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Amino-Acylation Site Mutations in Amino Acid-Activating Domains of Surfactin Synthetase: Effects on Surfactin Production and Competence Development in *Bacillus subtilis*

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The part of the *srfA* operon of *Bacillus subtilis* that contains the region required for competence development is composed of the first four amino acid-activating domains which are responsible for the incorporation of Glu, Leu, D-Leu, and Val into the peptide moiety of the lipopeptide surfactin. Ser-to-Ala substitutions were made in the amino-acylation site of each domain, and their effects on surfactin production and competence development were examined. All of the mutations conferred a surfactin-negative phenotype, supporting the finding that the conserved Ser in the amino-acylation site is required for peptide synthesis. However, none of the mutations affected significantly competence development or the expression of a *lacZ* fusion to the late competence operon *comG*. This, coupled with recent findings that only the fourth, Val-activating, domain is required for competence, suggests that some activity, other than amino-acylation and perhaps unrelated to peptide synthesis, possessed by the fourth domain is involved in the role of *srfA* in regulating competence development.

A number of bacterial and several lower eukaryotic species produce an abundance of peptide special metabolites in response to growth-limiting conditions (16, 35, 48). These are often synthesized through a mechanism that does not involve the cell's translation machinery but instead utilizes large multienzyme complexes called peptide synthetases (16, 17, 22, 24, 52). Many of these enzymes are thought to utilize the multienzyme thiotemplate mechanism to catalyze peptide synthesis (17, 22, 24). In this model, the multienzyme complex is composed of amino acid-activating domains that catalyze the adenylylation of the constituent amino acid (9) and the covalent attachment of the amino acid to the enzyme by a carboxyl thioester at the site of an enzyme-associated sulfhydryl (10, 15). The domains are organized such that they are colinear with the sequence of the cognate amino acids in the oligopeptide. The growing peptide chain is then transferred from one amino acid domain to the next, where a peptide bond is formed. This translocation process is carried out with the aid of a 4'-phosphopantetheine cofactor (13, 24). Synthesis is then terminated by cyclization of the peptide or its release from the thiotemplate by a thioesterase.

The recent isolation and primary structural analysis of several peptide synthetase genes (8, 11, 26, 28, 40, 42, 46, 50, 52) have shown that their products are composed of tandem domains that share significant amino acid sequence similarity to each other. Within these domains are conserved core sequences, some of which resemble motifs of known function such as sites of ATP binding (26). The DNA encoding these domains has been expressed in *Escherichia coli*, yielding a product that can catalyze activation of its cognate constituent amino acid (14, 21, 23). These domains also contain the site of carboxyl thioester formation between the constituent amino acid and the enzyme (41). The amino-acylation site of the amino acid-activating domain contains the sequence GG H/D S L/I, where the Ser is thought to be the attachment site for a cofactor, perhaps 4'-phosphopantetheine, to which the constituent amino acid is covalently linked following activation. All of these studies lend validity to the multienzyme thiotemplate model, but with the possible added feature of a pantetheine cofactor at each amino acid-activating domain instead of a single pantetheine cofactor as originally proposed.

The *srfA* operon of *Bacillus subtilis* encodes at least some of the enzymes that catalyze surfactin biosynthesis (8, 11, 30, 45). Surfactin is a cyclic lipopeptide special metabolite composed of seven amino acids in the order Glu-Leu-D-Leu-Val-Asp-D-Leu-L-Leu and a β-hydroxytetradecanoic acid (47). As with other lipopeptides (3), surfactin is synthesized by the multienzyme thiotemplate mechanism (18, 44, 47). In addition to *srfA*, another gene, *sfp* (29, 34), is required for surfactin production, but its primary function is unknown. Recent sequence analysis (8, 11) has shown that *srfA* contains four open reading frames. *srfAA* contains the amino acid-activating domains for Glu, Leu, and D-Leu; *srfAB* contains the amino acid-activating domains for Val, Asp, and D-Leu; and *srfAC* encodes the enzyme that contains the amino acid-activating domain for L-Leu. *srfAD* encodes a product (11) with significant sequence similarity to the *grsT* gene product (20) that resembles in primary structure a family of thioesterases.

In addition to its role in the biosynthesis of surfactin, *srfA* functions in the development of genetic competence in *B. subtilis* (5, 30, 46), a process of cell specialization whereby a fraction of a population of cells becomes endowed with the capacity to internalize exogenous DNA in response to conditions encountered in stationary-phase cultures of glucose-grown cells. *srfA* serves a regulatory function in the process, as the transcription of late competence genes requires *srfA* expression (5, 12, 36). The *srfA* operon is regulated by the ComP-ComA signal transduction system (5, 32, 33, 37) in which active phosphorylated ComA positively controls *srfA* transcription by binding to the ComA boxes located upstream of the *srfA* promoter. A transposon Tn*917* insertion in the region between the sequences encoding the

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TABLE 1. *B. subtilis* strains

