JOURNAL OF BACTERIOLOGY, Dec. 1999, p. 7346–7355 Vol. 181, No. 23
0021-9193/99/$04.00+0

Genes of the *sbo-alb* Locus of *Bacillus subtilis* Are Required for Production of the Antilisterial Bacteriocin Subtilosin

GUOLU ZHENG,[1] LIANG Z. YAN,[2] JOHN C. VEDERAS,[2] AND PETER ZUBER[1]*

Department of Biochemistry and Molecular Biology, Oregon Graduate Institute of Science and Technology, Beaverton, Oregon 97006-8921,[1] and Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2, Canada[2]

Received 8 July 1999/Accepted 3 September 1999

***Bacillus subtilis* JH642 and a wild strain of *B. subtilis* called 22a both produce an antilisterial peptide that can be purified by anion-exchange and gel filtration chromatography. Amino acid analysis confirmed that the substance was the cyclic bacteriocin subtilosin. A mutant defective in production of the substance was isolated from a plasmid gene disruption library. The plasmid insertion conferring the antilisterial-peptide-negative phenotype was located in a seven-gene operon (*alb*, for antilisterial bacteriocin) residing immediately downstream from the *sbo* gene, which encodes the precursor of subtilosin. An insertion mutation in the *sbo* gene also conferred loss of antilisterial activity. Comparison of the presubtilosin and mature subtilosin sequences suggested that certain residues undergo unusual posttranslational modifications unlike those occurring during the synthesis of class I (lantibiotic) or some class II bacteriocins. The putative products of the genes of the operon identified show similarities to peptidases and transport proteins that may function in processing and export. Two *alb* gene products resemble proteins that function in pyrroloquinoline quinone biosynthesis. The use of *lacZ-alb* and *lacZ-sbo* gene fusions, along with primer extension analysis, revealed that the *sbo-alb* genes are transcribed from a major promoter, residing upstream of *sbo*, that is very likely utilized by the σ^A form of RNA polymerase. The *sbo* and *alb* genes are negatively regulated by the global transition state regulator AbrB and are also under positive autoregulation that is not mediated by the subtilosin peptide but instead requires one or more of the *alb* gene products.**

Polypeptide antibiotics possess bacteriocidal, fungicidal, metal-chelating, and immunomodulating activities. They are frequently found as secondary metabolites or small, secreted proteins produced by various microorganisms, such as the gram-positive bacteria of the genus *Bacillus*, lactic acid bacteria, and the genus *Streptomyces* (22, 23, 25, 27, 60). In *Bacillus subtilis*, some polypeptide antibiotics, such as bacteriocins, are gene encoded and are synthesized ribosomally while others are produced nonribosomally by the multienzyme thiotemplate mechanism (60). Bacteriocins such as nisin, produced by *Lactococcus lactis*, can be used in foods as antimicrobial agents to replace chemical preservatives, such as nitrite (35), that are potentially hazardous or carcinogenic. As demonstrated in studies of subtilin (*B. subtilis*), nisin (*L. lactis*), pediocin (*Pediococcus acidilactici*), and other known bacteriocins, the bacteriocins of gram-positive bacteria are typically first formed as precursors with reduced biological activity (6, 21, 38). The C-terminal ends of the precursors are then cleaved from the N-terminal leader sequences to yield the mature, active bacteriocins. In some cases, the precursor polypeptide undergoes posttranslational modifications. The formation of lanthionine and thiazole or oxazole adducts is characteristic of the maturation processes of class 1 bacteriocins (lantibiotics) and microcins of the gram-negative bacterium *Escherichia coli*, respectively (4, 32). Transport of the peptides to the external environment is carried out by ATP-dependent efflux protein complexes that are membrane associated (16). Genes involved in the biosynthesis of bacteriocins are typically organized into operons (21, 28) which include the bacteriocin structural gene and genes whose products function in bacteriocin maturation, export, immunity, and, in some cases, the regulation of operon expression. The operons that encode proteins that function in lantibiotic biosynthesis also contain genes encoding simple signal transduction systems composed of two-component regulatory proteins (6, 26, 29). These genes mediate a form of positive-feedback regulation that is induced in response to the presence of the lantibiotic (nisin or subtilin).

