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Transcription Initiation Region of the *srfA* Operon, Which Is Controlled by the *comP-comA* Signal Transduction System in *Bacillus subtilis*

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***srfA* is an operon required for the production of the lipopeptide antibiotic surfactin, competence development, and efficient sporulation in *Bacillus subtilis*. The expression of *srfA* is induced after the end of exponential growth and is dependent on the products of late-growth regulatory genes *comP*, *comA*, and *spo0K*. To begin to understand the mechanism of *srfA* regulation, the *srfA* promoter region was identified and characterized. To examine *srfA* promoter activity, the *srfA* promoter was fused to *lacZ* and inserted into the *B. subtilis* chromosome as a single copy at the SPβ prophage. The location of the transcription start site of *srfA* was determined by primer extension analysis and shown to be preceded by a sequence that resembles the consensus promoter recognized by the σ^A form of RNA polymerase. The *srfA* operon was found to have a sequence corresponding to a long, untranslated leader region of the *srfA* mRNA (300 bp). A nucleotide sequence and mutational analysis of the promoter identified a region of dyad symmetry required for *srfA-lacZ* expression. A similar sequence is found in the region upstream of the *degQ* promoter, transcription from which is also regulated by ComA. This region of dyad symmetry found upstream of these promoters may be the target for ComA-dependent transcriptional activation.**

srfA was first identified as a locus required for the production of the *Bacillus subtilis* lipopeptide antibiotic surfactin (19). It has since been determined to be an operon of over 25 kb and has been shown to function in cell specialization and differentiation (18). Two genes, *csh-293* (13) and *comL* (30), were independently identified and were found to be located in the *srfA* operon (18). Of the *srfA* operon DNA that has been isolated thus far, all of it is required for surfactin production and efficient sporulation, whereas competence requires only the 5′ half of *srfA* (18). The gene product of the first open reading frame (ORF1) of *srfA* was shown to be a subunit of an antibiotic synthetase by amino acid sequence homology to another peptide antibiotic synthetase, tyrocidine synthetase 1 (18), and by the correspondence between the deduced amino acid sequence of ORF1 and the amino-terminal sequence of a purified subunit of surfactin synthetase enzyme (32). *srfA* is thought to be located at an intermediate position in a regulatory pathway which senses an environmental condition and responds by transferring a signal to the apparatus that controls the expression of genes involved in competence development and cellular differentiation (5, 6, 18, 26, 30).

An important part of this pathway is the ComP-ComA signal transduction system, which is thought to function in sensing the nutritional environment. ComP and ComA are believed to be partners in a two-component regulatory system (1, 4, 31). ComP is homologous to the histidine kinase or sensor class of two-component proteins and contains a membrane-spanning domain (35). The *comA* gene product is homologous to the response regulator class and contains three highly conserved aspartic acid residues in the N-terminal portion of the protein which may be the targets for ComP-catalyzed phosphorylation (11, 34). The presence of a possible helix-turn-helix domain in ComA suggests that ComA is a DNA-binding protein (34). *srfA* transcription is induced at the end of exponential growth (T_0) and is dependent on the ComP-ComA system (20, 21, 30). The presence of excess glucose and glutamine in the medium synergistically represses *srfA* transcription (20).

Taken together, these results suggest that ComP senses glucose and glutamine levels in the medium and responds by autophosphorylating. The phosphoryl group on ComP is then transferred to ComA, which then binds to the *srfA* promoter in order to activate transcription initiation, perhaps by interacting with RNA polymerase. In order to begin to understand the details concerning the ComP-ComA system regulation of *srfA* expression, a study was initiated to isolate the *srfA* promoter and identify the sequences required for *srfA* transcriptional regulation.

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* Δ*M15*)] was used as a host for the phage M13 cloning-sequencing vector. AG1574 [*araD139*Δ(*ara leu*)*7697* Δ*lacX74 galUK* r$^-$ m$^+$ *strA recA56 srl*] was used as a host for the propagation of other plasmid constructions in *E. coli*. NK7085 [Δ(*lac pro*) *nalA* r/F *lacZYA536 proA*$^+$ *proB*$^+$ *mutS104*::Tn*5*] was used for oligonucleotide site-directed mutagenesis. *B. subtilis* strains and plasmids used in this work are listed in Table 1. The mutation in OKB167, previously called *srfB* (20), was found to have lost both the *comP* and *comA* genes. Two plasmid derivatives of PZΔ327 (37), pZΔ328 (18) and pTKlac (14), were used as promoter probe vectors. Various deletion derivatives of the *srfA* promoter were first inserted into the promoter probe plasmids, which were then propagated in *E. coli* and later integrated as single copies by homologous