Strain	Genotype	Relevant features	Source and/or reference
OKB105	*pheA sfp*		28
BD1512	*hisA1 leuA8 metB5 comG12-lacZ* (Cm^r)		1, 2
LAB 848	*pheA sfp* Δ*srfA*::pNAC14(Phleo^r)	*srfA* deletion mutant	This study
LAB960	*pheA sfp* Δ(pCD23) *srfAA1* (S→A)	1st domain mutation	49
LAB 961	*pheA sfp comG-lacZ* (Cm^r)		This study
LAB 962	*pheA sfp* Δ*srfA*::pNAC14 (Phleo^r) *comG-lacZ*	*srfA* deletion mutant	This study
LAB963	*pheA sfp comG-lacZ* (Cm^r) Δ(pCD23)	Srf$^+$ segregant of 1st domain integrant	This study, 49
LAB965	*pheA sfp comG-lacZ* (Cm^r) Δ(pCD23) *srfAA1* (S→A)	1st domain mutant	This study, 49
LAB1194	*pheA sfp srfA*::pCD36 (Cm^r)		This study
LAB1195	*pheA sfp* Δ(pCD36) *srfAA3* (S→A)	3rd domain mutant	This study
LAB1252	*pheA sfp comG-lacZ* (Cm^r) Δ(pCD36)	Srf$^+$ segregant of 3rd domain integrant	This study
LAB1253	*pheA sfp comG-lacZ* (Cm^r) Δ(pCD36) *srfAA3* (S→A)	3rd domain mutant	This study
LAB1255	*pheA sfp srfA*::pCD51 (Cm^r)		This study
LAB1292	*pheA sfp* Δ(pCD51) *srfAB1* (S→A)	4th domain mutant	This study
LAB1297	*pheA sfp comG-lacZ* (Cm^r) Δ(pCD51)	Srf$^+$ segregant of 4th domain integrant	This study
LAB1298	*pheA sfp srfA*::pMMN130 (Cm^r)		This study
LAB1307	*pheA sfp comG-lacZ* (Cm^r) Δ(pCD51) *srfAB1* (S→A)	4th domain mutant	This study
LAB1309	*pheA sfp* Δ(pMMN130) *srfAA2* (S→A)	2nd domain mutant	This study
LAB1310	*pheA sfp* Δ(pMMN130) *comG-lacZ* (Cm^r)	Srf$^+$ segregant of 2nd domain integrant	This study
LAB1311	*pheA sfp comG-lacZ* (Cm^r) Δ(pMMN130) *srfAA2* (S→A)	2nd domain mutant	This study
LAB1334	*pheA sfp* Δ(pCD23)	Srf$^+$ segregant of 1st domain integrant	This study
LAB1335	*pheA sfp* Δ(pMMN130)	Srf$^+$ segregant of 2nd domain integrant	This study
LAB1336	*pheA sfp* Δ(pCD36)	Srf$^+$ segregant of 3rd domain integrant	This study
LAB1337	*pheA sfp* Δ(pCD51)	Srf$^+$ segregant of 4th domain integrant	This study

first and second amino acid-activating domains of *srfAB* (the fourth and fifth amino acid-activating domains of surfactin synthetase) has no effect on competence development but abolishes surfactin production (8, 30, 31). Hence, a region within the part of *srfA* encoding the first four domains is required for competence development. Recent studies (45) strongly suggest that only the fourth amino acid-activating domain of SrfA is required for competence, since DNA encoding the fourth domain when present in multiple copy number can complement a *srfA* mutant with respect to competence development.

In this report, experiments which were designed to determine if the amino-acylation motif is required for peptide synthesis in vivo and to determine if the peptide-synthesizing capacity of *srfA* is involved in its role in competence development are described. This was done by creating amino acid substitutions in the amino-acylation sites of the first four domains of *srfA*. This is the first report describing the effects of peptide synthetase active site mutations in vivo.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* MV1190 [Δ(*lac pro AB*) *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. AG1574 [*araD139* Δ(*ara leu*)*7697* Δ*lacX74 galUK* r$^-$ m$^+$ *strA recA56 srl*] was used for routine propagation of other plasmids in *E. coli*. NK7085 [Δ(*lac pro*) *nalA*/F′ *lacZYA536 proA$^+$ proB$^+$ mutS104*::Tn*5* (25)] was used as a host for oligonucleotide-directed mutagenesis. The *B. subtilis* strains used in this work are listed in Table 1. The plasmids and phage M13 strains used in this work are listed in Table 2.

Culture media. YT broth (2×), LB, and DSM agar media, prepared as described previously (31), were used for routine culture of *B. subtilis* and *E. coli* cells. Blood agar solid medium was used for detection of surfactin production (31). Chloramphenicol-resistant (Cm^r) cells were selected as described previously (31). One-step competence medium was used for growing strains bearing *lacZ* fusions (6).

Oligonucleotide-directed mutagenesis. The mutagenesis was performed by the gapped-duplex procedure (19, 53). The oligonucleotides used for the substitution of the serine codon by the alanine codon in the respective amino-acylation motifs of the first four domains of *srfA* were as follows:

TABLE 2. Plasmids and phage M13 strains

Plasmid or M13 strain	Construction and characteristics
mCD31	M13mp9 with 1.3-kb *Hin*dIII-*Eco*RI pMMN40 fragment
mCD33	mCD31 (S→A) by oligonucleotide-directed mutagenesis
mCD39	M13mp9 with 2-kb pNAC13 *Hin*dIII fragment
mCD46	mCD39 (S→A) by oligonucleotide-directed mutagenesis
pMMN124	pUC18 with 2-kb mCD46 *Hin*dIII fragment
pMMN125	pUC18 with pMMN40 1.7-kb *Hin*dIII-*Eco*RV fragment
pMMN127	pMMN125 0.48-kb *Nae*I fragment replaced by 0.48-kb *Nae*I fragment of pMMN124
pMMN130	pMMN127 with 1.6-kb *cat* gene containing *Eco*RI fragment from pMMN13 (30)
pCD36	pMMN13 with 0.4-kb *Sst*I-*Eco*RI mCD33 fragment and an 0.75-kb *Eco*RI-*Hin*dIII pMMN38 (30) fragment
pCD44	pDH88 (51) with 0.7-kb p120-21E (30) *Bgl*II fragment
mCD45	M13mp9 with 0.7-kb pCD44 *Cla*I-*Sma*I fragment
mCD47	mCD45 (S→A) by oligonucleotide-directed mutagenesis
pCD48	pMMN13 with 3.9-kb p120-21E *Cla*I fragment
pCD51	pCD48 0.7-kb *Bgl*II fragment replaced by 0.7-kb mCD47 *Bgl*II fragment