In *B. subtilis*, production of and resistance to antibiotics are regulated by the *spo0-abrB* system of control. The Spo0 phosphorelay is activated by conditions of nutritional stress and high cell density (2, 17, 18). The signals derived from these conditions are integrated into the phosphorelay and promote the accumulation of Spo0A phosphate, which activates sporulation gene transcription and represses the transcription of the transition state regulatory gene *abrB* (11, 48, 51). Mutations in *spo0A* render *B. subtilis* cells unable to produce certain antibiotics and confer sensitivity to those antibiotics. A mutation in *abrB* suppresses this *spo0A* phenotype (13, 14, 19, 54), indicating that AbrB exerts negative control of antibiotic production and resistance. AbrB is known to interact directly with the promoter regions of several genes that are normally induced in the transition from exponential growth to stationary phase (44, 48–50). The *tycA* operon, encoding the enzyme tyrocidine synthetase, which catalyzes the synthesis of a cyclic peptide antibiotic, is but one operon that is repressed by AbrB (8, 33).

Antimicrobial substances produced by a wild strain of *B. subtilis* isolated from an Oriental fermented food (57a, 58) are currently under investigation in our laboratory. One of these substances was initially identified as a bacteriocin endowed with activity against *Listeria monocytogenes* and *Bacillus cereus*. As detailed in this report, an operon required for the observed activity has been identified by insertion mutagenesis. The operon (*alb*, for antilisterial bacteriocin) consists of seven genes and is preceded by the gene *sbo*, encoding subtilosin, a

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Biology, Oregon Graduate Institute of Science and Technology, 20000 N.W. Walker Rd., Beaverton, OR 97006-8921. Phone: (503) 748-7335. Fax: (503) 748-1464. E-mail: pzuber@bmb.ogi.edu.

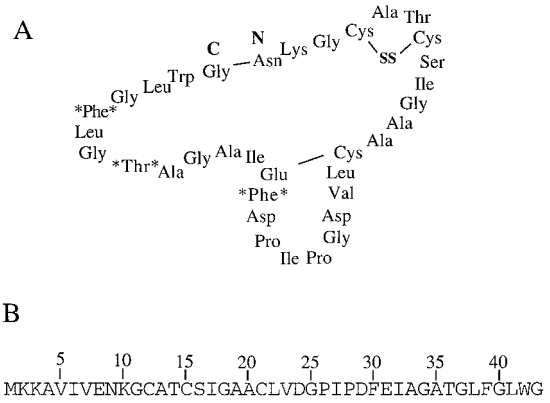


FIG. 1. (A) Proposed structure of subtilosin (1). The boldfaced N and C mark the N and C termini of the prosubtilosin peptide (presubtilosin leader peptide removed), respectively. The proposed link between Glu31 and Cys21 (1) is shown. The asterisks on either side of an amino acid indicate residues that have likely undergone chemical modification. SS, disulfide link between Cys12 and Cys15. (B) Amino acid sequence of the presubtilosin peptide, deduced from the nucleotide sequence of the *sbo* coding region.

modified antimicrobial peptide originally identified by Kurahashi and coworkers (1). The *sbo* gene resides in the vicinity of *fnr* and *argS* (encoding arginyl-tRNA synthetase) (30). The peptide product is composed of 32 common amino acids and some unusual residues that are likely the result of posttranslational modifications (Fig. 1). Comparison of the presubtilosin and mature subtilosin sequences suggests that the Sbo primary translation product may undergo novel modifications. The regulation of *sbo-alb* was also investigated by using *alb*- and *sbo-lacZ* fusions. A novel form of autogenous regulation that does not involve the product of *sbo* but instead requires an *alb* operon product(s) was uncovered.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains used in this study are listed in Table 1. Strain 22a is a wild strain of *B. subtilis* isolated from an Oriental fermented soybean product (58). With the exception of wild strain 22a, all strains constructed are derivatives of *B. subtilis* JH642. The indicator bacterium, *L. monocytogenes* F4244, was provided by M. Slavik (University of Arkansas). The gene disruption plasmid library, in which 0.5- to 2-kb genome DNA fragments of JH642 were randomly inserted into the vector pJPM1 (31, 47), was obtained from A. L. Sonenshein (Tufts University). ZB449 is an SPβ-cured strain bearing a frameshift mutation in the *abrB* gene (59). TT71 (a gift from T. Tanaka) carries an insertion of a neomycin resistance (Neo^r) cassette (*neo*) in the *abrB* gene.

