and LAB1876, respectively. To further examine the expression of *comS* in its normal context within *srfB*, PCR products from *srfB* DNA were used to create *comS′*::*′lacZ* and *srfB′*::*′lacZ* translational fusions. The oligonucleotides used for PCR were OL16, 5′-GGCGAGAAAGCTTGTCG AACAT-3′, and OL17, 5′-GCTGATAAGAAGCTTGCCTG AT-3′. The PCR (VENT DNA polymerase; New England BioLabs, Inc., Beverly, Mass.) with plasmid pCD65 (bearing a 3.9-kb *Cla*I fragment of the *srfB* gene) as the template generated a 1.7-kb fragment of *srfB* bearing the N-terminal portion of the *comS* ORF. This fragment was digested with *Hin*dIII and inserted into *Hin*dIII-cleaved pZΔ329, placing the 6th codon of *comS* (Lys) in frame with the 16th codon of *lacZ* to give plasmid pCD103 (Fig. 2B). The *srfB′*::*′lacZ* translational fusion was constructed by digesting pCD103 with *Sal*I, treating the digestion with mung bean nuclease, and religating the plasmid to yield plasmid pCD108. Plasmids pCD103 and pCD108 were used to transform competent cells of OKB105 (Srf^+). DNA was isolated from Srf^- transformants that arose as a result of the integration of pCD103 or -108 into *srf* and was used to transform competent

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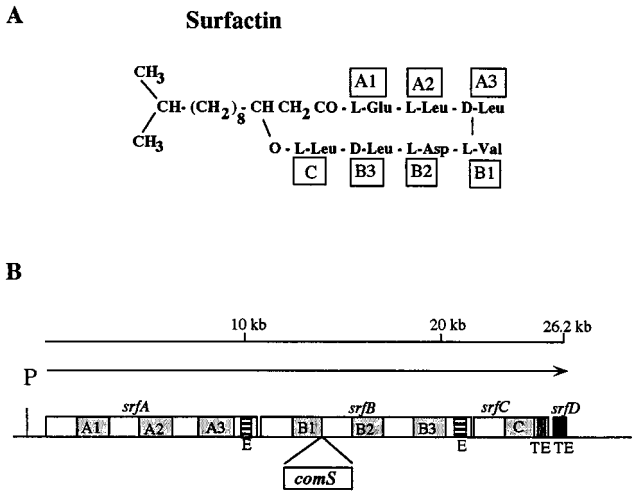

FIG. 1. Surfactin and the organization of the *srf* operon. (A) The structure of the lipopeptide antibiotic surfactin. The amino acid sequence is shown along with the names of the Srf amino acid-activating domains that catalyze the incorporation of each substrate amino acid. (B) The *srf* operon. The locations of the *srfA*, *srfB*, *srfC*, and *srfD* genes are indicated by the open boxes, within which are shown the locations of the amino acid-activating domains indicated by the shaded boxes. E, epimerase domains; TE, location of thioesterase active sites; P, site of the *srf* promoter region. The location of *comS* within *srfB* is indicated.

cells of JH642 with selection for Cm^r to create strains LAB1891 (*srf*::pCD103) and LAB1935 (*srf*::pCD108).

Cells of LAB1771 showed a significant level of β-galactosidase activity, indicating that the 46-amino-acid *comS* ORF was indeed expressed in *B. subtilis* (Fig. 3A). As expected, the expression was dependent on *comP* and *comA* (13, 28) (Fig. 3A). Strain LAB1891 had a fourfold higher β-galactosidase activity than LAB1876 (Fig. 3). This difference could be attributed to the possible differential stabilities of the two different fusion proteins. Similar activity was observed in LAB1876 cells compared with the cells bearing the pCD96 fusion introduced at the *srf* promoter (LAB1771) (Fig. 3A). That *comS′*::*′lacZ* exhibited a similar level of activity whether at the *srf* promoter or in *comS* indicated that the DNA used to construct the fusion of pCD96 contained all the elements necessary for the translation of the ORF coded for by *comS*.

A *srfB*::*lacZ* translational fusion was also examined in order to compare its temporal expression with that of *comS′*::*′lacZ*. Expression of both fusions increased with culture cell density, although LAB1935 cells had an eightfold higher activity than those bearing the *lacZ* fusion to the sixth codon of *comS* (LAB1891) (Fig. 3B). This difference may be due to differential stabilities of the fusion proteins, a difference in the rates of translation of the two genes due to a difference in the translational regulation, or inhibition of *comS* expression by translation through *srfB*.