domain I: 5′ TGG CGG ACA TGC TTT AAA AGC CAT GA 3′
domain II: 5′ GCG GAC ATG CAT TAG CAG G 3′
domain III: 5′ CCT TTG ATA GCA TCT CCG CC 3′
domain IV: 5′ GAT CGG CGG CCA TGC ATT GAA AG 3′.

Southern hybridization analysis. The hybridization analysis was performed by a previously described protocol (30).

Integration plasmid curing protocol. Two independent Cm^r isolates of the *B. subtilis* strain to be cured were grown in two separate tubes containing 2 ml of 2× YT medium at 37°C to late log phase and subcultured a number of times. An overnight grown culture from each tube was diluted 100-fold, and chloramphenicol resistance was induced at a concentration of 0.5 μg/ml for 1 h and was followed by growth at a selective concentration of 5 μg/ml for another hour. The actively growing Cm^r cells were then killed by both the addition of ampicillin at a concentration of 1 mg/ml and further growth for 5 to 6 h. Since chloramphenicol is bacteriostatic, the culture was enriched for the Cm^s cells. The cells were washed with 2× YT, appropriately diluted, and plated on a nonselective medium such as LB or DSM agar. Colonies obtained for each culture were then screened for Cm^s.

***B. subtilis* transformation.** *B. subtilis* cells were rendered competent by the method of Niaudet and Ehrlich (38). Transformation frequency was determined by adding chromosomal DNA from BD1512 carrying the *comG-lacZ* (Cm^r) fusion to 1 ml of competent cells. Cells were selected for Cm^r by plating onto DSM-chloramphenicol. The numbers of the transformants were normalized to the total number of CFU obtained on LB agar to estimate transformation frequency of the respective strain.

β-Galactosidase assays. The β-galactosidase activity in *lacZ* fusion-bearing strains was determined as described previously (31).

RESULTS

Our earlier studies had shown that a region within the 5′ half of the *srfA* operon, upstream of the Tn*917* ΩOK120, was required for competence development (30). We hypothesized that the peptide-synthesizing capacity of this region of *srfA* might play a role in cell specialization. Nucleotide sequence analysis followed by prediction of the amino acid sequence revealed that this region would contain the first four amino acid-activating domains required for surfactin biosynthesis (8). The amino acid sequence comparisons between *srfA* and other peptide synthetases such as those for gramicidin, tyrocidine (43) and enterobactin (40, 42) revealed the presence in *srfA* of a number of conserved motifs (26). One of these, the GG H/D S I/L motif seen in the putative 4′-phosphopantetheine binding regions of fatty acid and polyketide synthetases (39), was shown to be required for surfactin peptide synthesis, since a serine-to-alanine mutation in this motif in the first domain of *srfAA* resulted in the loss of surfactin production as observed on blood agar indicator plates (49). This mutation resulted in loss of Glu-adenylylating activity (49), consistent with the assignment of the first domain of *srfAA* in the activation of the first amino acid (Glu) of the surfactin peptide moiety. In order to determine whether the amino-acylation sites of the remaining three domains were required for surfactin synthesis and whether a peptide synthesized by *srfA* is required for competence development, we proceeded to construct Ser-to-Ala mutations in the amino-acylation motifs of the remaining three consecutive domains of the *srfA* competence region and determine their effects on surfactin production and competence development. This was done because Ala substitutions of Ser tend to be more tolerable then other substitutions with respect to protein conformation (4).

Construction of mutations in amino-acylation motifs of *srfA* and their effects on surfactin production. The construction of the domain I (SrfAA1 [Fig. 1]) Ser-to-Ala mutant has already been described (49). We have also shown that the mutation abolished surfactin production (49). Note that the mutants and Srf$^+$ segregants were respectively given separate strain LAB numbers, although they were derived from the same Cm^r plasmid integrants.

