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Genetics

Identification of *comS*, a gene of the *srfA* operon that regulates the establishment of genetic competence in *Bacillus subtilis*

(peptide antibiotic biosynthesis/genetic competence/transcriptional regulation)

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ABSTRACT **Genetic competence (the ability to internalize exogenous DNA) in *Bacillus subtilis* is dependent on a regulatory pathway that activates the expression of a battery of competence-specific genes. The *srfA* operon, encoding the subunits of surfactin synthetase, which catalyzes the nonribosomal synthesis of the peptide antibiotic surfactin, also functions in the competence regulatory pathway. The DNA encoding only one of the seven amino acid-activating domains of surfactin synthetase, the valine-activating domain (*srfAB1*), is necessary for competence. Deletion analysis revealed that a 569-bp fragment of *srfAB1*, fused to the *srfA* promoter, complements a *srfA* deletion mutation (Δ*srfA*) with respect to competence. This fragment contains an open reading frame consisting of 46 amino acids (*orf46*), which is out of frame with *srfAB1*. A frameshift mutation in *srfAB* upstream of *orf46* has no effect on competence but a frameshift and nonsense mutation in *orf46* resulted in failure to complement the Δ*srfA* mutation. These results indicate that *orf46* encodes the *srfA*-associated competence regulatory factor. Computer-aided analysis of the putative *orf46* product (ComS) shows similarity to the homeodomain of the POU domain class of eukaryotic transcriptional regulators.**

Genetic competence in *Bacillus subtilis* is established when cells of a culture, having reached a high cell density, become able to internalize DNA (1). The establishment of competence occurs in only a fraction of the total cell population and is initiated in response to the accumulation of an extracellular peptide factor encoded by the *comX* gene (2). The peptide is thought to interact with the Spo0K peptide permease complex (3), resulting in the activation of the ComP–ComA signal transduction system (1). ComP is a histidine protein kinase and a member of the sensor class of two-component regulatory proteins (4). ComA is its cognate receiver that, when phosphorylated, becomes a transcriptional activator (5). There is evidence that ComA phosphate interacts with the promoter region of the *srfA* operon (6), encoding the subunits of the peptide synthesizing complex surfactin synthetase (7, 8), thereby activating *srfA* transcription (9). *srfA* is a 27-kb transcription unit required not only in the biosynthesis of the lipopeptide surfactin (Fig. 1) but also in the transcription of the late competence genes encoding the protein components of the DNA import apparatus (1, 7, 10–12).

Surfactin (Fig. 1) is a cyclic compound composed of seven amino acids and a β-hydroxy fatty acid (13). The three subunits of surfactin synthetase required to form the peptide moiety can be divided into seven amino acid activating domains (AA1, AA2, AA3, AB1, AB2, AB3, and AC) that function in the incorporation of the surfactin constituent amino acids (refs. 8, 14, and 15; Fig. 1). Our studies indicate that the peptide synthetic capacity of *srfA* is not required for competence development (16). Furthermore, the region of *srfA* encoding the fourth domain (SrfAB1; valine-activating domain) was shown to be required for competence and it was proposed that its activity functions in competence development (11). However, it was recently shown that a triple mutant of *srfAB1* that no longer activates valine retained competence-regulating activity.† In this report, evidence is presented demonstrating that a small gene, *comS*, nested within *srfAB* is required for the development of genetic competence.‡

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *Escherichia coli* MV1190 [Δ(*lac proAB*) *thi supE* Δ(*srl-recA*)306::Tn*10*(Tet^r) (F′ *traD36 proAB lacI^q lacZ* ΔM15)] was used as a host for the phage M13 derivatives (9). AG1574 [*araD139* Δ(*ara leu*) 7697 Δ*lacX74 galUK* r$^-$m$^+$ *strA recA56 srl* (from A. Grossman)] was used for propagation of plasmids in *E. coli* (9). NK7085 (17) [Δ(*lac pro*) *nalA*/F′ *lacZYA536 proA$^+$ proB$^+$ mutS104*::Tn*5*] was the host for M13 clones used in oligonucleotide-directed mutagenesis. *B. subtilis* strains were derivatives of JH642 (*trpC2 pheA*). ZB307A is a prototrophic derivative of JH642, which is lysogenic for SPβ*c2del2*::Tn*917*::pSK10Δ6 (18). Strain LAB848 bears the Δ*srfA*::pNAC14 mutation, which is a 19-kb deletion of *srfAA* and *srfAB* DNA (15).

