Identification of a Genetic Locus Required for Biosynthesis of the Lipopeptide Antibiotic Surfactin in *Bacillus subtilis*

MICHIKO M. NAKANO,¹* MOHAMED A. MARAHIEL,² AND PETER ZUBER¹†

Department of Botany and Microbiology, Oklahoma State University, Stillwater, Oklahoma 74078,¹ and Institut für Biochemie und Molekulare Biologie, Technische Universität Berlin, 1000 Berlin 10, Federal Republic of Germany²

Received 13 June 1988/Accepted 14 September 1988

Surfactin is a lipopeptide antibiotic produced by the cells of *Bacillus subtilis* ATCC 21332. A genetic locus responsible for surfactin production (*sfp*) was transferred by transformation from ATCC 21332 to JH642, a derivative of the standard *B. subtilis* 168. To study the *sfp* locus at the molecular level, a Tn917 insertion mutant that was blocked in surfactin production (*srf*) was isolated. The *srf*::Tn917 mutation was found to be closely linked to *sfp*, and both loci mapped by PBS1 phage transduction to the chromosomal region between *arol* and *mtlB*. These studies suggest that JH642, a strain which is not a producer of surfactin (genotypically *sfp*⁰), contains at least some of the genes encoding surfactin production. Expression of the *srf* gene(s) was examined in both *sfp* and *sfp*⁰ cells by assaying β -galactosidase activity encoded by a promoterless *lacZ* gene that was fused to the *srf*::Tn917 insertion. In cells of both strains, *srf*-directed β -galactosidase activity increased when cells entered the stationary phase of the growth curve, but the activity in *sfp* cells was higher than that in *sfp*⁰ cells. *srf*-*lacZ* expression was partially impaired by a mutation in *spo0A*. In *sfp*⁰ cells, this dependence on the *spo0A* gene product could be entirely bypassed by an *abrB* suppressor mutation. In the *sfp* cells, the *abrB* mutation could not restore the defect conferred by the *spo0A* mutation. These data suggest that the *sfp* locus, which is responsible for surfactin production, alters the transcriptional regulation of *srf* in JH642 cells.

A lipopeptide antibiotic, surfactin, produced by *Bacillus subtilis* was first identified as a potent inhibitor of fibrin clotting (1) and later found to lyse erythrocytes as well as spheroplasts and protoplasts of some bacterial species (3). Surfactin is also the most powerful biosurfactant known (lowering the surface tension of water from 72 to 27 mN/m) (6) and as such may have many potential industrial applications, which include uses related to oil recovery, emulsion stabilization, etc. (5). Biosurfactants provide an advantage over synthetic surfactants because most are biodegradable and, hence, less toxic.

In *B. subtilis*, the production of secondary metabolites such as antibiotics is but part of a complex response to conditions of nutritional deprivation. These conditions will also induce a process of cellular differentiation called sporulation, which gives rise to the formation of a highly resistant and metabolically inert cell type called the endospore (17, 23). Genetic studies have provided strong evidence that the expression of genes encoding sporulation-related functions is under common mechanisms of control. The genetic examination of antibiotic biosynthesis and its regulation is one approach to understanding these control mechanisms. However, as compared with studies of endospore formation and sporulation-related proteases, there have been very few investigations into the molecular genetics of antibiotic production in *B. subtilis*. Only the gene for the DNA-encoded antibiotic subtilin has been molecularly cloned (2). Recently, a genetic locus involved in the biosynthesis of the dipeptide antibiotic bacilysin was mapped in the *B. subtilis* 168 genome (11). The antibiotic biosynthesis genes encoding gramicidin S synthetase 2 (15) and tyrocidine synthetase 1 (19) from *Bacillus brevis* have been cloned, as have the genes for bacitracin biosynthesis from *Bacillus licheniformis* (L. Kor-

sens, M. O. Gulliksen, A. Sundan, and A. Nerland, Third International Conference on Genetics and Biotechnology of Bacilli, p. 65, 1985), which have also been mapped (25). Antibiotic biosynthesis genes of *Streptomyces* spp. have been successfully cloned. For example, the genes encoding the enzymes that catalyze actinorhodin biosynthesis were cloned from *Streptomyces coelicolor*. The introduction of the cloned genes to the heterologous nonproducer host *Streptomyces parvulus* led to the production of the antibiotic (18). *B. subtilis* may offer a useful system for studying the molecular genetics of antibiotic production. Simple and efficient gene transfer systems such as generalized transduction and transformation have led to the construction of an extensive genetic map of the *B. subtilis* genome. Additionally, powerful strategies can be designed for studying the regulation of gene expression in *B. subtilis* with the recent development of new techniques such as transposon mutagenesis (29) and gene fusion analysis (32).

The production of surfactin is easily detected, since a colony of producer cells will generate a zone of lysis on solid medium containing erythrocytes. We have taken advantage of this property to initiate a genetic study of surfactin production which is aimed at the identification and isolation of the surfactin biosynthesis genes. In this report, we describe the discovery of two genetic loci that are involved in surfactin production.

MATERIALS AND METHODS

Bacterial strains and plasmid. Bacterial strains are listed in Table 1. All *B. subtilis* strains except ATCC 21332 are derivatives of strain 168. The plasmid pTV55 (31) was obtained from P. Youngman.