To construct the *sbo*::*neo* mutant, an *Eco*RI-*Hin*dIII fragment containing the *sbo* gene along with 512 bp of sequence upstream and 578 bp of sequence downstream of *sbo* was obtained by PCR with primers osboP1 and osboP2 (Table 2) and was then inserted into *Eco*RI- and *Hin*dIII-cleaved pUC18 (57). The resulting plasmid, pUC-sboEH, was used as template for a PCR using two partially complementary oligonucleotides (osboP3 and osboP4) specifying a *Bam*HI site within the *sbo* coding sequence 43 bp downstream of the ATG start codon. The PCR DNA product was then cleaved with *Bam*HI and subjected to intramolecular ligation, yielding pUC-sboEBH. A *Bgl*II-*Bam*HI fragment bearing the *neo* gene from pDG782 (12) was inserted into the *Bam*HI site, and the resulting ligation mixture was used to transform competent cells of *E. coli*, with selection for Neo^r (50 μg/ml). The plasmid was purified from the Neo^r transformants and used to transform cells of JH642 to create mutant strains ORB3148 (*sbo*::*neo-1*) and ORB 3149 (*sbo*::*neo-2*). The plasmid DNA recombined with the *sbo* gene DNA by a double-crossover mechanism by virtue of the homologous *sbo* DNA fragments flanking the *neo* insertion cassette. The insertion was confirmed by PCR analysis. The plasmid pE1 was acquired by a spontaneous loop-out recombination event that occurred in the isolate obtained in the plasmid disruption library mutagenesis. It contains a 1,178-bp fragment of the *alb* locus extending from 472 bp upstream to 646 bp downstream of *albB* (*ywhR*). Plasmid pE1 was used to transform competent cells of JH642 to yield the *alb*::pE1 insertion mutant ORB3146.

To construct pMUPE1, a *Hin*dIII-*Bam*HI fragment containing the 1,178-bp segment of *alb* DNA in pE1 was inserted into *Hin*dIII- and *Bam*HI-cleaved pMUTIN2 (55). This plasmid was used to transform JH642 and mutant derivatives, with selection for erythromycin resistance. Plasmid pMUALBG, another derivative of pMUTIN2, was constructed by inserting a *Hin*dIII-*Bam*HI PCR fragment (generated with primers oywhm-U and oywhm-L) containing bp 22 to 465 of the *albG* (*ywhM*) coding sequence into *Hin*dIII- and *Bam*HI-cleaved pMUTIN2. The resulting recombinant plasmid was used to transform competent cells of JH642, with selection for erythromycin resistance, yielding strain ORB3231.

Plasmids pTK-sboEH, pTK-sboEBH, pTK-sboΔEB, and pTK-sboΔBH, all derivatives of pTKlac (24), were used to construct transcriptional *lacZ* fusions for analyses of promoter activity and regulation of the *sbo* gene and *alb* operon. The *Eco*RI-*Hin*dIII fragments released from pUC-sboEH (carrying the intact *sbo* gene) and pUC-sboEBH (containing the *sbo Bam*HI allele [see below]) were inserted into *Eco*RI- and *Hin*dIII-cleaved pTKlac to generate pTK-sboEH and pTK-sboEBH, respectively. pTK-sboΔEB was a derivative of pTK-sboEBH with a deletion of the *Eco*RI-*Bam*HI fragment, while pTK-sboΔBH was a derivative of pTK-sboEBH with a deletion of the *Bam*HI-*Hin*dIII fragment. The plasmid pTK-sboΔEB contains the 3′ half of the *sbo* gene and the 5′ end of *albA* (to bp 442).

The *sbo-lacZ* fusion plasmids derived from pTKlac were introduced into prophage SPβ*c2del2*::Tn*917*::pSK10Δ6 of strain ZB307 by transformation as previously described (59). Heat-induced lysates containing fusion-bearing phages were obtained and were used to transfer the *sbo-lacZ* fusions to mutant derivatives of JH642 by specialized transduction.

Isolation of an antilisteria-protein-negative mutant. A plasmid gene disruption library was obtained from L. Sonenshein (47). The plasmid library was used to transform JH642, with selection for chloramphenicol resistance. Total chromosomal DNA was purified from the pool of transformants and used to transform competent cells of *B. subtilis* 22a. Chloramphenicol resistant (Cm^r) transformants were then screened for loss of activity against *L. monocytogenes* F4244. After the transformants were patched onto yeast extract-glucose (YG) plates and incubated for 20 to 24 h at 30°C, the YG cultures were then overlaid with brain-heart infusion semisoft agar (0.8% agar; 25 μg of nalidixic acid/ml) containing 0.1 ml of an overnight culture of *L. monocytogenes* and incubated at 37°C for 18 to 24 h. Of 3,400 resultant colonies, 7 had no zone of inhibition when overlayed with a suspension of *L. monocytogenes*. To confirm the phenotypic linkage of antilisterial activity and Cm^r, chromosomal DNA was prepared from the mutants and used to transform *B. subtilis* 22a. Mutants that showed 100% transformation linkage between the antilisterial phenotype and plasmid-associated drug resistance were chosen. One of the mutations was transferred to strain JH642 by transformation, using mutant chromosomal DNA, with selection for Cm^r. The mutation also conferred an antilisterial-protein-negative phenotype in JH642.