In order to mutagenize the amino-acylation motif of the second domain (SrfAA2), a 2-kb *Hin*dIII fragment (Fig. 1) from pNAC13 was inserted into the *Hin*dIII site of M13mp9 to yield mCD39 (Table 2 and Fig. 1), the single-stranded form of which was used for in vitro mutagenesis (Materials and Methods). The mutagenic oligonucleotide was designed so as to create an *Nsi*I site as an outcome of the Ser-to-Ala codon change. Mutant clones were screened by an *Nsi*I restriction endonuclease digestion. The clone mCD46 (Table 2) was sequenced to verify that the only nucleotide changes were those created by the oligonucleotide. Sequencing was done only up to the 624th base away from the multiple cloning site of the template DNA. This was done so that a *Nae*I fragment that was well contained within the 624-base region could later be used to replace a fragment of identical size in the plasmid bearing the wild-type sequence and thus preclude the need to sequence the entire 2-kb fragment used for the mutagenesis. For introduction of a *srfA* fragment bearing the Ser-to-Ala mutation into *B. subtilis*, the plasmid pMMN130 (Fig. 1) was constructed as shown in Table 2. This was used to transform OKB105 (31) with selection for Cm^r to yield the strain LAB1298. Two different isolates of this strain were subjected to a plasmid-curing protocol (Materials and Methods) to obtain independent Cm^s segregants, some of which were Srf$^-$ (LAB1309 series) and others of which were Srf$^+$ (LAB1335 series). In order to verify the presence of the mutation in the Srf$^-$ segregants and its absence in the Srf$^+$ segregants, Southern hybridization was performed. Chromosomal DNAs from OKB105 (wild type), LAB1335-1 (Srf$^+$ segregant) and independently isolated mutants LAB1309-2, -4, -5, -6, and -7 were digested with *Nsi*I. Following blotting, the digested DNA was hybridized to an RNA probe synthesized with linearized pMMN40 (30) (Fig. 1) and T7 RNA polymerase. The mutation resulted in the appearance of a 0.54-kb fragment which was found to be absent in the Srf$^+$ segregant and the wild type and thus was a diagnostic feature of the mutants (Fig. 2). The RNA probe also hybridized to other fragments of 7.6 and 6.0 kb in the Srf$^+$ species and to 7.6-, 5.5-, and also 6.0-kb fragments arising because of partial digestion at the mutation site.

For the mutagenesis of the amino-acylation motif of the third domain, a 1.3-kb *Hin*dIII-*Eco*RI fragment (Fig. 1) of pMMN40 was inserted into M13mp9 to yield mCD31 (Table 2, Fig. 1), the single-stranded form of which was used for the oligonucleotide-directed mutagenesis. The mutagenic oligonucleotide was designed so that the codon change of Ser to Ala would result in the loss of a *Hin*fI site in the mutated sequence. The construction of the plasmid (pCD36 [Fig. 1]) used to deliver the Ser-to-Ala mutation in the *B. subtilis* chromosome is shown in Table 2. The same procedure as was used in creating the Ser-to-Ala mutation in *srfAA2* was followed. Southern hybridization was employed to verify the presence of the mutation as shown in Fig. 3. The 0.78-kb band in the Srf$^+$ species is shifted to the 1.1-kb position in the Srf$^-$ species on account of the loss of the *Hin*fI site as a

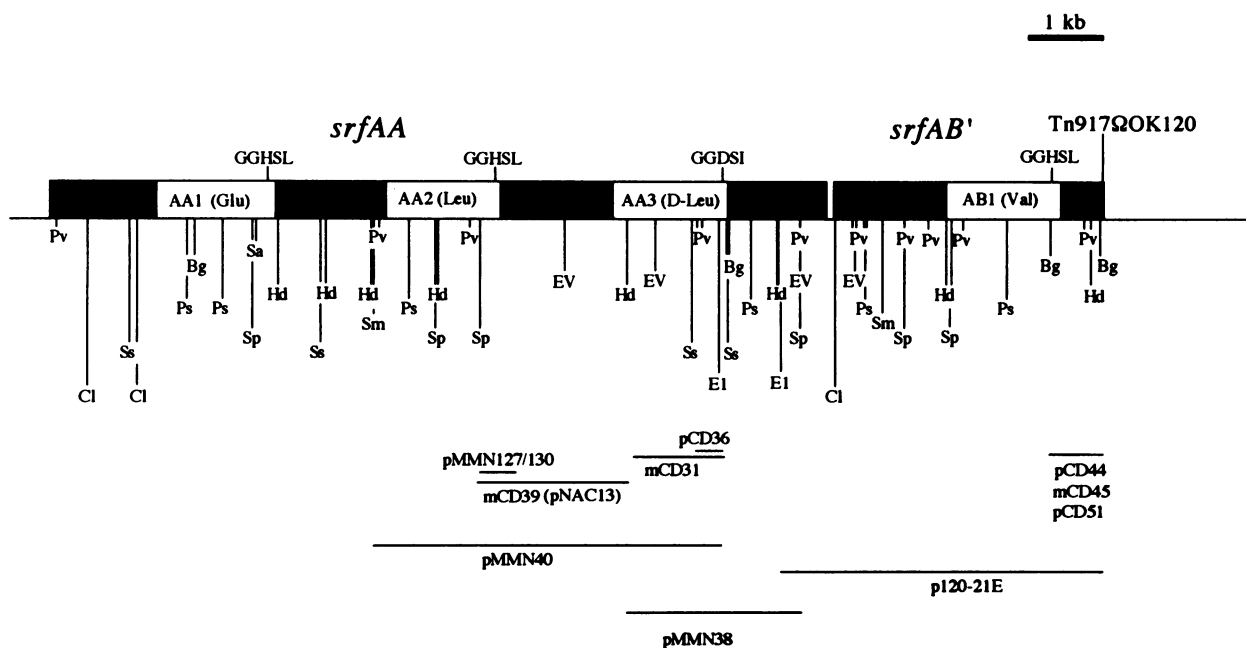

FIG. 1. Organization of the 5′ end of the *srfA* operon and the gene products encoded therein. Included is a diagram showing the restriction map of the 5′ end of *srfA* encoding the first four amino acid-activating domains of surfactin synthetase. The first gene (*srfAA*) and part of the second gene (*srfAB*) are indicated, as is the approximate site of the Tn*917* ΩOK120 insertion. Restriction endonuclease sites: Bg, *Bgl*II; Cl, *Cla*I; EV, *Eco*RV; Hd, *Hin*dIII; Ps, *Pst*I; Pv, *Pvu*II; Sa, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; Ss, *Sst*I. The inserts of the plasmids used in the study are indicated by the lines below the *srfA* restriction map. The white boxes indicate the locations within the *srfA* gene products of the amino acid-activating domains, whereas the black boxes indicate the location of the nonhomologous spacers. The boundaries for the domains correspond to the domain homologies described by Turgay et al. (43). The amino-acylation sites (GGHSL or GGDSI [41]) of each amino acid-activating domain are shown.

result of the mutation. The disappearance of some bands in lane 5 (LAB1195-23) suggested that the particular strain contained a deletion in its chromosome.