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TABLE 1. *B. subtilis* strains

Strain	Genotype	Reference
OKB105	*pheA sfp*	19
OKB125	*pheA sfp srfA*::Tn*917*ΩOK120 *lac*(pTV55) (Cm^r)	19
OKB167	*pheA sfp* Tn*917lac*ΩOK167 Δ(*comP comA*) (Erm^r)	20
LAB353	*pheA sfp srfA*::pXL3 (*lac*) (Cm^r)	This work
LAB358	*pheA sfp* SPβ *c2del2*::Tn*917*::pXL5 (Cm^r)	This work
LAB359	*pheA sfp* Tn*917lac*ΩOK167 Δ(*comP comA*) (Erm^r) SPβ *c2del2*::Tn*917*::pXL5 (Cm^r)	This work
LAB362	*pheA sfp srfA*::pXL3 (*lac*) (Cm^r) Tn*917lac*ΩOK167 Δ(*comP comA*) (Erm^r)	This work
LAB554	*pheA sfp* pMMN94 (Nm^r)	This work
LAB555	*pheA sfp srfA*::Tn*917*ΩOK120 *lac*(pTV55) (Cm^r) pMMN94 (Nm^r)	This work
LAB589	*pheA sfp srfA*::Tn*917*ΩOK120 *lac*(pTV55) (Cm^r) pMMN99 (Nm^r)	This work

recombination into the SPβ prophage of the *B. subtilis* chromosome as previously described (37). pBD64 (10) is a *B. subtilis* multicopy plasmid (a pUB110 derivative with a copy number of approximately 40) (7) harboring kanamycin and chloramphenicol resistance (Km^r and Cm^r) genes.

Culture media. YT liquid medium (2×) and LB agar medium, prepared as described previously (19), were used for the routine culture of *B. subtilis* and *E. coli*. DS medium (19) was used in the cultivation of bacteria for β-galactosidase assays and for RNA isolation. Competence medium (8) was used for β-galactosidase assays of strains which bear the neomycin resistance (Nm^r) gene. Selection for antibiotic resistance was described in a previous report (18, 19).

β-Galactosidase assay. β-Galactosidase activity was determined as described previously (19).

DNA sequencing. The *srfA* promoter (P*srfA*) was cloned into M13mp18 and M13mp19, and the nucleotide sequence was determined by the dideoxynucleotide chain termination procedure (27) with T7 DNA polymerase (Sequenase; United States Biochemical Corporation, Cleveland, Ohio) and [α-^{35}S]dATP. Both strands were sequenced to confirm the P*srfA* sequence.

RNA isolation and primer extension. RNA was prepared from cells which were grown in DS medium to T_2 of the growth curve (2 h after the end of the exponential-growth phase, when *srfA* transcription was known to reach its maximum) (19). RNA was extracted as described by Igo and Losick (12). For primer extension analysis, the primer 5′-GCAGGCTGCCGTCAGTCAGCATTGC-3′ was used. The primer was labeled with [γ-^{32}P]ATP by polynucleotide kinase. About 10 ng of labeled primer was mixed with 50 μg of total RNA in 10 μl of hybridization buffer (50 mM Tris [pH 8.3]–100 mM KCl). The mixture was heated at 90°C for 1 min, heated at 60°C for 2 min, and finally incubated on ice for 15 min. Two microliters of 5× reverse transcriptase buffer, with a mixture of four deoxyribonucleotide triphosphates and RNase inhibitor RNasin (Promega Corp., Madison, Wis.), and 2 μl of avian myeloblastosis virus reverse transcriptase (8 U/μl; Promega) were added to 6 μl of annealing reaction mixture, and the mixtures were incubated at 42°C for 1 h. The reaction mixture was treated with RNase (preheated at 100°C for 15 min), followed by phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation. An analysis of the extended product was carried out by electrophoresis in 6% polyacrylamide–urea gels.

Oligonucleotide-directed mutagenesis. The target DNA carrying the minimal region required for *srfA* transcription and regulation was inserted into M13mp9, which requires a *supE44* host. One microgram of single-stranded template DNA from the recombinant phage and 10 μg of 5′-end-labeled oligonucleotide were hybridized with 0.2 μg of the replicative form of M13mp9* (a mutant derivative of M13mp9 which does not require a *supE44* host) (38), which is digested with the same restriction enzymes used for the cloning of P*srfA* into M13mp9. The oligonucleotide used, 5′-CGGCA TCCCTTAAAAAATATTGCTG-3′, contains part of the region of dyad symmetry and extends through the region corresponding to the sequence that lies 3′ of the region of dyad symmetry. Hybridization to the M13mp9-P*srfA* clone results in a 2-bp mismatch in the region of dyad symmetry. T7 DNA polymerase (Sequenase) was used to synthesize a complement to the circular DNA by using the oligonucleotide as a primer. The mixture was ligated and transformed into *E. coli* NK7085, which is *sup*^{0} and defective in mismatch repair (15). The mutagenized clones were screened by nucleotide sequencing. Approximately 70% of the resulting clones had the desired mutation.