Plasmid pCD65 was constructed by inserting the *Hin*cII fragment of p120-21E (7) [containing 733 bp of the 3′ end of *srfAA* and a 3.9-kb fragment of the 5′ end of *srfAB* including *srfAB1* (encoding the valine-activating domain; Fig. 2)] into pMMN46 (7), which contains the *srfA* promoter (P*srfA*) region plus 3.0 kb of DNA encoding the N terminus of SrfAA. This placed *srfAB1* under the control of the P*srfA* promoter. Plasmid pCD77 contains 0.7 kb of the P*srfA* region plus 38 codons of *srfAA* (9) joined to the 1112-bp *Pst* I/*Hin*dIII fragment of *srfAB1* (see Fig. 2). Plasmids pMMN166 and pMMN167 are mutated derivatives of pCD77 (see *Results*). Plasmid pMMN174 contains the 569-bp *Bgl* II/*Hin*dIII encoding Δ*srfA*-complementing activity. Plasmid pCD86 is a derivative of pMMN13 (7) containing the 0.7-kbp P*srfA* fragment flanked by multiple restriction endonuclease cleavage sites and was used as a vector for testing mutated derivatives of the 569-bp *Bgl* II/*Hin*dIII fragment for Δ*srfA*-complementing activity. Plasmids pCD94 and pCD95 are pCD86 derivatives containing the 569-bp *Bgl* II/*Hin*dIII fragment bearing the *srfABtt1* or *srfABtt2* insertion mutations

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Abbreviations: X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; ORF, open reading frame.

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†Sinderen, D.v., Eshuis, H., Jongbloed, J., Venema, G., Kong, L., Luttinger, A., Dubnau, D. & Hamoen, L., International Meeting on Bacillus, July 18–24, 1993, Paris, France.

‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. U10926).

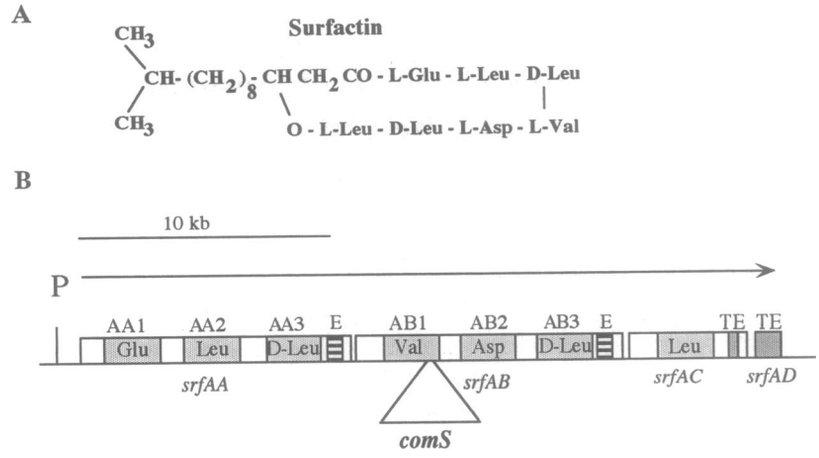

FIG. 1. (*A*) Structure of surfactin. (*B*) The *srfA* operon. The four genes *srfAA*, *srfAB*, *srfAC*, and *srfAD* are shown, as are the putative epimerase (E) and thioesterase (TE) sites. Amino acid-activating domains (AA1, AA2, AA3, AB1, AB2, AB3, AC) are indicated, as are their cognate substrate amino acids incorporated into the surfactin peptide. Location of the *comS* gene is indicated.

(see *Results*), respectively. Plasmid pCD101 contains the 569-bp fragment with an A to T substitution at nucleotide 16 of *orf46* resulting in an amber mutation.

Culture Media. YT broth (2×), LB, and DSM agar media, prepared as described (19), were used for routine culture of *B. subtilis* and *E. coli* strains. One-step competence medium was used for growing strains bearing *lacZ* fusions (20) and to make CM (competence medium) agar (1.2% agar). 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) was added to make CM X-Gal plates, which were used to determine the Lac phenotype of *comG::lacZ* fusion-bearing cells.