Culture media. 2xyt medium contained 16 g of Bacto-Tryptone (Difco Laboratories), 10 g of yeast extract (Difco), and 5 g of NaCl per liter. Luria-Bertani (LB) medium was as previously described (21). DSM medium was Difco sporulation medium, which contains Difco nutrient broth with 0.6 ml of 1 M NaOH, 10 ml of 1.2% MgSO₄, and 10 ml of 10%

* Corresponding author.

† Present address: Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, Kings Highway, P.O. Box 33932, Shreveport, LA 71130-3932.

TABLE 1. *B. subtilis* strains

Strain	Genotype	Source or reference
ATCC 21332	<i>sfp</i>	6
IA4	<i>aroI906 dal-1 purB33 trpC2</i>	Bacillus Genetic Stock Center
IA54	<i>mtlB1 narB1 sacA321</i>	Bacillus Genetic Stock Center
IS251	<i>trpC2 spo0BΔPst</i>	I. Smith
JH642	<i>trpC2 pheA1</i>	J. Hoch
MI100	<i>trpC2 pheA1 sigB::cat</i>	M. Igo (13)
ZB369	<i>trpC2 pheA1 spo0AΔ204 abrB703</i>	20
ZB480	<i>trpC2 pheA1 spo0HΔHind Tn917 HU146</i>	
ZB515	<i>trpC2 pheA1 spo0AΔ204</i>	
OKB105	<i>pheA1 sfp</i>	This paper
OKB120	<i>pheA1 srf::Tn917 sfp</i>	This paper
OKB121	<i>pheA1 sigB::cat sfp</i>	This paper
OKB122	<i>aroI906 dal-1 purB33 trpC2 sigB::cat</i>	This paper
OKB125	<i>pheA1 srf-lacZ (pTV55) sfp</i>	This paper
OKB126	<i>mtlB1 narB1 sacA321 sigB::cat</i>	This paper
OKB127	<i>mtlB1 aroI906 dal-1 purB33 trpC2 sigB::cat</i>	This paper
OKB142	<i>pheA1 srf::Tn917</i>	This paper
OKB143	<i>pheA1 srf-lacZ (pTV55)</i>	This paper
OKB145	<i>trpC2 pheA1 spo0AΔ204 abrB703 srf-lacZ</i>	This paper
OKB146	<i>trpC2 pheA1 spo0AΔ204 srf-lacZ</i>	This paper
OKB148	<i>trpC2 spo0BΔPst srf-lacZ</i>	This paper
OKB152	<i>pheA1 spo0AΔ204 sfp srf-lacZ</i>	This paper
OKB153	<i>spo0BΔPst sfp srf-lacZ</i>	This paper
OKB154	<i>pheA1 spo0HΔHind sfp srf-lacZ</i>	This paper
OKB159	<i>trpC2 pheA1 spo0AΔ204 abrB703 sfp srf-lacZ</i>	This paper
OKB162	<i>trpC2 pheA1 spo0HΔHind srf-lacZ</i>	This paper

KCl per liter. After autoclaving it was supplemented with 1 ml of 0.01 M MnCl_2 , 1 M $\text{Ca}(\text{NO}_3)_2$, and 0.001 M $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ per liter. LB agar and DSM agar media were made by supplementing LB and DSM media with 1.2% agar. To make minimal medium (MM) agar, 10 ml of 1.2% MgSO_4 , 10 ml of 50% glucose, 100 ml of 10xT base [20 g of $(\text{NH}_4)_2\text{SO}_4$, 140 g of K_2HPO_4 , 60 g of KH_2PO_4 , and 10 g of sodium citrate per liter], and 20 ml of 0.5% glutamate were added to 1 liter of 1.2% agar after autoclaving.

The resistance to macrolides, lincosamides, and streptogramin B (MLS^r) conferred by Tn917 was induced by incubation in media containing sublethal levels of erythromycin. In 2xyt medium, cultures were incubated for 1 h at 37°C with erythromycin at 0.15 μg/ml. MLS^r was selected with erythromycin at 1 μg/ml and lincomycin at 25 μg/ml. For direct plating on agar plates, cells were plated with LB soft agar overlay supplemented with 4 μl of erythromycin (1 mg/ml) for induction. After induction for 1.5 h at 37°C, a second overlay of LB soft agar with 40 μl of erythromycin (1 mg/ml) and lincomycin (25 mg/ml) was applied. Chloramphenicol resistance (Cm^r) was selected on LB agar medium with chloramphenicol at 5 μg/ml. Cm^r was induced by the addition of chloramphenicol to 0.1 μg/ml.

Surfactin production was assayed on blood agar plates made by pouring 5 ml of MM soft agar overlay supplemented with 0.5 ml of bovine blood onto MM agar with nutritional requirements. Antibiotic production was tested on LB agar plates overlaid with 3 ml of LB soft agar containing Spo0A (ZB515) or *Staphylococcus aureus* cells.

Transformation and transduction. Competent cells of *B. subtilis* were prepared and transformed by the method of

Gryczan and Dubnau (9). Transduction by PBS1 phage was performed as described by Hoch et al. (12).

Isolation of Tn917 insertion mutants defective in surfactin production. Surfactin-producing OKB105 cells were transformed with DNA from a Tn917 insertion library prepared by Sandman et al. (26). MLS^r was induced in 2xyt medium as described above. MLS^r *srf* transformants were selected on blood agar plates containing selecting levels of erythromycin and lincomycin.

Construction of *spo0 srf-lacZ* strains. Various *spo0 srf-lacZ* strains bearing either *sfp* or *sfp*⁰ were constructed. These were isogenic except for strains that had lost an auxotrophic marker in congression experiments.