The experiments discussed above and the mutational analysis of *comS* (5, 15) provide only circumstantial evidence for the involvement of the *comS* ORF in the development of competence. To demonstrate the requirement of the *comS* product for genetic competence and rule out the involvement of a regulatory RNA species, suppression of *comS*(Am) (5) by the ochre suppressor *sup-3* (11) encoding a mutant lysyl-tRNA (24) was examined. Strains bearing plasmid pCD101 (5) containing the *comS*(Am) allele (introduced within the *srf* promoter), the Δ*srf* mutation ($comS^-$), and *comG*::*lacZ* (1) to serve as an indicator of competence development were constructed. *comG* is a late competence operon, the expression of which is dependent on *comS*. The plasmid bearing the *comG-lacZ* fusion had integrated into the 5′ end of *comG* without disrupting the *comG* operon. These strains also bear the *metB* and *thr-5* mutations, which are suppressible by *sup-3*, thus confirming the presence of the suppressor allele. Strain LAB1903 bearing *sup-3* had at least 20- to 38-fold higher levels of competence and *comG-lacZ* expression compared with those of LAB1909 bearing the corresponding wild-type allele (Table 2). The activity in LAB1903 was considerably higher (28- to 100-fold) than that in the *sup-3* Δ*srf* strain (LAB1807), suggesting that *comS*(Am) was indeed suppressed (Table 2). The incomplete restoration of competence levels observed as a result of suppression, compared with those of the strain that is wild type with respect to *srf* (*comS*) (LAB1808), can be attributed to the relatively low degree of suppression of the amber mutation by the ochre suppressor.

The *comS*(Am) mutation was introduced into a surfactin-producing strain, OKB105, to determine if it affected surfactin

TABLE 1. *Bacillus subtilis* strains

Strain	Relevant genotype	Reference or source
1A14	*metB5 thr-5 leuB8 sup-3*	11
JH642	*trpC2 pheA1*	J. Hoch
OKB105	*sfp pheA1*	26
LAB1564	*pheA sfp comG*::*lacZ* (Campbell) Δ*srf*::*phleo*	5
LAB1567	*comG*::*lacZ* (Campbell, Spc^r) SPβ*c2del2*::Tn*917*::pSK10Δ6	This study
LAB1771	*trpC2 pheA1* P*srf*::pCD96	This study
LAB1799	*trpC2 pheA1* P*srf*::pCD96 Δ(*comP comA*)::Tn*917lac* ΩOK167	This study
LAB1807	*metB5 thr-5 leuB8 sup-3* Δ*srf*::pNAC14 ($Phleo^r$) *comG*::*lacZ* (Spc^r)	This study
LAB1808	*metB5 thr-5 leuB8 sup-3 comG*::*lacZ* (Spc^r)	This study
LAB1809	*metB5 thr-5 leuB8* Δ*srf*::pNAC14 ($Phleo^r$) *comG*::*lacZ* (Spc^r)	This study
LAB1876	*trpC2 pheA1 srfB*::pCD96 (Cm^r)	This study
LAB1891	*trpC2 pheA1 srfB*::pCD103 (Cm^r)	This study
LAB1903	*metB5 thr-5 leuB8 sup-3* Δ*srf*::pNAC14 ($Phleo^r$) *srf*::pCD101 [Cm^r, *comS*(Am)] *comG*::*lacZ* (Spc^r)	This study
LAB1909	*metB5 thr-5 leuB8* Δ*srf*::pNAC14 ($Phleo^r$) P*srf*::pCD101 [Cm^r, *comS*(Am)] *comG*::*lacZ* (Spc^r)	This study
LAB1935	*trpC2 pheA1 srfB*::pCD108 (Cm^r)	This study
LAB1974	*pheA1 sfp srf*::pMMN238	This study
LAB1990	*pheA1 sfp comG*::*lacZ* (Spc^r)	This study
LAB2019	*pheA1 sfp comS*(Am)	This study
LAB2020	*pheA1 sfp comS*(Am) *comG*::*lacZ* (Spc^r)	This study