Construction of *srfA-lacZ* plasmid integrant. A *Pvu*II-*Sma*I *srfA* fragment adjacent to one of the Tn*917* inserts (Tn*917*Ω LA223) that we reported previously to be located approximately 25 kb downstream from the promoter (18) was cloned into the *Sma*I site of pZΔ328. One of the resultant plasmids (pXL3), when integrated into OKB105 by Campbell-type recombination, allowed *lacZ* expression to be under the control of P*srfA*. pXL3 was also integrated into OKB167 [Δ(*comP comA*)].

Propagation of the P*srfA* on a multicopy plasmid. A fragment containing P*srfA* was recovered from pMMN92 by digestion with *Eco*RI and *Hin*dIII. The fragment was cloned into *Eco*RI-*Hin*dIII-digested pUC8. The resultant plasmid (pMMN93) was digested with *Eco*RI and ligated with *Eco*RI-digested pBD64 to construct pMMN94. Plasmid pMMN99 is identical to pMMN94 but contains an *Eco*RI-*Hin*dIII fragment bearing the mutated P*srfA*. This was done by inserting the fragment into pUC8 and then ligating the resulting pUC8 derivative with pBD64, as in the construction of pMMN94. The chimeric plasmids (pMMN94 and pMMN99) were first isolated in *E. coli* after selection for ampicillin resistance (Amp^r) coded for by pUC8 and later transformed into *B. subtilis* OKB105 and OKB125 with selection for Nm^r.

Nucleotide sequence accession number. The nucleotide sequence of P*srfA* (see Fig. 3) has been assigned GenBank accession no. M64702.

RESULTS

Isolation of P*srfA*. We have previously reported the isolation of the *srfA* operon by cloning the DNA flanking *srfA*::Tn*917* insertions followed by chromosome walking (18). The direction of transcription of *srfA* was also determined (19). The promoter region was first identified by subcloning *srfA* fragments into integrative plasmids (18), followed by transformation of surfactin-producing cells (Srf^+; OKB105) to see whether the integration of the integrative plasmids resulted in a surfactin-negative phenotype (Srf^-). Integration of a plasmid containing an internal *srfA* fragment disrupts *srfA* and thus results in a Srf^- phenotype. Integration of a plasmid containing an end of the transcription unit results in a wild-type (Srf^+) phenotype. By this

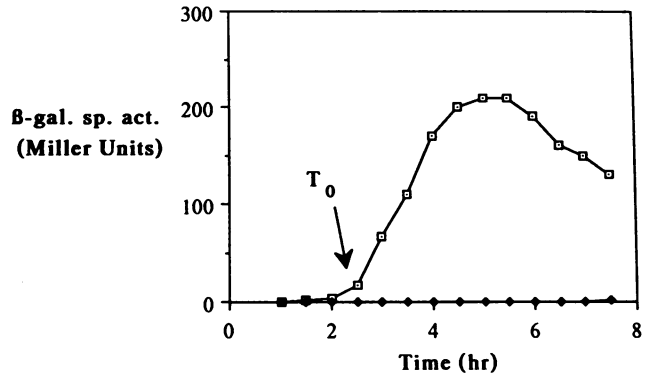

FIG. 1. Effect of Δ(*comP comA*) mutation on *srfA*-directed β-galactosidase synthesis. Time course of β-galactosidase production in wild-type and Δ(*comP comA*) cells which were lysogenized with the SPβ transducing phage carrying *srfA-lacZ* propagated in DS medium. One-milliliter samples were collected at 30-min intervals and assayed for β-galactosidase specific activity. T_0 represents the end of the exponential growth phase. Symbols: ⊡, LAB358 (wild type); ◆, LAB359 [Δ(*comP comA*)].

method, an *Eco*RV-*Sal*I fragment (3.5 kb) was shown to contain a *srfA* transcription initiation region (18). To confirm this, a transcriptional *srfA-lacZ* fusion was constructed by subcloning the *Eco*RV-*Sal*I fragment into the promoter probe vector plasmid pZΔ328 (18), which carries a promoterless *E. coli lacZ* gene. pZΔ328 derivatives can be propagated in *E. coli* and then transferred by transformation into a lysogen of the SPβ phage derivative SPβ *c2del2*::Tn*917*:: pSK10Δ6 (39). The *lacZ* fusion will integrate into the SPβ prophage by homologous recombination.