*spo0A sfp*⁰ and *spo0A abrB sfp*⁰ strains carrying *srf-lacZ*, called OKB146 and OKB145, respectively, were isolated by transforming ZB369 (*spo0A abrB*) with OKB143 DNA, selecting for Cm^r, and screening for *spo0A* and *spo0A abrB* phenotypes. The screening was carried out by examining colonies on DSM agar plates for sporulation and antibiotic production as described above; 98 and 2% of the transformants were *spo0A abrB* and *spo0A*, respectively. A *spo0B sfp*⁰ strain carrying *srf-lacZ*, OKB148, was constructed by transforming strain IS251 (*spo0B*) with OKB145 DNA with selection for Cm^r. Screening of the transformants for sporulation and antibiotic production revealed that the transformation resulted in *spo0B* at the frequency of 82%, *spo0B abrB* at 7%, *spo*⁺ at 9%, and *spo0A spo0B* at 2%. A *spo0H srf-lacZ sfp*⁰ strain, OKB162, was made by transforming ZB480 (*spo0H*) with OKB143 DNA with selection for Cm^r. The *spo0H* phenotype was screened by examining colonies for sporulation and antibiotic production.

A *spo0A sfp* strain carrying *srf-lacZ*, called OKB152, was constructed by transforming OKB105 (*sfp*) cells with OKB125 (*srf-lacZ sfp*) DNA at a low concentration and ZB515 (*spo0A*) DNA at a high concentration. Transformants were selected for Cm^r and screened for the sporulation-negative (Spo⁻) and antibiotic production negative (Ab⁻) phenotypes; 1% of the transformants were *spo0A* congressants. A *spo0A abrB sfp* strain carrying *srf-lacZ*, OKB159, was constructed by transducing ZB369 (*spo0A abrB*) with a PBS1 lysate of OKB125 (*srf-lacZ sfp*). Transductants were selected for Cm^r and screened for the *spo0A abrB* phenotype (Spo⁻ Ab⁺) as described above. A *spo0B srf-lacZ sfp* strain, OKB153, was constructed by transforming OKB105 (*sfp*) with OKB125 (*srf-lacZ sfp*) DNA at a low concentration and IS251 (*spo0B*) DNA at a high concentration. Cm^r transformants were screened for the *spo0B* phenotype; 1% of the transformants were *spo0B* congressants. A *spo0H srf-lacZ sfp* strain, OKB154, was constructed by transforming OKB105 (*sfp*) with a low concentration of OKB125 DNA and a high concentration of ZB480 (*spo0H*) DNA; 5% of the Cm^r transformants were found to be *spo0H*, and 2% were MLS^r (conferred by the Tn917 transposon that was linked to *spo0H*). These three strains, OKB152, OKB153, and OKB154, carrying *srf-lacZ* were confirmed to have *sfp* by purifying DNA from each strain and then transforming JH642 with selection for Trp⁺ and screening for surfactin production. As OKB159 was still auxotrophic for tryptophan, the *sfp* genotype was confirmed by transforming IA4 by using congression of *aroI*⁺.

β-Galactosidase assay. Cells were grown overnight on LB agar medium at 37°C. Culture tubes containing 2 ml of DSM supplemented with 0.002% tryptophan and 5 μg of chloramphenicol per ml were inoculated with a single isolated colony. Serial 1/10 dilutions to 10⁻⁵ were made of the initial inoculated tube, and all were incubated overnight at 37°C with shaking. Cells (0.5 ml) were transferred from the tube

FIG. 1. Surfactin production assayed on MM blood agar plates prepared as described in Materials and Methods. (A) ATCC 21332, (B) JH642, (C) OKB105, (D) OKB120.

containing a log-phase culture to 50 ml of the same medium and grown at 37°C with vigorous aeration. Samples were taken at regular intervals for determination of optical density (600 nm) and β -galactosidase specific activity. β -Galactosidase assays were done as previously described (21).

Purification of surfactin. Cells were grown in Landy medium (20 g of glucose, 5 g of glutamate, 0.5 g of MgSO_4 , 0.5 g of KCl, 1 g of KH_2PO_4 , 0.15 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.005 g of MnSO_4 , 0.16 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and 0.1% yeast extract per liter [pH 7.0]) (16) at 33°C for 30 h. Surfactin was purified as described by Cooper et al. (6). The cultured broth was brought to pH 2.0 by HCl, which resulted in a precipitate. This was dissolved in distilled water (adjusted to pH 7.0 with NaOH). The suspension was kept overnight at 4°C and then extracted with the same volume of dichloromethane (Merck Sharp & Dohme) for 30 min at room temperature. The dichloromethane phase was resolved by centrifugation and evaporated. The white precipitate was then dissolved in 0.5 to 1 ml of ethanol and used for chromatography and infrared spectrophotometry. Thin-layer chromatography was done with silica-gel plates (Schleicher & Schuell, Inc.). The solvent was chloroform-methanol-water (65:25:4, vol/vol). Components were visualized by spraying the plates with concentrated H_2SO_4 and heating at 110°C for 45 min. Infrared spectra were obtained on a Beckman IR 4230 spectrophotometer.