In order to mutagenize the amino-acylation motif of the fourth domain, a 0.69-kb *Cla*I-*Sma*I fragment (Fig. 1) of pCD44 (Table 2) was inserted into M13mp9 to create mCD45 (Table 2), and the single-stranded form of mCD45 was used for the mutagenesis. The mutagenic oligonucleotide creates an *Nsi*I site as an outcome of the Ser-to-Ala codon change. Plasmid pCD51 (Fig. 1 and Table 2) was used to deliver the mutation to the *srfAB1* domain by transformation of OKB105. The mutants were created and identified as described above. Southern hybridization analysis confirmed the presence of the mutation in the Srf$^-$ species and its absence in the Srf$^+$ species (Fig. 4). The presence of the mutation in the Srf$^-$ species was shown by the release of a 0.55-kb fragment which was not observed in the case of the wild-type and Srf$^+$ segregants. The probe also hybridizes to a 7.6-kb band in the Srf$^+$ species, which is reduced to 7.1 kb in the Srf$^-$ species (Fig. 4).

These results indicate that the Ser-to-Ala mutations in the amino-acylation motifs of the first four domains of *srfA* lead to the defect in surfactin production.

Effect of the mutations in the amino-acylation motifs on competence development. Since we showed that the mutations in the amino-acylation motifs abolish the ability to synthesize surfactin, we next determined if these mutations affect competence development. The mutants and the Srf$^+$ segregants of each domain along with the wild-type (OKB105) and a Δ*srfA* strain (LAB848) were transformed with chromosomal DNA from strain BD1512 which bears a *lacZ* fusion to the late competence operon *comG* (Materials and Methods). Cells of strain LAB848 have undergone a deletion of DNA from the site of the Tn*917* ΩOK120 insertion to the *Sal*I site within the sequence of *srfAA* encoding the SrfAA1 (Glu) domain. This region has been replaced with DNA bearing a phleomycin resistance (Phleo^r) marker. The transformation frequencies of these strains were then determined. Cells of the wild type and the Δ*srfA* strain served as positive and negative controls, respectively. The competence levels of the independent mutants and Srf$^+$ segregants of each domain were found to be comparable to that of the wild type, indicating that the Ser-to-Ala mutations in the amino-acylation motifs in *srfA* have no effect on competence (Table 3). The above strains were in an *sfp* genetic background. The *sfp* gene has been shown to be required for surfactin production (29, 34). In order to test the possibility that there might be a genetic background-dependent difference in competence levels of the wild type and the mutants, the effect of the mutation in the first domain was analyzed in an *sfp*° background (data not shown). No such difference was observed.

Effect of the amino-acylation site mutations on *comG-lacZ* expression. The confirmation of the observation that the amino-acylation site mutations in the *srfA* competence region have no significant effect on transformation ability was obtained by the assay of β-galactosidase-specific activity in cells bearing the *comG-lacZ* fusion (Fig. 5). *comG* is an operon whose products function in late stages of competence development (1, 2, 5). The β-galactosidase activities in mutant strains are comparable to those in wild-type and Srf$^+$ segregants.

All the above results show that the Ser-to-Ala mutations in the amino-acylation motifs of the first four amino acid-

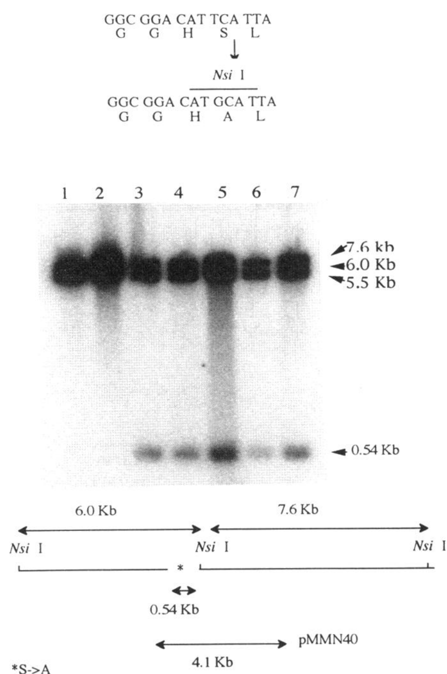

FIG. 2. Construction of the amino-acylation site mutation of the second domain (Leu) encoded by *srfA*. At the top are the nucleotide sequences and corresponding amino acid sequences (below each nucleotide sequence) of the wild type and the mutant amino-acylation sites. At the bottom is the *Nsi*I map of the region encompassing the amino-acylation site. The 0.54-kb fragment indicated is generated by the cleavage at the new *Nsi*I site created by the in vitro-generated mutation (*). Also shown is the autoradiograph of the Southern blot analysis of *Nsi*I-cleaved chromosomal DNA. A [^{32}P]RNA transcript of the pMMN40 (30) insert (indicated at the bottom) was used as a probe. Lane 1, OKB105 (wild type); lane 2, LAB1335-1 (Srf$^+$ segregant); lanes 3 through 7, independently isolated mutants LAB1309-2, -4, -5, -6, and -7, respectively.