***In Vitro* Mutagenesis.** Oligonucleotide-directed mutagenesis was performed by the gapped-duplex procedure (21). OL13 (5′-GGAAGAGTTAACGAAAGCAAGG-3′) was used to insert two T residues 9 bases upstream of the putative ribosome-binding site (Shine–Dalgarno) of *orf46* to create *srfABtt1*. OL14 (5′-GCAGACGTTTGTTAACCGATC-3′) was used to insert two T residues downstream of the *orf46* initiation codon, thus creating *srfABtt2*. Both OL13 and OL14 introduce *Hpa* I restriction cleavage sites. The amber mutation *orf46am* was created by using the oligonucleotide OL15 (5′-CCGATCAGGCTAGCATCTTATC-3′). mMMN175, an M13 clone containing the 569-bp *Bgl* II/*Hin*dIII fragment bearing *orf46* DNA, was used as the template for the mutageneses.

***B. subtilis* Transformation and Transduction.** *B. subtilis* cells were rendered competent by the method of Niaudet and Ehrlich (22). The phage PBS-1 transduction (19) and the SPβ phage procedures (18) have been described.

Analysis of DNA Complementing a Δ*srfA* Mutation. A congression protocol was initially adopted to carry out complementation experiments. Congression is a procedure in which competent cells are transformed with two genetic markers, one for selection and another that is identified by screening the transformants. First, competent cells of strain JH642 were transformed with pCD65 (or deletion derivatives; Fig. 2) with selection for chloramphenicol resistance (Cm^r) conferred by the plasmid-encoded chloramphenicol acetyltransferase gene. Chromosomal DNA from transformants and from Δ*srfA* (LAB848) cells was used to transform LAB1567, a ZB307A derivative ($SrfA^+$) bearing the *comG::lacZ* fusion. Cm^r transformants were screened for phleomycin resistance ($Phleo^r$) and β-galactosidase expression on

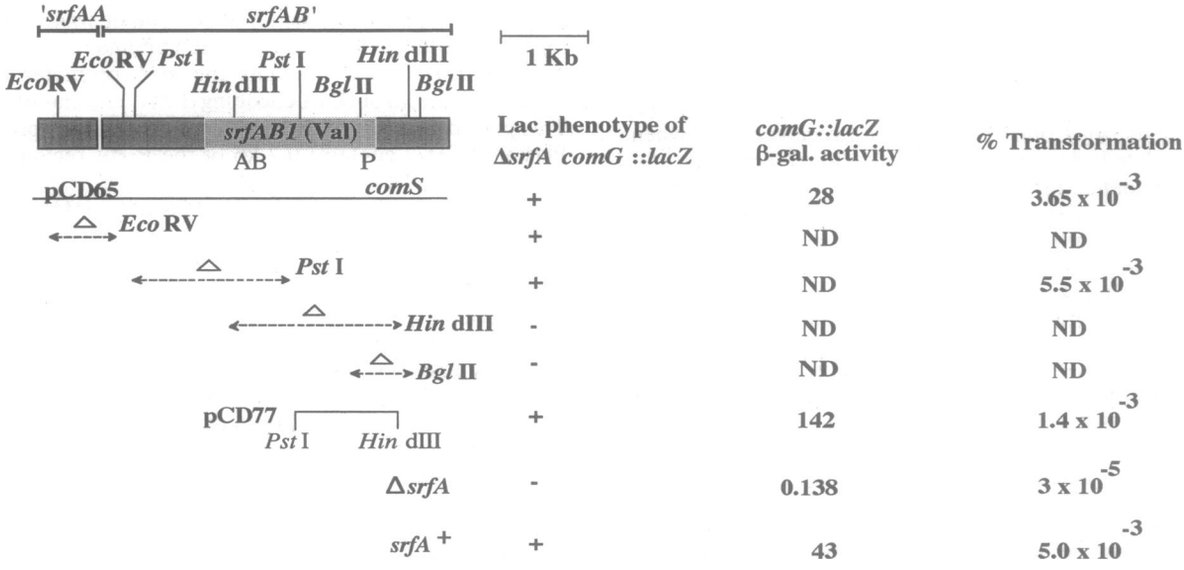

FIG. 2. Complementation of Δ*srfA comG::lacZ* cells with pCD65 and deletion derivatives. The insert of plasmid pCD65 is shown containing the 3′ end of *srfAA* and *srfAB1* DNA encoding the valine-activating domain. AB represents the ATP-binding region and P is the 4′-phosphopantetheine cofactor site. Deleted regions are indicated by a Δ above the dashed arrows. Lines labeled pCD65 and pCD77 represent inserts of plasmids used for complementation. Results of the complementations are shown to the right and include the Lac phenotype, *comG*-directed β-galactosidase activity, and transformation efficiency. ND, not determined.