RESULTS

Transfer of surfactin production (*sfp*) locus to strain JH642. *B. subtilis* ATCC 21332 produces high levels of surfactin that are easily detected by lysis of erythrocytes (6) on a solid medium (Fig. 1). Since ATCC 21332 was difficult to render competent and is genetically uncharacterized, the locus responsible for surfactin production (*sfp*) was transferred from ATCC 21332 to cells of the strain 168 derivative JH642, which can be genetically manipulated and is a member of an isogenic collection of strains that includes *spo0* mutants.

A surfactin-producing derivative of JH642 was constructed by transformation with ATCC 21332 DNA with selection for Trp^+ and screening for surfactin production. A surfactin-producing clone (designated OKB105) was identified by replica plating of Trp^+ clones onto MM-blood agar plates (Fig. 1). The hemolytic activity of OKB105 was identified as surfactin by thin-layer chromatography and the characteristic infrared spectrum (Fig. 2) of surfactin as previously reported (1).

Isolation of Tn917-mediated *srf* mutant. Total DNA from a Tn917 insertion library prepared by Sandman et al. (26) was used to transform OKB105. Tn917 insertion mutants were selected for MLS^r , the antibiotic resistance phenotype conferred by Tn917. Among the colonies of MLS^r clones, those containing cells that lost the ability to synthesize surfactin were identified by examination on blood agar. The *srf* mutant OKB120 produced no surfactin, as judged by its phenotype on blood agar (Fig. 1). Antibacterial activity of OKB120 against *S. aureus* and *spo0A* cells was not affected by the insertion of Tn917.

Mapping of the *sfp* locus and the Tn917 insertion of the *srf* mutant. PBS1 transduction was performed to map both the *srf::Tn917* insertion and the *sfp* locus. Initial transduction experiments revealed that *srf::Tn917* was linked 20% (co-transduction frequency) to *sigB* (4) and 75% to *aroI*, or in an area near 40° on the *B. subtilis* genetic map (24). For three-factor crosses, strain OKB122 (*aroI sigB*) was constructed by transforming cells of strain IA4 with DNA from MI100 (13) with selection for Cm^r . PBS1 transducing lysates prepared from OKB120 cells were used to transduce OKB122 cells with selection for MLS^r or *aroI*⁺. At least 100 trans-

FIG. 2. (a) Thin-layer chromatography of hemolytic activities from ATCC 21332 (lane 1) and OKB105 (lane 2) with a standard surfactin (lane 3). (b) Infrared spectrum of surfactin extracted from OKB105. Surfactin was purified and assayed as described in Materials and Methods.

TABLE 2. PBS1-mediated three-factor crosses

Donor and selection	Recipient	Cotransduction					% Cotransduction
		MLS ^r	Cm ^r	<i>sfp</i>	<i>aroI</i>	<i>mtlB</i>	
OKB120 (1) ^a	OKB122 (0) ^b						
Selection for MLS ^r		1	1		1		6
		1	1		0		2
		1	0		1		68
		1	0		0		24
Selection for <i>aroI</i> ⁺		1	1		1		2
		1	0		1		74
		0	1		1		1
		0	0		1		23
OKB121 (1)	IA4 (0)						
Selection for Cm ^r			1	1	1		4
			1	1	0		26
			1	0	1		2
			1	0	0		68
OKB120 (1)	OKB127 (0)						
Selection for MLS ^r		1			1	1	46
		1			1	0	32
		1			0	1	11
		1			0	0	11
Selection for <i>aroI</i> ⁺		1			1	1	39
		1			1	0	28
		0			1	1	3
		0			1	0	30
Selection for <i>mtlB</i> ⁺		1			1	1	30
		1			0	1	17
		0			1	1	0
		0			0	1	53

^a 1, Donor phenotype.^b 0, Recipient phenotype.

ductants were tested to determine cotransduction frequencies in each cross. Based on the data summarized in Table 2, the order of the markers can be either *aroI*-Tn917-*sigB* or Tn917-*aroI*-*sigB*.

It was necessary to determine the genetic map position of *sfp*, since it was possible that *srf*::Tn917 insertion had occurred at a position that is not linked to *sfp*. This was carried out by PBS1 transduction with strain OKB121 as a donor. OKB121 was constructed by transforming OKB105 cells with DNA of MI100. IA4 cells were transduced with selection for Cm^r by using a PBS1 lysate of OKB121 cells. Transductants were tested for the presence of the *aroI* and *sfp* markers (Table 2). The *sfp* locus mapped in the same region of the chromosome as *srf*::Tn917. Based on the three-factor cross data, the order of the three markers can be either *aroI*-*sfp*-*sigB* or *sfp*-*aroI*-*sigB*.

To more accurately determine the arrangement of markers in the region where the surfactin loci mapped, an *aroI* *mtlB* (mannitol utilization) strain was constructed and used in further transduction experiments. Cells of strain IA54 were transformed with DNA from MI100 with selection for Cm^r. A transformant, designated OKB126 (*mtlB* *sigB*::*cat*), was used to generate a PBS1 lysate. Cells of strain IA4 were then transduced with the lysate with selection for Cm^r. One of the transductants, designated OKB127 (*aroI* *mtlB* *sigB*::*cat*), was a recipient in transductional mapping experiments with a PBS1 lysate generated from cells of strain OKB120. The data from the three-factor crosses (Table 2 and Fig. 3) showed that the Tn917 insertion causing the *srf* phenotype was mapped between *aroI* and *mtlB*.