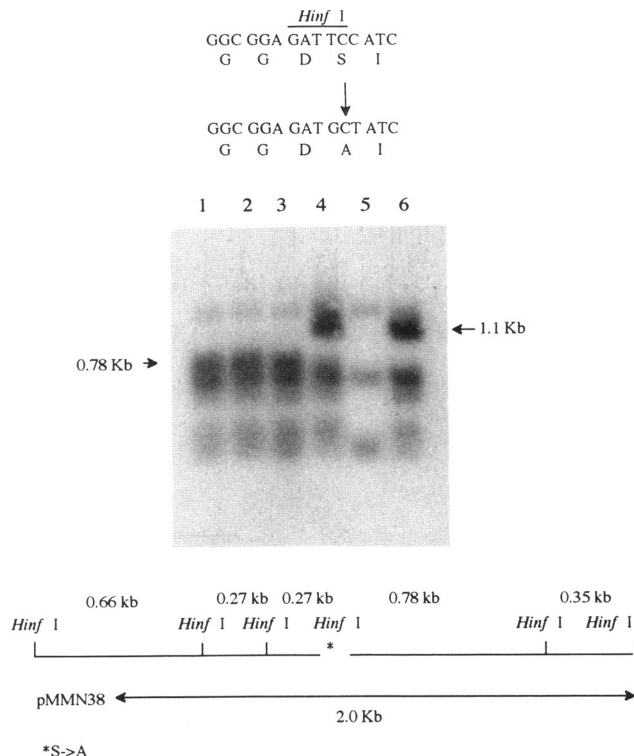

FIG. 3. Construction of amino-acylation site mutation in the third amino acid-activating domain (D-Leu) of *srfA*. At the top are the nucleotide sequences and the corresponding amino acid sequences (below the nucleotide sequence) of the wild type and the Ser-to-Ala mutant. At the bottom is the *Hin*fI restriction map of the region encompassing the third domain amino-acylation site. The *Hin*fI site that is lost by creation in vitro of the Ser-to-Ala mutation is indicated (*). This results in the formation of a 1.1-kb fragment as indicated in the autoradiograph of the Southern analysis of *Hin*fI-cleaved chromosomal DNA shown in the figure. A [^{32}P]RNA transcript of the pMMN38 (30) insert (indicated at the bottom) was used as a probe. Lane 1, OKB105 (wild type); lanes 2 and 3, LAB1336-1 and LAB1336-18 (independently isolated Srf$^+$ segregants), respectively; lanes 4, 5, and 6, LAB1195-2, -23, and -24 (independently isolated mutants), respectively. Mutant LAB1195-23 had undergone a deletion and was not used for further analysis.

activating domains of *srfA*, while causing a loss of surfactin production, have no effect on competence development.

DISCUSSION

The part of *srfA* that lies upstream of the site of Tn*917* ΩOK120 was subjected to site-directed mutagenesis in order to determine if the motifs previously known to be the amino-acylation sites of peptide synthetases were required for peptide antibiotic synthesis in vivo. This part of *srfA* encodes the first four amino acid-activating domains of surfactin synthetase and contains the region required for competence development (8, 30). The conserved Ser codon within the amino-acylation motifs of the four domains was changed to an Ala codon. Each of the four mutations abolished surfactin production (49) but had no effect on the development of genetic competence, nor did they affect significantly the expression of the late competence operon *comG*, as judged by examining the activity of a *comG-lacZ* fusion.

The amino-acylation sites of peptide synthetases were identified when proteolytic fragments of gramicidin synthetase 2 that were covalently bound to ^{14}C-labeled constituent amino acids of gramicidin were isolated and subjected to sequence analysis (41). Vater and coworkers (41) determined that the site contained the motif GG H/D S L/I, a sequence similar to conserved active sites of polyketide synthetases and acyl carrier proteins of fatty acid synthetases (39). They proposed that this may be the site of the 4′-phosphopantetheine cofactor known to be involved in peptide synthesis and fatty acid synthesis. The results presented here show that the conserved Ser is required for peptide synthesis in the case of the lipopeptide surfactin which, like gramicidin, is synthesized by the multienzyme thiotemplate mechanism (17, 22, 24). If, as proposed, the conserved Ser is the site of attachment of a 4′-phosphopantetheine prosthetic group, then the original thiotemplate model of peptide synthesis may have to be modified to include the feature of multiple pantetheine cofactors associated with the peptide synthetase complex. One could imagine that the activated amino acids would be covalently bound to the Ser–4′-phosphopantetheine group by a carboxyl thioester. Translocation of the growing peptide chain may then involve the exchange of the oligopeptide from one domain pantetheine group to the next with concomitant peptide bond formation. Further in vitro characterization of