The plasmid pXL5 is a pZΔ328 derivative that contains the putative *srfA* promoter in the orientation in which it will direct *lacZ* expression in *B. subtilis* (Fig. 1). Expression of the SPβ-borne fusion was observed to be identical to that of the previously described *srfA*::Tn*917lacZ* fusion located within the *srfA* locus (19). The expression of both fusions increased dramatically after the end of exponential phase (T_0) and was dependent on the *comA* gene product. *srfA-lacZ* expression was not observed when the fragment was inserted in the opposite orientation into pZΔ328 (data not shown). Thus, it was concluded that the *Eco*RV-*Sal*I fragment carries the P*srfA* region.

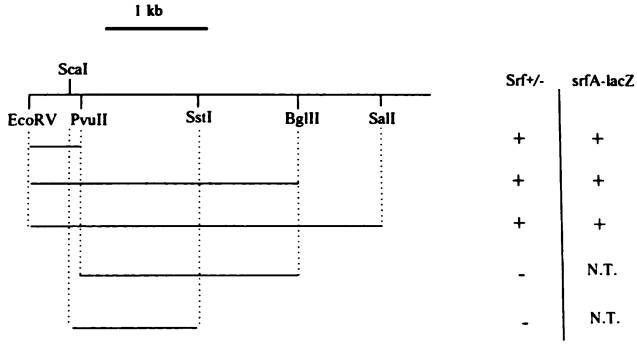

FIG. 2. Localization of P*srfA*. The restriction enzyme map of the 5′ end of *srfA* is shown at the top. The fragment carrying the 5′ end was identified by using the integrative plasmid carrying the various *srfA* fragments represented by the lines under the restriction enzyme map. Surfactin-positive plasmid integrants (+) and surfactin-negative phenotypes (−) are indicated. The promoter activity of the fragments was determined by cloning each into the promoter probe vector pZΔ328. A Lac$^+$ phenotype on DS agar containing the chromogenic indicator X-Gal (+) is shown. N.T., not tested.

FIG. 3. Nucleotide sequence of P*srfA*. The putative polypeptide-coding region is shown by italic letters. The sequence is numbered from the start of transcription, as indicated by an asterisk above the sequence. The presumed Shine-Dalgarno sequence is underlined (RBS, ribosome-binding site), and −10 and −35 regions are boxed. Regions of dyad symmetry which are located upstream of P*srfA* are indicated by arrows under the sequence. A probable rho-independent terminator is shown by arrows above the sequence.

Nucleotide sequence of the P*srfA* region. P*srfA* was further localized either by subcloning fragments of the promoter region into the integration plasmid vector or into the promoter probe vector as described above. The fragments were then tested for their capacity to disrupt *srfA* by insertional inactivation and to direct the expression of *lacZ*. By using these methods, a 700-bp *Eco*RV-*Pvu*II fragment was found to possess P*srfA* activity (Fig. 2). The nucleotide sequence of the fragment (Fig. 3) included the amino-terminal coding end of *srfA* ORF1, which encodes a subunit of surfactin synthetase (18). The ATG start codon was preceded by the potential ribosome-binding site.

Deletion analysis of the *srfA* promoter. A series of deletion derivatives of P*srfA* were created by using a promoter probe vector plasmid, pTKlac (14). pTKlac is identical to pZΔ328 except that it carries the *trpA* transcriptional terminator followed by a multiple cloning site. SPβ-borne fusions carrying the various fragments of the P*srfA* region were transferred into the Δ(*comP comA*) strain (OKB167) as well as the wild-type strain (OKB105). The β-galactosidase activity of each strain was measured to determine the region required for ComA-dependent transcription. Figure 4 shows a com-