Segregation of *sfp* from the Tn917 insertion. Transduction of OKB122 cells with PBS1 phage that were propagated in

FIG. 3. Genetic map of *srf*::Tn917. The data used here were derived from Table 2. Arrows point from unselected to selected markers. Figures represent 100 minus the percent cotransduction.

cells of OKB120 gave rise to *aroI*⁺ transductants that were MLS^s and produced surfactin. OKB122 does not produce surfactin; therefore, the surfactin production phenotype must have resulted from the segregation of the *sfp* locus present in OKB120 from the *srf*::Tn917 insertion. This strongly indicated that the impairment of surfactin production in OKB120 was caused by the Tn917 insertion itself and not by replacement of the *sfp* DNA of OKB105 with *sfp*⁰ DNA from the transposon library that was used to construct OKB120. Although *sfp* and *srf*::Tn917 are linked by transduction, infrequent recombinations can be detected which cause the two to segregate. This strongly suggests that at least some of the genes required for surfactin production were present originally in JH642 cells, which do not produce surfactin.

It was necessary to construct a *srf*::Tn917 *sfp*⁰ strain to examine the effect of *sfp* on the expression of *srf*-*lacZ* in the experiments described below. JH642 was transformed with OKB120 DNA with selection for MLS^r and Trp⁺ (the latter was required for subsequent congression experiments described below). We predicted that many of the transformants would be *sfp*⁰, since previous transformation experiments which introduced Tn917 into OKB105 (*sfp*) cells failed to yield *sfp*⁰ recombinants. To test this possibility, five MLS^r Trp⁺ transformants were picked, and chromosomal DNA was prepared from each and used to transform JH642. Transformants were selected for Trp⁺ and screened for surfactin production. OKB120 (*sfp* *srf*::Tn917) DNA was also used to transform JH642 cells as a control. If the donor cells were *sfp*, then transformation of JH642 cells would give rise to surfactin-producing congressants. Surfactin-producing (*sfp*) cells were detected among the Trp⁺ colonies after transformation with DNA from one of the five isolates and from OKB120. However, DNA from the remaining four strains did not give rise to *sfp* congressants. We obtained the same results when the congression experiments were repeated three times and concluded that the four MLS^r Trp⁺ transformants were *sfp*⁰. One of the four strains (OKB142) was chosen for further experiments.

Construction of Tn917-mediated *srf*-*lacZ* fusion. The direction of transcription was determined by introducing a promoterless *lacZ* gene of plasmid pTV55 (31) into the *srf*::Tn917 insertion. OKB120 and OKB142 were transformed with pTV55 that had been cleaved at the unique *Pst*I site. Transformants were selected for Cm^r and were grown at 45°C, which prevents autonomous replication of pTV55. Since the transformants were also MLS^s, they must have arisen as a result of the marker-replacement recombination between the plasmid DNA and the transposon (Fig. 4). Colonies of the Cm^r MLS^s transformants of OKB120 and OKB142 were blue on agar plates containing the chromogenic indicator of β-galactosidase activity, 5-bromo-4-chloro-3-indolyl-β-galactoside, showing that a promoter was directing transcription into the *lacZ*-proximal end of the Tn917-*lac* construction. The observed *lacZ* expression is the

FIG. 4. Formation of *srf::Tn917 lacZ* fusion. The *srf::Tn917 lacZ* fusion was constructed by the method of Youngman et al. (31) as described in the text. P indicates the putative *srf* promoter, and the arrow shows the direction of transcription.

result of transcription that is directed into the *srf* locus, presumably from the *srf* promoter(s). The formal possibility that the expression observed is due to transcription in the direction opposite to that of *srf* transcription is highly unlikely, as predicted from the studies of Perkins and Youngman (22). Expression of *srf-lacZ* was monitored by assaying β -galactosidase activity in the cells of two transformants, designated OKB125 (*srf srf-lacZ*, derived from strain OKB120) and OKB143 (*sfp⁰ srf-lacZ*, derived from OKB142) (Fig. 5). The specific activity of β -galactosidase directed by the putative *srf* promoter in the cells of both strains increased gradually through exponential growth and then dramatically increased after T_0 (the beginning of the stationary phase). Activity reached a maximum level an hour after T_0 and remained at approximately the same level throughout the stationary phase. An almost twofold higher level of enzyme activity was observed in OKB125 (*srf*) cells than in OKB143 (*sfp⁰*) cells.

Effect of the *spo0* mutation on the expression of the *srf-lacZ* fusion. To study the regulation of the *srf-lacZ* expression, we introduced the *srf-lacZ* fusion into early blocked sporulation mutants (*spo0A*, *spo0B*, and *spo0H*) as described in Materials and Methods. The *srf-lacZ* expression in *sfp⁰* cells was

FIG. 5. β -Galactosidase activity of *B. subtilis* cells grown in DSM sporulation medium. T_0 represents the end of the exponential growth phase. Symbols: $\square$, OKB125 (*srf-lacZ sfp*); $\blacklozenge$, OKB143 (*srf-lacZ sfp⁰*).

unaffected by *spo0B* and *spo0H* mutations (Fig. 6A); however, in *sfp* cells, both mutations resulted in an approximately twofold decrease in *srf-lacZ* expression (Fig. 6B). The *spo0A* mutation caused a more severe decrease in *srf-lacZ* expression in both *sfp* and *sfp⁰* cells (Fig. 7).