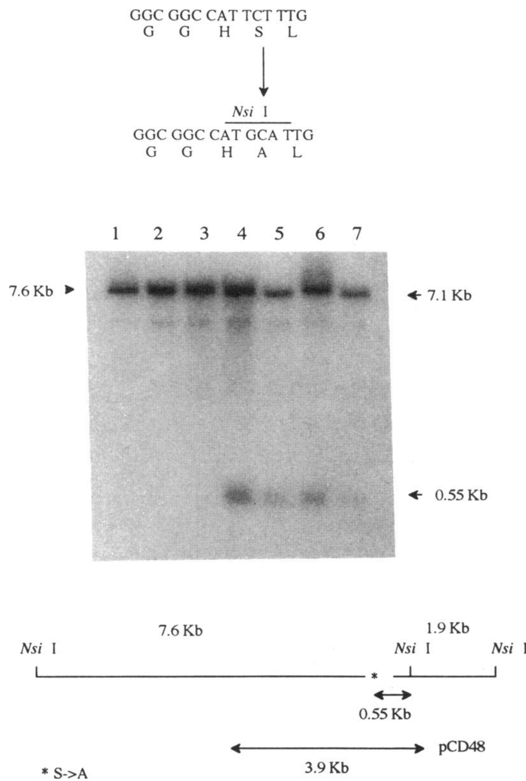

FIG. 4. Construction of the amino-acylation site mutation in domain four (Val) of *srfA*. At the top are the nucleotide sequences and the corresponding amino acid sequences (below the nucleotide sequence) of the wild type and the Ser-to-Ala mutant. At the bottom is the *Nsi*I restriction map of the region encompassing the amino-acylation site of the fourth domain. The 0.55-kb *Nsi*I fragment is generated by the creation of the *Nsi*I site in the construction of the Ser-to-Ala mutation (*). Also shown is the autoradiograph from the Southern analysis of *Nsi*I-cleaved chromosomal DNA. A [^{32}P]RNA transcript of the pCD48 insert (indicated at the bottom) was used as a probe. Lane 1, OKB105; lanes 2 and 3, LAB1337-5 and 1337-10 (Srf$^+$ segregants), respectively; lanes 4 through 7, LAB 1292-1, -2, -3, and -4 (mutants), respectively.

the surfactin synthetase complex of the Ser-to-Ala mutants will likely provide important information regarding the multienzyme thiotemplate mechanism.

The part of the 5′ end of *srfA* that was subjected to mutagenesis contains the region required for the development of genetic competence (8, 30). Recent studies of van Sinderen et al. (11, 45) suggest that only the region of *srfA* encoding the fourth domain (SrfAB domain 1) is required for competence development. The results presented here show that the amino-acylation site mutation of the fourth domain, although conferring a Srf$^-$ phenotype on blood agar, does not affect competence or *comG-lacZ* expression.

Some insight into how *srfA* may function in competence development is gained from a potentially interesting comparison between the primary structure of the Ser-to-Ala allele of strain LAB1292 and the product of the *angR* gene (7) of marine bacterium *Vibrio anguillarum*, which is required for the production of anguibactin, an iron siderophore. AngR shows significant sequence similarity to the amino acid-activating domains of SrfA and other peptide synthetases (8). At its amino-acylation motif is the sequence LSGGDA̲Y, indicating that the conserved Ser found in peptide synthetases is replaced by an Ala (underlined), the exact substitution made in the mutants described in this report. Other adenylylating enzymes, such as the product of *entE* (42) which functions in the synthesis of the iron siderophore enterobactin, also resemble in primary structure the peptide synthetase amino acid-activating domains but are truncated homologs that lack the amino-acylation motif (43). There is evidence that amino acid-activating domains of peptide synthetases that lack amino-acylation sites can catalyze adenylylation reactions. A fragment of the Pro-activating domain of gramicidin synthetase 2, although lacking the amino-acylation motif, could still catalyze the adenylylation of proline (14, 23). This fragment also lacked a bound 4′-phosphopantetheine prosthetic group. Interestingly, when this fragment is combined with gramicidin synthetase I, the dipeptide Phe-Pro is made. More recently, Marahiel and coworkers (27) have created the Ser-to-Ala mutation in the amino-acylation motif of the amino acid-activating domain of TycA and examined its activity by using the ATP-PP_i exchange reaction. This mutation had no effect on ATP-PP_i exchange. However, the Ser-to-Ala mutation in the amino-acylation motif of the *srfAA1*-encoded domain of *srfA* results in loss of glutamate-dependent ATP-PP_i exchange activity (49). Because of these contradictory results, it is not possible at this time to determine if the in vitro analysis of the active site mutations by the ATP-PP_i exchange assay provides an accurate reflection of the in vivo situation. It is possible that the SrfAB1 (Val) domain, in a manner analogous to the Pro-activating fragment of gramicidin synthetase 2, catalyzes a Val-adenylylation reaction that somehow serves as a signal or is followed by the attachment of Val to an amino

TABLE 3. Transformation frequency of the amino-acylation site mutants

Domain and strain[a]	Transformation frequency (10^3)[b]
SrfAA1	
OKB105 (wild type)	0.13, 0.12
LAB848 (Δ*srfA*)	0.0005, <0.0001
LAB1334 (Srf$^+$ segregant)	0.06, 0.03
LAB960-4[b] (S-to-A mutant)	0.02, 0.04
LAB960-7 (S-to-A mutant)	0.19, 0.06
SrfAA2	
OKB105 (wild type)	1.5, 2.2
LAB848 (Δ*srfA*)	0.0003, 0.0004
LAB1309-2 (S-to-A mutant)	1.4, 2.6
LAB1309-4 (S-to-A mutant)	1.3, 2.3
LAB1335-1 (Srf$^+$ segregant)	0.80, 2.1
SrfAA3	
OKB105 (wild type)	0.23, 0.08
LAB848 (Δ*srfA*)	0.0001, 0.0002
LAB1195-2 (S-to-A mutant)	0.23, 0.15
LAB1195-24 (S-to-A mutant)	0.21, 0.32
LAB1336-1 (Srf$^+$ segregant)	0.16, 0.70
LAB1336-18 (Srf$^+$ segregant)	0.02, 0.14
SrfAB1	
OKB105 (wild type)	0.90, 0.38
LAB848 (Δ*srfA*)	<0.0001, <0.0001
LAB1292-2 (S-to-A mutant)	0.15, 0.37
LAB1292-4 (S-to-A mutant)	0.13, 0.06
LAB1337-5 (Srf$^+$ segregant)	0.18, 0.17

[a] Hyphenated strain name indicates independently isolated mutant.
[b] Data from two experiments are presented.