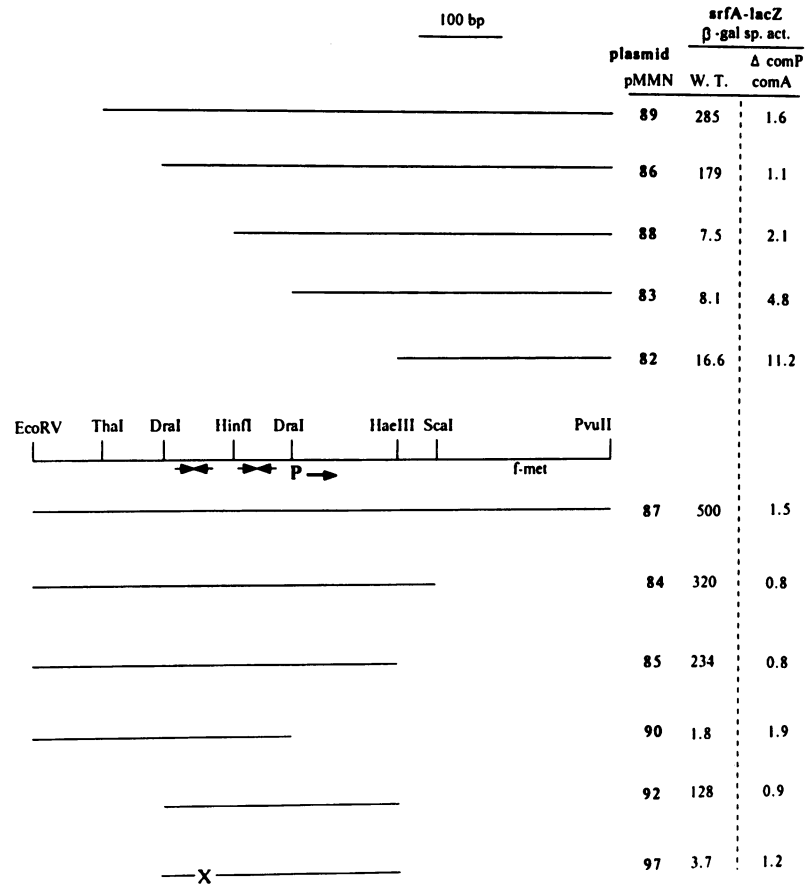

FIG. 4. β-Galactosidase activity directed by various deletion derivatives of P*srfA*. The lines above and below the restriction map represent the fragments that were inserted into pTKlac. The resultant plasmids are indicated by their pMMN numbers. These were integrated into the SPβ prophage by homologous recombination. β-Galactosidase activity in strains carrying SPβ P*srfA-lacZ* fusions was determined in wild-type (OKB105) and Δ(*comP comA*) (OKB167) backgrounds, and the maximum activity is shown in Miller units. The approximate locations of the translation start site (f-met), the transcription start site (P), and the two dyad symmetries (converging arrows) are shown under the restriction map. X (in the cloned fragment of pMMN97) indicates the site of the mutation that was generated in vitro as described in the text. W.T., wild type.

parison of the maximal β-galactosidase activity for *srfA-lacZ* fusion derivatives in wild-type and Δ(*comP comA*) mutant strains. A higher activity was detected in the strain carrying pMMN87, which has the *Eco*RV-*Pvu*II fragment, compared with the strain carrying pXL5, which contains the original *Eco*RV-*Sal*I fragment. The reason for this difference is not known, but it is not due to the different promoter probe vectors used to create the *srfA-lacZ* fusion derivatives. The *Eco*RV-*Pvu*II fragment after being subcloned into pZΔ328, which was used for construction of pXL5, directed higher *lacZ* activity than the *Eco*RV-*Sal*I fragment. Successive deletions originating from either the 5′ or 3′ end gradually decreased *srfA-lacZ* expression, but in each case *srfA* transcriptional activity was still dependent on *comA* and was induced after T_0. The 3′-end deletion to the downstream *Dra*I site (pMMN90) severely impaired *srfA-lacZ* activity, and no stimulation by ComA was detected. The 5′-end deletion to the *Hin*fI site (pMMN88) strongly reduced *srfA-lacZ* activity, which showed a slight dependence on ComA. Thus, the region primarily required for the transcription of *srfA* was localized within a 300-bp region between the 5′-end *Dra*I and the *Hae*III sites (the fragment of plasmid pMMN92). We cannot rule out the possibility that sequences outside this fragment also participate in the regulation of *srfA* transcription. The deletion which removes DNA from the *Eco*RV site to the *Tha*I site reduces *srfA* transcription up to 57% (pMMN87 versus pMMN89), and a larger deletion to the *Dra*I site (pMMN86) reduces transcription 36%. A twofold reduction of *srfA*-directed β-galactosidase activity was also detected in pMMN92 compared with that in pMMN85, with the former having a deletion between the *Eco*RV and *Dra*I sites.

Deletions located 3′ to the major transcriptional start site also caused a twofold reduction in *srfA-lacZ* expression (pMMN87 versus pMMN84 and pMMN85). This could be due to the removal of an important regulatory sequence from the nontranslated leader region or to differences in the stability of the deletion mutant transcripts.