The suppressor mutation of *spo0A*, *abrB*, is known to overcome the *spo0A* effect on antibiotic production in *B. subtilis* (10, 27) and the transcription of an antibiotic biosynthesis gene (20). The mutation *abrB703* overcame the impairment of *srf-lacZ* expression caused by a *spo0A* mutation in

FIG. 6. Effect of *spo0B* and *spo0H* mutations on β -galactosidase activity of *B. subtilis* cells. (A) Symbols: $\square$, OKB143 (*spo⁺ srf-lacZ sfp⁰*); $\blacklozenge$, OKB148 (*spo0BΔPst srf-lacZ sfp⁰*); $\blacksquare$, OKB162 (*spo0H ΔHind srf-lacZ sfp⁰*). (B) Symbols: $\square$, OKB125 (*spo⁺ srf-lacZ sfp*); $\blacklozenge$, OKB153 (*spo0BΔPst srf-lacZ sfp*); $\blacksquare$, OKB154 (*spo0HΔHind srf-lacZ sfp*).

FIG. 7. Effect of *spo0A* and *abrB* mutations on β -galactosidase activity of *B. subtilis* cells. (A) Symbols: $\square$, OKB143 ($spo^+ srf-lacZ sfp^0$); $\blacksquare$, OKB146 ($spo0A\Delta204 srf-lacZ sfp^0$); $\blacklozenge$, OKB145 ($spo0A\Delta204 abrB703 srf-lacZ sfp^0$). (B) Symbols: $\square$, OKB125 ($spo^+ srf-lacZ sfp$); $\blacklozenge$, OKB152 ($spo0A\Delta204 srf-lacZ sfp$); $\blacksquare$, OKB159 ($spo0A\Delta204 abrB703 srf-lacZ sfp$).

*sfp*⁰ cells (Fig. 7A). However, no increase in *srf-lacZ* expression was observed in *spo0A srf* cells when the *abrB* mutation was introduced (Fig. 7B).

The results of the *srf-lacZ* expression experiments prompted us to study the effect of *spo0* mutations on surfactin production in *sfp* cells. The surfactin production locus (*sfp*) was introduced into *spo0* mutants, and surfactin production was examined on blood plates. *spo0B* and *spo0H* mutations had no effect on surfactin production; however, colonies of *sfp spo0A* cells showed a slight decrease in surfactin production (data not shown).

DISCUSSION

A genetic study of surfactin biosynthesis was initiated with the ultimate goal of understanding how antibiotic biosynthesis gene expression is regulated. A genetic locus (*sfp*) responsible for surfactin production in *B. subtilis* was transferred from strain ATCC 21332 to cells of the nonproducer strain JH642. The *sfp* cells were then used to identify a Tn917 insertion mutation, *srf::Tn917*, which impairs surfactin production. Both *sfp* and *srf::Tn917* mapped to the *aroI* region, which lies in a section of the chromosome where Tn917 insertions had not been identified previously (26, 28).

Some of the surfactin biosynthesis genes probably exist in the chromosome of nonproducing 168-derived strains. The *srf::Tn917* insertion was isolated from the transposon library that was constructed with cells of strain PY79, a derivative of 168. The *srf::Tn917* mutation and *sfp* will segregate in transductional and transformational crosses involving cells of JH642, which is itself a 168 derivative. Therefore, the transposon must have inserted in DNA that is required for

surfactin production. At present, it is not known what *srf* and *sfp* encode. The surfactin DNA of 168 cells could be silent, or perhaps these cells lack the gene(s) of the *sfp* locus which may encode enzymes needed to complete the surfactin biosynthetic pathway. We cloned a part of *srf* DNA by the method of Youngman et al. (30). A preliminary Southern hybridization experiment with the cloned DNA as a probe showed no difference in the hybridization patterns between JH642 and OKB105 chromosomal DNAs (data not shown). These results suggest either that there are no gross changes between the chromosomal DNAs of the surfactin producer (OKB105) and the nonproducer (JH642) or that the changes attributed to the *sfp* locus are located far from the DNA flanking the *srf::Tn917* insertion which was used as the probe in the Southern hybridization experiments. We cannot exclude the latter explanation, because almost 10 kilobases of the *srf* DNA have been cloned and the 5' and 3' ends of the *srf* transcriptional unit have yet to be identified by gene disruption experiments (M. Nakano and P. Zuber, unpublished data). Genes involved in the biosynthesis of other peptide antibiotics, such as gramicidin S and tyrocidine, have been shown to be organized in clustered transcriptional units (M. Marahiel, personal communication). Based on the structure of surfactin, it is likely that it is synthesized through similar enzymatic reactions that catalyze the synthesis of these peptide antibiotics (nonribosomal synthesis). Indeed, a recent study indicated that a peptide-synthesizing multienzyme complex catalyzes the synthesis of surfactin (14); therefore, it is reasonable to presume that the surfactin biosynthesis gene(s) is organized in a way similar to those for tyrocidine and gramicidin biosynthesis.