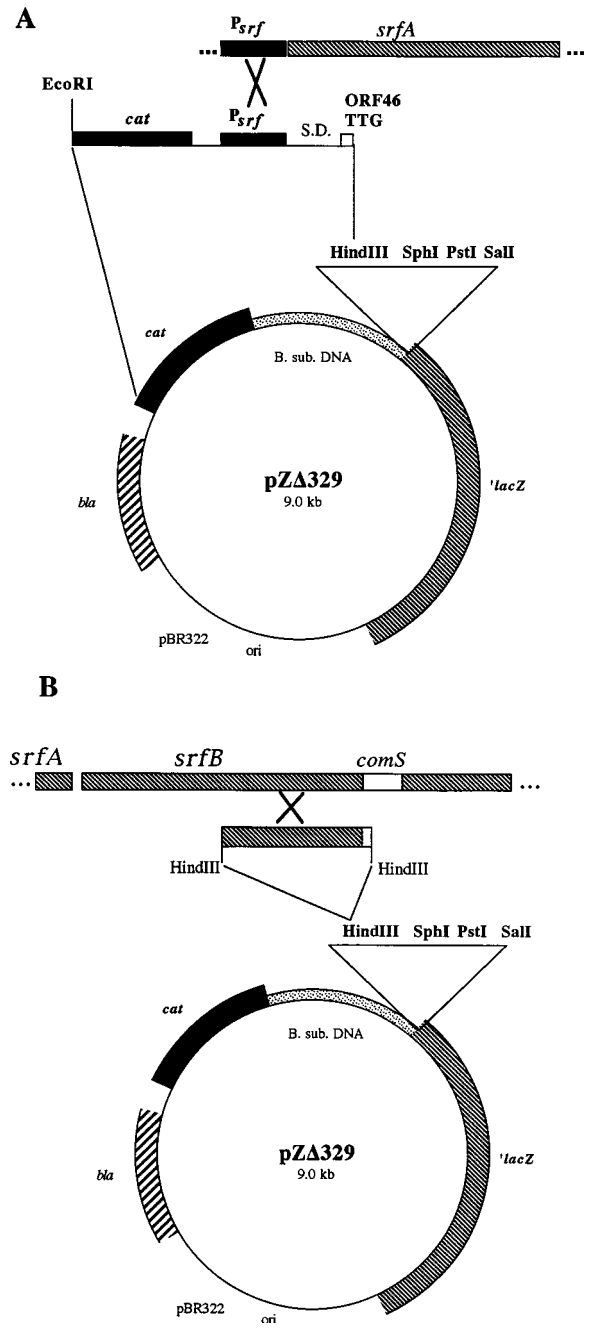

FIG. 2. *comS'::'lacZ* translational fusions. (A) Structure of pCD96 and its integration into the *srf* promoter region. B. sub., *B. subtilis*; S.D., Shine-Dalgarno. (B) Structure of pCD103 and its integration into the *comS* locus in *srfB*.