Transcription mapping. The transcriptional start site for the *srfA* operon was determined by primer extension analysis (Fig. 5). RNA was prepared from strains LAB353 (wild type) and LAB362 [Δ(*comP comA*)]. Both LAB353 and LAB362 have a promoterless *lacZ* gene located 25 kb downstream of the promoter region in the *srfA* operon. The expression of this 3′ *srfA-lacZ* sequence as well as its regulation is identical with that of the SPβ-borne *srfA-lacZ* fusion (Fig. 1) and *srfA*::Tn*917lacZ*, the site of which is located 10 kb upstream of the 3′ *srfA-lacZ* fusion (19).

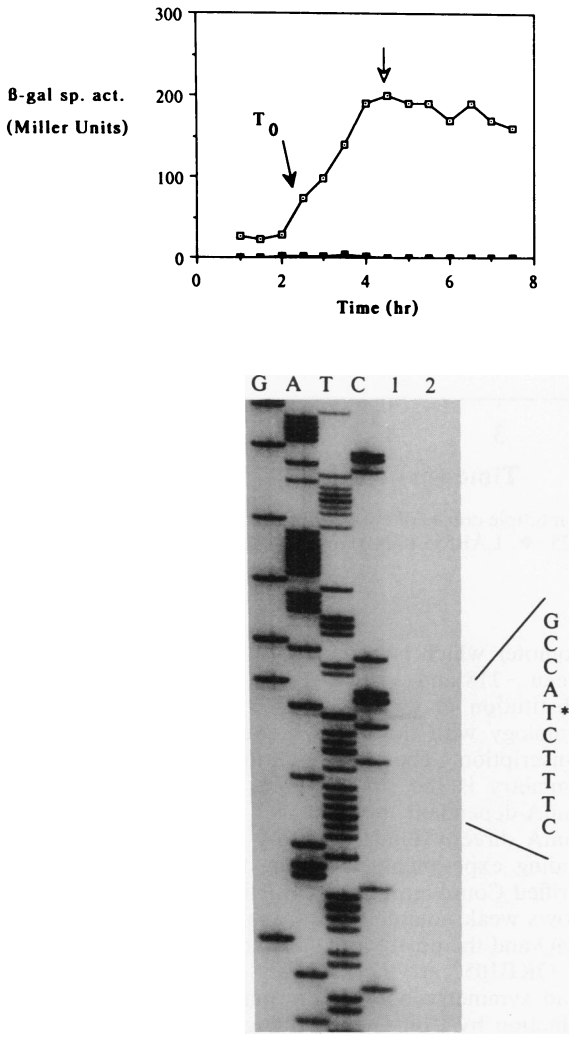

FIG. 5. Primer extension analysis of *srfA* transcription. A dideoxy-sequencing ladder obtained with the same primer used for primer extension analysis was resolved on a 7 M urea–polyacrylamide gel in parallel with the primer extension product by using RNA from wild type (LAB353; lane 1) and Δ(*comP comA*) (LAB362; lane 2). The nucleotide position of the start site is marked by an asterisk. The expression of β-galactosidase in LAB353 (□) and LAB362 (■) strains used for isolation of RNA is shown above. RNA was prepared from a sample of the cultures that was collected at the time shown (⏚).

Samples of the cultures from LAB353 and LAB362 strains were collected for RNA isolation and for assays of β-galactosidase specific activity (Fig. 5). A primer extension product which indicated a major transcriptional start site at approximately 290 bp upstream of the translational start site (Fig. 3 and 5) was detected. We cannot rule out the existence of other minor transcription start sites. There were no or only very weak primer extension signals observed when RNA from the Δ(*comP comA*) mutant was examined for *srfA* transcripts. This is in keeping with previous experiments in which *srfA-lacZ* expression was found to be severely impaired in Δ(*comA comP*) cells (20). A nucleotide sequence upstream of the start site resembled those of promoters which are recognized by the σ^A form of RNA polymerase holoenzyme, showing four of six base matches to both the −10 and −35 sequences.

The deletion mutant, pMMN88, which carries a fragment of the P*srfA* region that extends to the *Hin*fI site located at −75, directed a very reduced level of β-galactosidase activity. What activity remained showed a slight dependency on ComA-ComP. This indicated that a region between the *Dra*I and the *Hin*fI sites (−160 to −75) is required for the full expression of *srfA-lacZ*. A region of dyad symmetry (TTGCGG-N_4-CCGCAA) was found within this region (as shown by arrows in Fig. 3). Many DNA-binding proteins including positive activators of transcription have been shown to bind as dimers to DNA sequences possessing dyad symmetry (2). The importance of the upstream dyad symmetry was assessed by examining the effect of base substitutions in this region on *srfA-lacZ* expression. The *Dra*I-*Hae*III fragment carrying P*srfA* was subjected to site-directed mutagenesis by using an oligonucleotide designed to substitute the GC in the right half of the region of dyad symmetry with TT. The mutated promoter was inserted into pTKlac to create a mutated *srfA-lacZ* fusion derivative, pMMN97. When the fusion was introduced into *B. subtilis*, the mutation caused a severe reduction in promoter activity (Fig. 4). The upstream dyad symmetry region is thus required for optimal *srfA* transcription.