CM X-Gal plates. Using this procedure, plasmids that were tested for complementing activity were introduced into the *srfA* promoter region in a *srfA* deletion mutant derivative of ZB307A. The location of the plasmids was determined by linkage to *srfA* by cotransformation with the Phleo^r marker associated with the Δ*srfA* mutation. Southern blot analysis confirmed the presence of the *srfA* fragments in the wild type and in the Δ*srfA* plasmid complemented cells and their absence in the Δ*srfA* and the Δ*srfA* noncomplemented strains (data not shown).

The plasmid pCD65, its deletion-mutated derivatives (Fig. 2), and pCD77 were also used to transform Δ*srfA*::pNAC14 (Phleo^r) cells of strain LAB848 directly. Transformants were obtained at a very low frequency as expected since Δ*srfA* cells are Com$^-$. These plasmids do not replicate in *B. subtilis* but will integrate by homologous recombination into the *srfA* promoter region of the Δ*srfA* allele. This was confirmed by transformation linkage to the Phleo^r gene associated with the Δ*srfA*::pNAC14 mutation (data not shown). The generalized transducing phage of *B. subtilis* PBS1 was subsequently used to introduce the *comG*::*lacZ* (Spc^r) fusion into the plasmid-bearing Δ*srfA*::pNAC14 cells.

SPβ-mediated specialized transduction was used to introduce the smaller plasmids pCD86, pMMN174, pCD94, pCD95, and pCD101 into cells of LAB1708 (Δ*srfA*::pNAC14 *comG*::*lacZ trpC2 pheA*, a derivative of JH642) according to a published protocol (23). Complementation analysis of the Δ*srfA* mutant was attempted by using SPβ*c2del2*::Tn*917*::pSK10Δ6, a specialized transducing phage of *B. subtilis* (18). Plasmids described above will recombine with the prophage by virtue of the pBR322 homologous DNA within the prophage-borne Tn*917*. Such plasmids can then be transferred by specialized transduction.

β-Galactosidase Assays. The β-galactosidase activity in *lacZ* fusion-bearing strains was determined as described (24).

Computer Analysis. The FASTA algorithm of Lipman and Pearson (25) and interest level score calculation of Karlin and Altschul (26) were used in a computer-aided (DNAstar, Madison, WS) search for proteins with primary structures similar to the Orf46 amino acid sequence.

RESULTS

Localization of the Competence-Regulating Region Within *srfAB*. Complementation of the Δ*srfA* mutation by *srfAB1* on a multicopy plasmid had been reported previously (11). Complementation of Δ*srfA* by a single copy of srfAB1 was tested by using an integrative plasmid bearing *srfAB1* placed under the control of the *srfA* promoter (P*srfA*). This plasmid, pCD65 (Fig. 2), contains the 3′ end of the third domain of SrfAA and 3.9 kb of *srfAB1* (Fig. 2). The expression of *comG*::*lacZ* (gift from D. Dubnau), composed of the promoter region of the late competence operon *comG* (27) joined to a promoterless *lacZ* gene, is abolished in a Δ*srfA* background (1, 16). A single copy of pCD65 complements Δ*srfA* with respect to competence and *comG* expression (Fig. 2).

Restriction enzyme cleavage within *srfAB1* was followed by ligation to create derivatives of pCD65 bearing *srfAB1* deletion mutations (Fig. 2). These were analyzed for complementing activity in Δ*srfA comG*::*lacZ* cells by examining the Lac phenotype on CM X-Gal plates. The *Eco*RV deletion removes the 3′ *srfAA3* sequence and the translation start site of *srfAB*. The *Pst* I deletion removes the conserved, ATP-hydrolysis region of *srfAB1*.† Both of these mutations did not affect the complementation of Δ*srfA* (Fig. 2). Deletion of the *Bgl* II fragment resulted in loss of complementing activity. These results suggested that the DNA required for competence was in the vicinity of the *Bgl* II sites of *srfAB1* (Fig. 2).