Optimal transcription of *srf*, as judged by the results of the *srf-lacZ* experiments, is dependent on the *spo0A* locus. In *sfp*⁰ cells, the dependence of the *spo0A* gene product is entirely bypassed by an *abrB* suppressor mutation. This result was consistent with the model (33, 34) previously proposed that the *spo0A* gene product serves to overcome an *abrB*-dependent mechanism which represses transcription of sporulation genes like *spoVG* and antibiotic genes like *tycA*, a structural gene for tyrocidine synthetase I (20). In the latter case, the *abrB* mutation caused *tycA-lacZ* fusion to be transcribed constitutively. However, in the case of *srf-lacZ* in *sfp*⁰ cells, the expression was still induced postexponentially. In contrast, the *abrB* mutation could not overcome the effect of *spo0A* on *srf-lacZ* expression of *sfp* cells. In this case, it appears that *srf-lacZ* expression is dependent on *spo0A* to activate *srf* transcription by a means other than through a mechanism involving *abrB*. There are several possible explanations to account for the difference between *sfp* and *sfp*⁰ cells with respect to *srf-lacZ* expression. One possibility is that the expression of *srf-lacZ* was directed by different promoters in *sfp* and *sfp*⁰ cells. Introduction of the *sfp* locus may have placed an additional promoter upstream of the *srf* genes or may have changed the sequence of the *srf* promoter that is utilized in *sfp*⁰ cells. Another possibility is that surfactin production in 168 cells requires additional factors, such as a positive activator of transcription that is encoded by a gene of the *sfp* locus. The level of *srf-lacZ* expression in *spo0H sfp* or *spo0B sfp* cells is equal to that observed in *sfp*⁰ cells, yet *sfp* cells of the *spo0* mutants produce surfactin and *sfp*⁰ cells do not. Hence, it is probable that the surfactin production phenotype conferred by *sfp* is not simply the result of enhanced *srf* transcription. The molecular cloning and characterization of *sfp* and the corresponding DNA of *sfp*⁰ cells will be necessary to understand the mechanism of surfactin production and the biosynthetic pathway which has yet to be elucidated.

The deletion of *spo0H* (encoding σ^H [7]) caused an approximately 50% decrease in the level *srf*-directed *lacZ* expression as compared with the level in *spo*⁺ cells. This result, together with the finding that *sfp spo0H* cells produce surfactin, suggests that σ^H may not play a major role in the transcription of surfactin production genes or that the transcription of *srf* is initiated from several promoters, one of which is utilized by σ^H . Alternatively, the effect of the *spo0H* mutation on *srf-lacZ* expression might be more indirect, due to the sporulation defect that is conferred (a similar explanation may apply to the *spo0B* effect). The transcription of *aprE* was recently reported to be similarly affected by *spo0H* mutations (8). Additionally, the cells of the *sigB::cat* strain, which lacks σ^B (4, 13), also produce surfactin. Therefore, it seems likely that a sigma factor other than σ^H and σ^B is primarily responsible for transcription initiation of genes involved in surfactin production.

ACKNOWLEDGMENTS

We thank Philip Youngman for the Tn917 library and the pTV55 plasmid, which made the experiment described in this paper possible. We thank Issar Smith and Michele Igo for gifts of *B. subtilis* strains, Joachim Vater for supplying surfactin for biochemical tests, and Gregor Czekay for infrared spectrophotometric analysis.

This work was supported by funding from More Oklahoma Science and Technology to P.Z., Public Health Service grant AI39479-01 to P.Z. from the National Institute of General Medical Sciences, and grant FGS/B2 from TU-Berlin to M.A.M.