Effect of plasmid amplification of P*srfA* on *srfA* transcription. The results described above show that *srfA* transcription requires the presence of sequences upstream of the promoter. This suggests that a positive activator (perhaps ComA) might be involved in stimulating transcription of *srfA*. This observation prompted an experiment designed to examine the effects of P*srfA* amplification on surfactin production and *srfA* transcription. The presence of the P*srfA* DNA in multiple copies might result in the titration of a positive transcription factor. Strains LAB555 and LAB589 were constructed by transformation of OKB125 cells, which carry *srfA*::Tn*917lacZ* (19), with pMMN94 and pMMN99, multicopy plasmids containing, respectively, P*srfA* and the mutant P*srfA* derivative containing the 2-bp substitution in the upstream dyad symmetry region. β-Galactosidase activity was examined in OKB125, LAB555, and LAB589 cells propagated in competence medium (8). Neomycin was added to the LAB555 and LAB589 cultures, since neomycin resistance is conferred by the pBD64 moiety of pMMN94 and pMMN99. This medium was chosen because we observed that LAB555 and LAB589 cells did not grow in DS medium with neomycin. The expression of *srfA-lacZ* has been shown to be the same whether cells are cultivated in DS or competence medium (22). LAB555 and LAB589 cultures had almost identical growth rates in competence medium. OKB125 cultures had a growth rate slightly higher than those of LAB555 and LAB589. All three strains reached the same maximum cell density. The cells carrying the P*srfA* fragment in the multicopy plasmid showed a twofold reduction in β-galactosidase activity compared with the strain lacking the plasmid (Fig. 6). LAB589 cells (OKB125 cells harboring pMMN99) showed the same β-galactosidase activity as OKB125 cells (Fig. 6), indicating that the titration of a positive activator requires dyad symmetry. LAB554 (OKB105 carrying pMMN94) also showed partially reduced surfactin production (data not shown).

DISCUSSION

The transcription initiation region of the *srfA* operon was isolated, and its nucleotide sequence was determined. The

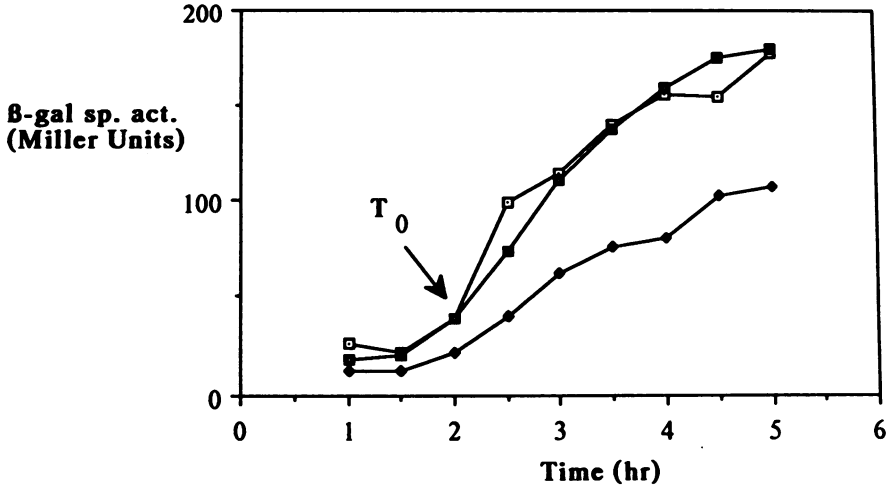

FIG. 6. Time course of β-galactosidase production showing the effect of multiple copies of P*srfA* on the expression of *srfA-lacZ*. Enzyme activity was measured in samples collected at 30-min intervals. ⊡, OKB125; ◆, LAB555 (OKB125 carrying multiple copies of P*srfA*); ■, LAB589 (OKB125 carrying multiple copies of the mutated P*srfA*).

sequenced region contains the first ORF of the operon, which is preceded by an approximately 300-bp transcribed leader region. In *B. subtilis*, the *trp* operon (29) and the *sacB* gene (28) possess leader regions which contain transcription attenuation sites. The *tycA* gene that encodes the antibiotic biosynthesis enzyme tyrocidine synthetase 1 of *Bacillus brevis* also has a 200-bp leader region which contains the site where the transition state regulator AbrB binds (9, 16, 25). It is not known whether the leader region of the *srfA* operon is involved in transcriptional regulation.