Plasmid pCD77, containing a 1.1-kb *Pst* I/*Hin*dIII fragment of *srfAB1* (Fig. 2) under the control of P*srfA* retained

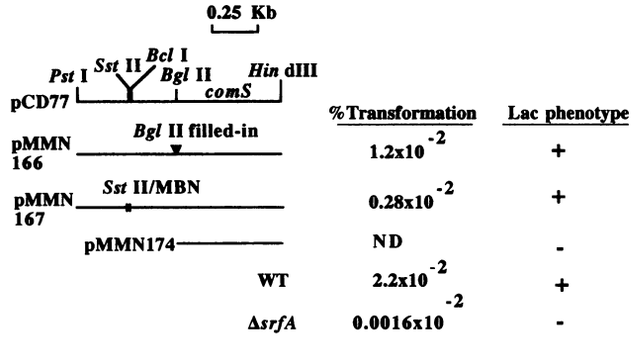

FIG. 3. Complementing activity of pCD77 and mutated derivatives. Line labeled pCD77 shows the plasmid insert and its restriction map. Lines below indicate the mutant derivatives of pCD77; pMMN166 was made by cleaving at the *Bgl* II site followed by fill-in synthesis and ligation; pMMN167 was made by cleaving at the *Sst* II site followed by mung bean nuclease (MBN) treatment and ligation. pMMN174 contains a 569-bp *Bgl* II/*Hin*dIII fragment. The transformation efficiency (number of transformants/total viable cell count) is shown for Δ*srfA comG*::*lacZ* cells with and without the plasmid constructs together with a wild-type (WT) control. Lac phenotype of plasmid-bearing Δ*srfA comG*::*lacZ* cells is also indicated.

Δ*srfA*-complementing activity. Unique sites (Fig. 3) were cleaved by restriction enzyme digestion followed by fill-in synthesis or by mung bean nuclease treatment, resulting in the creation of frameshift mutations in *srfAB*. These did not affect the ability of the 1.1-kb fragment to complement Δ*srfA* (Fig. 3). A 569-bp *Bgl* II/*Hin*dIII fragment from this region retained the ability to complement the Δ*srfA* mutation (Fig. 3; pMMN174).

Identification of the *srfAB* DNA Encoding the Competence-Regulating Activity. One open reading frame (ORF) was identified in the 569-bp fragment that was out of frame with *srfAB* and consisted of 46 amino acids (Fig. 4; *orf46*). A sequence resembling a ribosome-binding site [AAGGAGG, $\Delta G = -17.8$ kcal; 1 cal = 4.184 J (28)] was located 9 bp upstream of the ORF's TTG start codon (Fig. 4). A frameshift insertion mutation (*srfABtt1*) was introduced upstream of the putative ribosome-binding site of *orf46* and another (*srfABtt2*) was created after the initiation codon (Fig. 4). Plasmids pCD94 (bearing *srfABtt1*), pCD95 (bearing *srfABtt2*), and the vector pCD86 were introduced into LAB1708 (Δ*srfA comG*::*lacZ*) by SPβ specialized transduction. The SPβpCD94 lysogen (Lac$^+$) exhibited complementation of Δ*srfA*, while

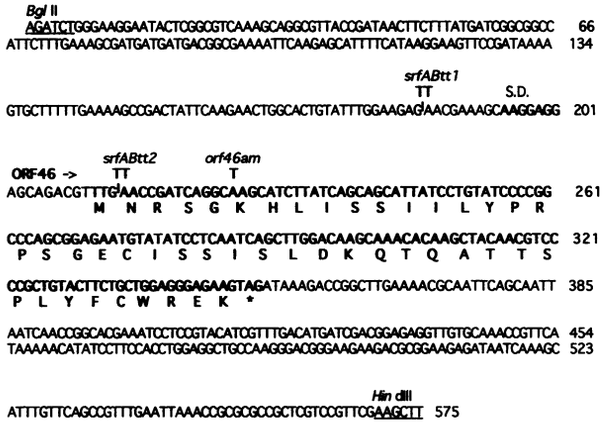

FIG. 4. Nucleotide sequence of the 569-bp fragment bearing *orf46*. Also shown are the sites of the dinucleotide insertions *srfABtt1* and *srfABtt2* as well as the site of the amber mutation *orf46am*. Shine–Dalgarno sequence (S.D.) of the *orf46* putative ribosome-binding site is indicated and the amino acid sequence of the putative *orf46* product is shown below the nucleotide sequence.