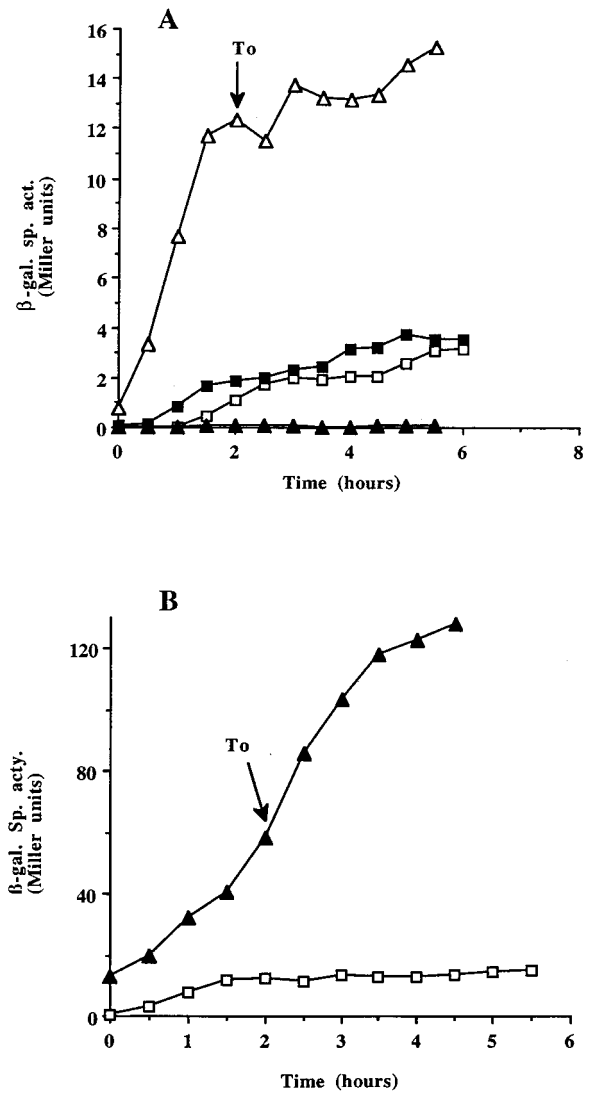

FIG. 3. Expression of *comS'::'lacZ* fusions. Time course of β-galactosidase (β-gal.) production in wild-type *comS'::'lacZ* and Δ(*comP comA*) *comS'::'lacZ* cells. Cultures were grown in one-step competence medium, and 1-ml samples were collected at 30-min intervals. β-Galactosidase specific activity (sp. act.) was determined for each sample (25). (A) Plasmid integrants LAB1876 [*comS'::'lacZ* (*srfB*::pCD96)], ■; LAB1771 [*comS'::'lacZ* (P*srf*::pCD96)], □; LAB1891 [*comS'::'lacZ* (*srfB*::pCD103)], △; and LAB1799 [*comS'::'lacZ* (P*srf*::pCD96) Δ(*comP comA*)], ▲. (B) Plasmid integrants LAB1891 [*comS'::'lacZ* (*srfB*::pCD103)], □, and LAB1935 [*srfB'::'lacZ* (*srfB*::pCD108)], ▲.

production. A 569-bp *Bgl*II-*Hin*dIII fragment of plasmid pCD100, a pUC18 derivative bearing a *comS* amber allele (5), was inserted into pG^+host6 (2), a plasmid that is temperature sensitive for replication in gram-positive bacteria. Plasmid pMMN238, thus created, was used to transform OKB105 with selection for erythromycin resistance (Erm^r). The transformant, LAB1974, was defective in surfactin production, indicating that the plasmid had integrated in the *srf* operon. LAB1974 was grown overnight in 2X YT broth medium (26) supplemented with erythromycin (1 μg/ml) and lincomycin (25 μg/ml) at 37°C. Cells were transferred (10^{-4} dilution) to 2X YT medium and incubated, nonselectively, overnight at 30°C. The cultures were diluted (10^{-3}) in 2X YT and incubated overnight at 37°C. Cells were appropriately diluted and plated onto DSM agar (26). Colonies were screened for Erm^s in order to isolate plasmid-cured segregants. Three Erm^s Srf^+ (LAB 2019) colonies were isolated from two independent cultures. OKB105 and LAB2019 were transduced with PBS1 phage lysate (16, 26) carrying *comG-lacZ* (Spc^r) prepared from LAB1567. Spc^r transductants of OKB105 and LAB2019 (LAB1990 and LAB2020, respectively) were isolated, and chromosomal DNA was prepared for PCR with two oligonucleotides (MN2, 5′-TGAGAGAATTTACCGCACTG-3′, and MN3, 5′-GTCTTC TTCCCGTCCCTTGG-3′) flanking the *comS* mutation site. The PCR products were digested with *Nhe*I, the restriction site generated by the mutation. The product of LAB2020 DNA was

TABLE 2. Suppression of *comS*(Am): competence and *comG*::*lacZ* data

Strain	Relevant genotype	*comG*-directed β-galactosidase sp act (Miller units)	% Transformation (10^4)[a]
LAB1807	Δ*srfA sup-3 comG*::*lacZ*	0.077	<0.048, 0.16
LAB1808	SrfA$^+$ *sup-3 comG*::*lacZ*	22.3	118, 57.9
LAB1903	*sup-3* Δ*srfA* P*srfA*::pCD101 *comS*(Am) *comG*::*lacZ*	2.17	10.1, 16.1
LAB1909	*sup-0* Δ*srfA* P*srfA*::pCD101 *comS*(Am) *comG*::*lacZ*	0.108	<0.063, 0.43

[a] Data are from two experiments.

shown to have an *Nhe*I site, but the one amplified from LAB1990 chromosomal DNA had no *Nhe*I site (data not shown), indicating that LAB2020 has the amber mutation in *comS*.

Competent cells from a wild-type strain (LAB1990), a Δ*srf* strain (LAB1564), and a *comS* amber mutant (LAB2020) were prepared and examined for transformation efficiency and expression of *comG-lacZ*. LAB1564 was impaired in transformation ability, with a transformation frequency less than 10^{-3} of that of the wild type. The transformation frequency of LAB2020 was also very low (10^{-2} of that of the wild type). Likewise, *comG-lacZ* expression was abolished by *comS*(Am), confirming previous work showing that *comS* is required for competence development in *B. subtilis* (5, 15). However, cells of strain LAB2020 showed the surfactin-producing (Srf$^+$) phenotype on DSM agar. Surfactin was purified from the cell-free media of the LAB1564 (Δ*srf*), LAB1990 (wild type), and LAB2020 [*comS*(Am)] cultures. No surfactin was detected from cultured medium of LAB1564 by thin-layer chromatography (Fig. 4). LAB1990 and LAB2020 produced almost equal amounts of surfactin. These results indicate that the *comS* mutation does not have a drastic effect on surfactin production.