The putative P*srfA* exhibits weak homology (4 of 6 bases) to the consensus sequence recognized by the major vegetative form of *B. subtilis* RNA polymerase ($E\sigma^A$). The poor homology to the consensus sequence suggests that P*srfA* is not sufficient to direct the initiation of *srfA* transcription by $E\sigma^A$ but that it requires the participation of a positive regulator (24). However, it is not known which form of RNA polymerase is responsible for *srfA* transcription.

P*srfA* may be activated through the interaction of a *comA*-dependent regulatory factor, perhaps the ComA protein itself, which is rendered active as cells enter the stationary phase of growth. This hypothesis was supported by mutational analysis of P*srfA*, which showed that a region of dyad symmetry upstream of the promoter is required for *srfA* transcription. The presence of dyad symmetry and its role in *srfA* transcription suggest that the positive regulator may bind to the sequence as a dimer, as is the case with other regulatory proteins that recognize regions of approximate dyad symmetry (23).

Three observations lend support to the hypothesis that there is direct interaction between P*srfA* and ComA. (i) The amino acid sequence of ComA contains a helix-turn-helix DNA-binding motif. (ii) *degQ*, a pleiotropic regulatory gene which affects the expression of genes encoding degradative enzymes (3, 36), has been shown to be subject to regulation by ComP-ComA (17). The target site for ComP-ComA-dependent regulation of *degQ* was reported to be located between −78 and −40 with respect to the transcription start site (17). We compared this region of *degQ* with the P*srfA* sequence (Fig. 7) and found an extensive region of homology between −80 and −28 of the two promoters. There is an imperfect dyad symmetry from −71 to −56 of the *degQ* promoter which is homologous to the dyad symmetry between −118 and −103 of P*srfA* (Fig. 3 and 7). (iii) A 2-bp substitution in the region of dyad symmetry that shows homology with the *degQ* sequence severely impairs *srfA* transcription. These observations suggest that the dyad symmetry is the site required in *cis* for activation by a ComA-dependent mechanism. The question of whether ComA directly binds to P*srfA* must await in vitro DNA-binding experiments including footprinting analysis using purified ComA protein. Interestingly, −74 to −59 of P*srfA* shows weak homology to the putative ComA target site of *degQ* and the upstream dyad symmetry region of *srfA* (Fig. 7). OKB105 carrying pMMN88, which contains the weak dyad symmetry but not the perfect upstream one, shows induction by ComA even though its activity is drastically reduced (data not shown). This might be explained by the weak interaction between the −74 to −59 region and the ComA-dependent factor. Experiments to examine the possibility of cooperative binding to the two dyad symmetry sequences are under way.

The involvement of a positive regulator was further examined in an experiment in which the P*srfA* region was propagated on a multicopy plasmid in *B. subtilis*. Multiple copies of P*srfA* partially inhibited *srfA* transcription as well as surfactin production, and no inhibitory effect on *srfA* tran-

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TAATGTTTAGTGGAAATGATTGCGGCATCCCGCAAAAAATATTG  -94  srfA
CTGTAAATAAACTGGAATCTTTCGGCATCCCGCATGAAACTTTT  -50  srfA
          TATTGAGATTTGCGGTGTCACGCAGGACTTTTTT  -47  degQ

                -35                  -10
CACCCATTTTTCGGTGATAAAAACATTTTTTTCATTTAAACTGAACGGTA  srfA
TGCATACTTTTCGGTGAAAAATGAGCCGAAAGCAGACACACTATTA  G  degQ
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FIG. 7. Homology between the two promoter regions, those of *srfA* and *degQ*. The *degQ* promoter sequence was reported by Yang et al. (36). The sequence is numbered from the start of transcription, as indicated by stars. Identical residues between the two sequences are dotted. Dyad symmetries are shown by arrows. −10 and −35 regions are boxed.

scription was detected when multiple copies of P*srfA* with the disrupted dyad symmetry were introduced, suggesting that there may be some titration of a regulatory factor by the amplified dyad symmetry sequence of P*srfA*. The fact that complete inhibition was not observed might be explained by an abundance of the positive factor or its reduced affinity for the plasmid-borne P*srfA* because of an alteration in the higher-order structure of the plasmid DNA. P*srfA* in multiple copies did not inhibit competence development (33).

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