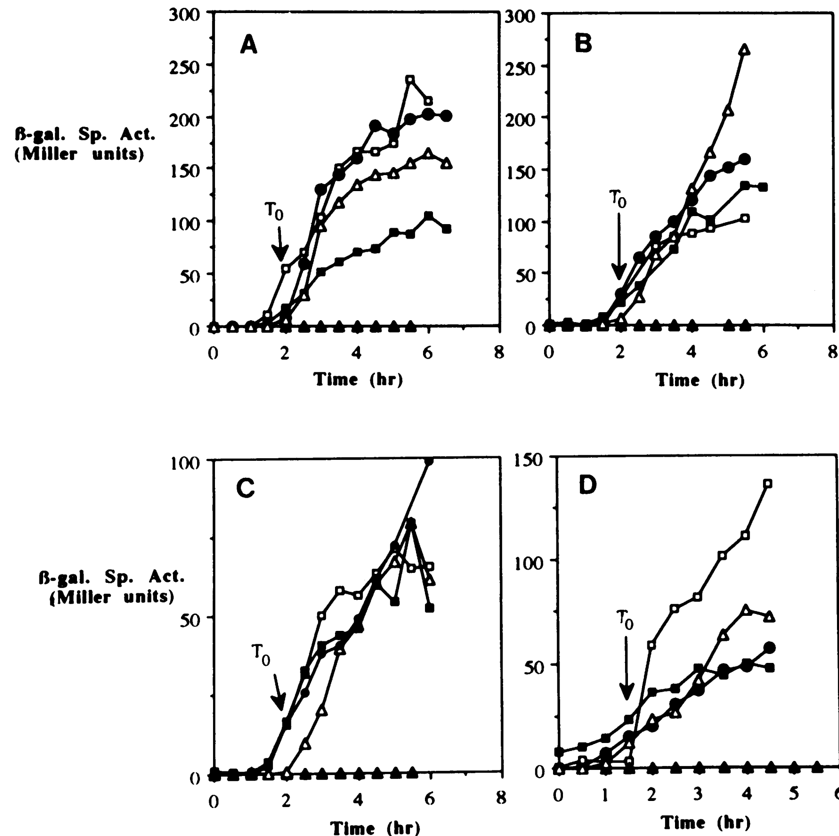

FIG. 5. Expression of *comG-lacZ* in each of the mutants bearing mutationally altered amino-acylation sites. Cells bearing the *comG-lacZ* fusion previously described (2) were grown in one-step competence medium (6). T_0 is the point in the growth of the cultures when exponential phase ends. β-Galactosidase specific activity is in Miller units and was determined as described previously. (A) *srfA* domain 1. LAB961 (*srfA*$^+$), □; LAB962 (Δ*srfA*::pNAC14), ▲; LAB963 [*srfA*$^+$ Δ(pCD23) Srf$^+$ segregant], ■; LAB965 (*srfAA1* S-A of LAB960-4 [49]), ●; LAB965 (*srfAA1* S-A of LAB960-7 [49]), △. (B) *srfA* domain 2. LAB961 (*srfA*$^+$), □; LAB962 (Δ*srfA*::pNAC14), ▲; LAB1310 [*srfA*$^+$ Δ(pMMN130) Srf$^+$ segregant], ●; LAB1311 (*srfAA2* S-A of LAB1304-2), △; LAB1311 (*srfAA2* S-A of LAB1304-4), ■. (C) *srfA* domain 3. LAB961 (*srfA*$^+$), □; LAB962 (Δ*srfA*::pNAC14), ▲; LAB1252 [*srfA*$^+$ Δ(pCD36) Srf$^+$ segregant], ■; LAB 1253 (*srfAA3* S-A of LAB1195-2), ●; LAB1253 (*srfAA3* S-A of LAB1195-24), △. (D) *srfA* domain 4. LAB961 (*srfA*$^+$), □; LAB962 (Δ*srfA*::pNAC14), ▲; LAB1297 [*srfA*$^+$ Δ(pCD51) Srf$^+$ segregant], ●; LAB1307 (*srfAB1* S-A of LAB1292-2), △; LAB1307 (*srfAB1* S-A of LAB1292-4), ■.

acid or peptide, perhaps the product of another peptide synthetase. The resulting signal molecule is recognized by the cell which responds by activating the transcription of late competence genes. This would presumably be an internal signal, since no extracellular complementation of a competence-negative *srfA* mutant by wild-type cells has been reported. It is also possible that SrfAB1 (Val) possesses some other activity that functions in competence gene regulation which is unrelated to peptide synthesis or amino acid activation. Possibly related to this was a report that the *angR* product also contained a sequence resembling the helix-turn-helix motif of phage P22 Cro protein (7), although no other evidence that AngR is a DNA binding protein is available. Further investigation of SrfAB1 may uncover a unique regulatory component of the pathway controlling cell specialization in prokaryotes.

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