the SPβpCD95 lysogen was Lac⁻. Cells of LAB1708 (Δ*srfA comG*::*lacZ*) and LAB1763 (Δ*srfA comG*::*lacZ* SPβpCD86) did not express *comG*::*lacZ* and exhibited significantly lower transformation efficiency compared to wild type (LAB1777) and the SPβpCD94 lysogen. The effect of the mutations was confirmed by a β-galactosidase assay and a competence assay (Table 1). The transformation efficiency of the SPβ lysogens is lower than that of SrfA⁺ cells (data not shown). This may be due to the induction of the SPβ phage, which results in part from the elevated expression of *recA* in cells undergoing competence development (29). This could also be related to the ectopic location of the P*srfA–orf46* construct or a requirement of *srfAB* DNA for optimal expression of *orf46*.

To further test the importance of *orf46* in regulating competence development, an amber mutation was introduced by substituting the A at nucleotide 16 with a T (Fig. 4), thus creating an *orf46* amber mutation. Cells containing the pCD86 derivative pCD101 bearing the *orf46* amber mutation showed a transformation efficiency 10- to 50-fold lower than that of the lysogen bearing wild-type *orf46* as well as diminished *comG*::*lacZ* expression (Table 1). These results suggest that the product of *orf46*, henceforth referred to as *comS*, is likely to be the competence factor encoded by *srfA*.

ComS Bears Similarity to the Homeodomain of POU Domain Proteins. A search of the GenBank data base revealed similarity between ComS and proteins with the POU (Pit-1, Oct-1, Unc-86) homeodomain. ComS shows 40.7% similarity to the homeodomain portion of the product encoded by the *Caenorhabditis elegans unc-86* gene required for neuronal development (30) (Fig. 5); 37% similarity was found to the homeodomain of *Drosophila melanogaster* I-POU, a POU domain protein that inhibits neuron-specific gene activation (36).

FIG. 5. Similarity of Orf46 with the homeodomain regions of POU domain proteins. Analysis is described in *Materials and Methods*. Underlined letters represent identities or conserved substitutions. Unc-86 and Mec-3 of *C. elegans* (30, 31), Pit-1 of rat (32), Oct-1 (33) and Oct-2 (34) of human, Oct-3 (35) of mouse, and IPOU of *D. melanogaster* (36).

DISCUSSION

The region of *srfA* required for development of genetic competence in *B. subtilis* had been localized to a DNA fragment containing the 5′ end of the *srfAB* coding region. This DNA encodes the SrfAB1 domain of surfactin synthetase, which functions in the incorporation of valine into the surfactin lipopeptide. Here we show that the *srfAB* coding sequence is not required for the development of competence but that a small DNA fragment containing a short ORF, *comS*, encoding a putative 46-amino acid product is necessary for expression of the late competence genes. This conclusion is based on the following evidence: (*i*) Deletion of the translation start signals and a large internal region of *srfAB1* had little or no effect on *comG*::*lacZ* expression or transformation efficiency. (*ii*) Insertion of 2 nucleotides (mutation *srfABtt1*) in the *srfAB* sequence upstream from *comS*, causing a frameshift mutation in *srfAB*, has no effect on *comG*::*lacZ* expression. (*iii*) The frameshift mutation, *srfABtt2*, and an amber mutation, *orf46am*, in *comS* abolished *comG*::*lacZ* expression and dramatically reduced transformation efficiency. In addition, a translational *lacZ* fusion was constructed with *comS* and was observed to be expressed in *B. subtilis*. This expression was under the control of the ComP–ComA system (C.D. and P.Z., unpublished data). These results strongly suggest that *comS*, encoding a 46-amino acid product, is the *srfA*-associated determinant of competence development in *B. subtilis*.