LITERATURE CITED

- Arima, K., A. Kakinuma, and G. Tamura. 1968. Surfactin, a crystalline peptidolipid surfactant produced by *Bacillus subtilis*: isolation, characterization and its inhibition of fibrin clot formation. *Biochem. Biophys. Res. Commun.* **31**:488-494.
- Banerjee, S., and J. N. Hansen. 1988. Structure and expression of a gene encoding the precursor of subtilin, a small protein antibiotic. *J. Biol. Chem.* **263**:9508-9514.
- Bernheimer, A. W., and L. S. Avigad. 1970. Nature and properties of a cytolytic agent produced by *Bacillus subtilis*. *J. Gen. Microbiol.* **61**:361-369.
- Binnie, C., M. Lampe, and R. Losick. 1986. Gene encoding the σ^{37} species of RNA polymerase σ factor from *Bacillus subtilis*. *Proc. Natl. Acad. Sci. USA* **83**:5943-5947.
- Cooper, D. G. 1986. Biosurfactants. *Microbiol. Sci.* **3**:145-149.
- Cooper, D. G., C. R. Macdonald, S. J. B. Duff, and N. Kosaric. 1981. Enhanced production of surfactin from *Bacillus subtilis* by continuous product removal and metal cation addition. *Appl. Environ. Microbiol.* **42**:408-412.
- Dubnau, E., J. Weir, G. Nair, L. Carter III, C. Moran, Jr., and I. Smith. 1988. *Bacillus* sporulation gene *spo0H* codes for σ^{30} (σ^H). *J. Bacteriol.* **170**:1054-1062.
- Ferrari, E., D. J. Henner, M. Perego, and J. A. Hoch. 1988. Transcription of *Bacillus subtilis* subtilisin and expression of subtilisin in sporulation mutants. *J. Bacteriol.* **170**:289-295.
- Gryczan, T. J., and D. Dubnau. 1978. Construction and properties of chimeric plasmids in *Bacillus subtilis*. *Proc. Natl. Acad. Sci. USA* **75**:1428-1432.
- Guespin-Michel, J. F. 1971. Phenotypic reversion in some early blocked sporulation mutants of *Bacillus subtilis*. *Mol. Gen. Genet.* **112**:243-254.
- Hilton, M. D., N. G. Alaeddinoglu, and A. L. Demain. 1988. *Bacillus subtilis* mutant deficient in the ability to produce the dipeptide antibiotic bacilysin: isolation and mapping of the mutation. *J. Bacteriol.* **170**:1018-1020.
- Hoch, J. A., M. Barat, and C. Anagnostopoulos. 1967. Transformation and transduction in recombination-defective mutants of *Bacillus subtilis*. *J. Bacteriol.* **93**:1925-1937.
- Igo, M., M. Lampe, C. Ray, W. Shafer, C. P. Moran, Jr., and R. Losick. 1987. Genetic studies of a secondary RNA polymerase sigma factor in *Bacillus subtilis*. *J. Bacteriol.* **169**:3464-3469.
- Kluge, B., J. Vater, J. Salnikow, and K. Eckart. 1988. Studies on the biosynthesis of surfactin, a lipopeptide antibiotic from *Bacillus subtilis* ATCC 21332. *FEBS Lett.* **231**:107-110.
- Krause, M., M. A. Marahiel, H. von Dohren, and H. Kleinkauf. 1985. Molecular cloning of an ornithine activating fragment of the gramicidin S synthetase 2 gene from *Bacillus brevis* and its expression in *Escherichia coli*. *J. Bacteriol.* **162**:1120-1125.
- Landy, M., G. H. Warren, S. B. Rosenman, and C. G. Golio. 1948. Bacillomycin: an antibiotic from *Bacillus subtilis* active against pathogenic fungi. *Exp. Biol. Med.* **67**:539-541.
- Losick, R., P. Youngman, and P. J. Piggot. 1986. Genetics of endospore formation in *Bacillus subtilis*. *Annu. Rev. Genet.* **20**:625-669.
- Malpartida, F., and D. A. Hopwood. 1984. Molecular cloning of the whole biosynthetic pathway of *Streptomyces* antibiotic and its expression in a heterologous host. *Nature (London)* **309**:462-464.
- Marahiel, M. A., M. Krause, and H.-J. Skarpeid. 1985. Cloning of the tyrocidine synthetase 1 gene from *Bacillus brevis* and its expression in *Escherichia coli*. *Mol. Gen. Genet.* **201**:231-236.
- Marahiel, M. A., P. Zuber, G. Czekay, and R. Losick. 1987. Identification of the promoter for a peptide antibiotic biosynthesis gene from *Bacillus brevis* and its regulation in *Bacillus subtilis*. *J. Bacteriol.* **169**:2215-2222.
- Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Perkins, J. B., and P. Youngman. 1986. Construction and properties of Tn917-lac, a transposon derivative that mediates transcriptional gene fusions in *Bacillus subtilis*. *Proc. Natl. Acad. Sci. USA* **83**:140-144.
- Piggot, P. J., and J. G. Coote. 1976. Genetic aspects of bacterial endospore formation. *Bacteriol. Rev.* **40**:908-962.
- Piggot, P. J., and J. A. Hoch. 1985. Revised genetic linkage map of *Bacillus subtilis*. *Microbiol. Rev.* **49**:158-179.
- Podlessek, Z., and M. Grabnar. 1987. Genetic mapping of the bacitracin synthetase gene(s) in *Bacillus licheniformis*. *J. Gen. Microbiol.* **133**:3093-3097.
- Sandman, K., R. Losick, and P. Youngman. 1987. Genetic analysis of *Bacillus subtilis* *spo* mutations generated by Tn917-mediated insertional mutagenesis. *Genetics* **117**:603-617.
- Trowsdale, J., S. M. H. Chen, and J. A. Hoch. 1979. Genetic analysis of a class of polymyxin resistant partial revertants of stage 0 sporulation mutants of *Bacillus subtilis*: map of the chromosomal region near the origin of replication. *Mol. Gen. Genet.* **8**:61-70.
- Vandeyar, M. A., and S. A. Zahler. 1986. Chromosomal insertions of Tn917 in *Bacillus subtilis*. *J. Bacteriol.* **167**:530-534.
- Youngman, P. J., J. B. Perkins, and R. Losick. 1983. Genetic transposition and insertional mutagenesis in *Bacillus subtilis* with *Streptococcus faecalis* transposon Tn917. *Proc. Natl. Acad. Sci. USA* **80**:2305-2309.
- Youngman, P. J., J. B. Perkins, and R. Losick. 1984. A novel method for the rapid cloning in *Escherichia coli* of *Bacillus subtilis* chromosomal DNA adjacent to Tn917 insertions. *Mol. Gen. Genet.* **195**:424-433.
- Youngman, P., J. B. Perkins, and K. Sandman. 1985. Use of Tn917-mediated transcriptional gene fusions to *lacZ* and *cat-86* for the identification and study of *spo* genes in *Bacillus subtilis*, p. 47-54. In J. A. Hoch and P. Setlow (ed.), *Molecular biology of microbial differentiation*. American Society for Microbiology, Washington, D.C.
- Zuber, P., and R. Losick. 1983. Use of a *lacZ* fusion to study developmental regulation by the *spo0* genes of *Bacillus subtilis*. *Cell* **35**:275-283.
- Zuber, P., and R. Losick. 1985. *Spo0H*: a developmental regulatory gene for promoter utilization in *Bacillus subtilis*. *Cold Spring Harbor Symp. Quant. Biol.* **50**:483-488.
- Zuber, P., and R. Losick. 1987. Role of *abrB* in *spo0A*- and *spo0B*-dependent utilization of a sporulation promoter in *Bacillus subtilis*. *J. Bacteriol.* **169**:2223-2230.