Two lines of evidence from the studies described above provide confirmation that *comS* is translated and that the *comS* product is required for competence. First, two *lacZ* translational fusions, one to the TTG start of *comS* and the other to the sixth codon of *comS*, are expressed in *B. subtilis*. The fact that a *comS*′::′*lacZ* fusion can be expressed ectopically (in the *srf* 5′ region) shows that the *comS* sequence contains all the signals required for translation. Secondly, the amber allele of *comS* can be suppressed by the *sup-3* nonsense suppressor with respect to competence development and *comG*::*lacZ* expression, proving that the translation of *comS* is needed for competence. The *comS* product does not appear to be required for the production of surfactin, although a regulatory role in *srf* expression cannot be ruled out at this time.

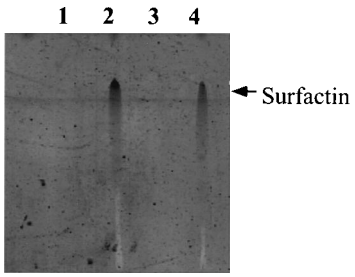

FIG. 4. Production of surfactin in *comS*(Am) cells. Thin-layer chromatography of purified surfactin (see text). Lane 1, JH642 Srf$^-$ (*sfp*0); lane 2, LAB1990 Srf$^+$ (*sfp*); lane 3, LAB1564 Srf$^-$ (Δ*srf sfp*); lane 4, LAB2020 Srf$^+$ [*comS*(Am) *sfp*].

Expression of *comS* depends on transcription from the *srf* promoter, and no significant contribution to its expression can be attributed to transcription initiation occurring internally within the *srf* operon. Its location within and out of frame with *srfB* may indicate that the translation of *srfB* functions in the optimal expression and, perhaps, regulation of *comS*. An attempt to address this involved the creation of a stop codon upstream of the *comS* start codon. A threefold reduction in the expression of *comS*′::′*lacZ* was observed in strains bearing the opal codon 136 bp upstream of the *comS* TTG start, suggesting that the translation of *srfB* enhances *comS* expression (data not shown). The translating ribosomes on the *srfB* coding sequence may unfold an mRNA secondary structure to allow translation initiation at the *comS* Shine-Delgarno sequence. Secondary structure prediction analysis can generate models in which the *comS* Shine-Delgarno sequence is sequestered in a double-stranded form which may be released by passing ribosomes. It is also possible that transcription termination is occurring within the 136 bp region between the *srfB* opal lesion and *comS*, perhaps by a rho-dependent mechanism.

What might be the function of the ComS protein in the development of competence? Mutations of *comS*, of the other early competence genes (8) (*comA*, *comP*, and *comQ*), and of *degU* (20) and *sinR* (34) are known to be suppressed by mutations in *mecA* and *mecB* (6, 17, 18, 23, 32). Unlike *comA*, *comP*, and *comQ* mutations, *degU* and *sinR* mutations do not appear to affect transcription initiation at the *srfA* promoter (6). Their effect on *comS* expression is now under investigation. The *mec* genes encode products that show significant similarities to proteins of the Clp family, consisting of putative chaperonins and ATP-dependent proteases (12). MecB resembles the ATPase subunit of Clp complexes (22), whereas MecA shows weak homology to the catalytic subunit of the *Escherichia coli* Clp protease, although it appears to lack important active-site residues (18). MecA and MecB can form a complex in vitro that can interact with ComK (17), a positive transcriptional regulator of the late competence genes (38). This binding is thought to sequester ComK (14, 17), which would impair late *com* gene transcription. One could imagine that ComS could bind to ComK, rendering it resistant to Mec-dependent inhibition. Another possibility is that ComS interacts directly with either MecA or MecB (ClpC). As has been pointed out by others (23), both *mecB* (*clpC*) mutants and the genetic competence of *B. subtilis* are temperature sensitive. Heat shock is known to increase the level of *mecB* (*clpC*) RNA, suggesting that *mecB* can be regulated at the level of transcription initiation (19). ComS appears not to affect *mecB* transcription (43), but it may function in conjunction with DegU, which is also needed for competence and whose function can be bypassed by *mec* mutations. This last possibility suggests another potential target of ComS, the response regulator protein DegU, which has been implicated not only in the regulation of competence development but also in the expression of several genes encoding extracellular degradative enzymes (20). Whether ComK, MecA, MecB, or DegU is the target of ComS, it is likely that the influence of *comS* extends beyond the regulation of genetic competence and could potentially affect other late growth processes.

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