The location of *comS* is unusual in that it lies within the sequence of the *srfAB* gene. Translation of the *comS* sequence would have to occur on an RNA that is undergoing translation of *srfAB*, unless there is a level of control (perhaps competence specific) affecting processing of the RNA or *srfAB* translation, either of which could render *comS* RNA accessible for translation initiation. A similar situation exists in the early competence gene cluster, where the translation initiation region of *comX*, which codes for the extracellular peptide that activates competence development, lies within the coding region of *comQ*, whose product functions in the export of ComX (2). It is also possible that the translation of *srfAB* is required for optimal *comS* expression, perhaps through the disruption of RNA secondary structure as the ribosomes traverse the *srfAB* mRNA.

The primary function of *comS* in activating late competence gene transcription is not known but recent reports provide clues to its role. The *srfA* operon is required for the transcriptional activation of *comK* (37), which in turn is needed for transcription of a number of late competence operons. *comK* transcription is positively autoregulated. It is possible that ComS positively regulates *comK* transcription or perhaps may associate with the ComK protein to promote *comK* transcription. Alternatively, it is possible that the ComS product antagonizes the activity of the Mec proteins (MecA and MecB; refs. 38–40), which, according to genetic studies, are thought to negatively regulate the *comK*-dependent transcriptional activation of the late competence operons. Mutations in either *mecA* or *mecB* suppress mutations in the early competence genes and in *srfA*. It has been proposed that the Mec proteins inactivate ComK by direct contact (41). ComS could counteract this inhibition by virtue of an interaction with ComK or by direct contact with the Mec proteins.

A computer-aided search for proteins showing primary structure homology detected potentially important similarities to the homeodomain of the POU class of eukaryotic transcriptional activators. Particularly interesting are the conserved phenylalanine and cysteine at positions 41 and 42, respectively (Fig. 5), which are required for interaction with the cognate binding sequence of POU protein-controlled

Table 1. *comG-lacZ* expression and transformation efficiency of *srfABtt1*, *srfABtt2*, and *orf46am* mutants

Strain	Relevant genotype	% transformation × 10^{-4}	Maximum β-galactosidase specific activity, Miller units
LAB1708	Δ*srfA*::pNAC14 *comG-lacZ*	0.33	<0.1
LAB1763	Δ*srfA*::pNAC14 *comG-lacZ* SPβpCD86(vector)	<0.0625	<0.1
LAB1765	Δ*srfA*::pNAC14 *comG-lacZ SPβpCD95*(*srfABtt2*)	0.23	<0.1
LAB1777	Δ*srfA*::pNAC14 *comG-lacZ* SPβpMMN174 (*orf46*⁺)	7.67	25, 15
LAB1764	Δ*srfA*::pNAC14 *comG-lacZ* SPβpCD94(*srfABtt1*)	11.4	29, 44
LAB1813	Δ*srfA*::pNAC14 *comG-lacZ* SPβpCD101(*orf46am*)	0.3	<0.1

All strains are derivatives of JH642 (*trpC2 pheA*).

genes (42). These are in the characteristic position with respect to the identities at positions 16–21, which is the turn located between the two helices of the homeodomain. However, there are significant differences at the proposed helix three region, which corresponds to the consensus WFXNR-RXR (T. Burglin, personal communication) and, in ComS, is YFCWREK. The N (replaced with a W in ComS) is strictly conserved among homoeodomain proteins and contacts the major groove of cognate regulatory nucleotide sequences. Furthermore, the highly conserved second R of the consensus is replaced with E and there is a P in ComS (position 38) located very near or within the region corresponding to helix three. If the similarity is meaningful with respect to ComS function, then it suggests that *comS* encodes a DNA-binding protein that functions in competence gene regulation, perhaps as a subunit of a larger complex. To our knowledge, this is the only example of a prokaryotic regulatory gene whose putative product resembles in primary structure a member of the homeodomain family of the POU class. If a connection can be made between the existence of such a factor in bacterial competence development and the role of POU domain proteins in eukaryotes, it is the resemblance of competence to the pathways of cell specification that are governed by the POU transcriptional regulators. Unc-86 of *C. elegans* functions in the development of neural cells (30); Oct-2 functions in lymphoid cell-specific transcription (34); Pit-I functions in pituitary cell-specific gene expression (32). Competence development can be thought of as the formation of a unique population of cells, having a specialized function, within the total cell population of a bacterial culture. This is akin to developmental pathways that give rise to specialized tissues in higher organisms.

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