To identify the gene(s) of the mutant locus affecting antilisterial activity in JH642, the integrated plasmid and flanking region were outcloned. Chromosomal DNA of a mutant was digested with *Eco*RI or *Hin*dIII, ligated at a low DNA concentration to facilitate intramolecular ligation, and then used to transform *E. coli* DH5α competent cells. However, the plasmid obtained, pE1, was not generated by restriction endonuclease digestion but rather was a product of a spontaneous loop-out recombination event. The pE1 insert was subjected to nucleotide sequence analysis.

Culture media. YG (2% glucose, 0.5% yeast extract, and 0.1% trace-metal solution [2.2 g of $ZnSO_4 \cdot 7H_2O$, 1.1 g of H_3BO_3, 0.5 g of $MnCl_2 \cdot 4H_2O$, 0.5 g of $FeSO_4 \cdot 7H_2O$, 0.16 g of $CoCl_2 \cdot 5H_2O$, 0.16 g of $CuSO_4 \cdot 5H_2O$, 0.11 g of $(NH_4)_5Mo_7O_{24} \cdot 4H_2O$, and 5.0 g of disodium EDTA in 100 ml]) was used to culture strain JH642 for the purification of subtilosin. *B. subtilis* cells were routinely grown on agar plates containing Difco sporulation medium (DSM) (15). Cells of *lacZ* fusion-bearing strains were grown on DSM plus 0.5% glucose (DSM-G) for the time course β-galactosidase assay experiments. Solid TSS minimal medium (7) was used to examine auxotrophic phenotypes. *E. coli* cells were routinely grown in 2× YT (yeast extract-tryptone) medium.

Transformation. Preparation of competent *B. subtilis* cells and genetic transformation were carried out as previously described (5). Preparation of *E. coli* competent cells and plasmid transformation were performed according to published procedures (45).

Assay of β-galactosidase activity. All inocula were grown overnight at 37°C on solid DSM supplemented with the appropriate antibiotics. The cells, harvested by washing the plate surface with 2 ml of DSM, then were used to inoculate batch cultures containing either DSM or DSM-G to an initial optical density at 595 nm of about 0.17 or an initial Klett value (red filter) of about 8. The cultures were grown at 37°C in a shaking water bath. Collection of 1-ml samples was started when the culture reached an optical density at 595 nm of OD_{595} 0.4 or had a Klett reading of around 20. Collection of samples for β-galactosidase activity assays continued at 30-min or 1-h intervals. Measurement of β-galactosidase activity has been described previously (36).

Purification of subtilosin. Two hundred milliliters of YG broth was inoculated with a single colony of *B. subtilis* JH642 and incubated at 32°C with shaking (200 rpm) for 36 h. The supernatant was collected by centrifugation (21,252 × *g*, 10 min) and adjusted with 1 M Tris buffer (pH 7.5) to 20 mM Tris-HCl (final concentration). The buffered supernatant was filtered through a 0.45-μm-pore-size syringe filter and subjected to chromatography with an anion-exchange cartridge (5-ml High Q; Bio-Rad). After sample application, the cartridge was washed with 20 mM Tris (pH 7.5) for 20 min (3 ml/min) followed by elution with

TABLE 1. Strains used in this study

Strain	Relevant genotype (phenotype)	Reference or source
L. monocytogenes F4224		M. Slavik
B. subtilis		
JH642	*trpC2 pheA1*	J. Hoch
22a	Wild strain	58
ZB449	*trpC2 pheA1 abrB703*	59
LAB2136	*trpC2 pheA1 fnr*::*spc*	31
TT71	*trpC2 abrB*::*neo*	T. Tanaka
ORB3146	*trpC2 pheA1 alb*::pE1 (Cm^r)	This study
ORB3147	*trpC2 pheA1 alb*::pMUPE1 (Erm^r)	This study
ORB3148	*trpC2 pheA1 sbo*::*neo-1*	This study
ORB3149	*trpC2 pheA1 sbo*::*neo-2*	This study
ORB3152	*trpC2 pheA1 sbo*::*neo-1 albABC*::pMUPE1 (Erm^r)	This study
ORB3153	*trpC2 pheA1 sbo*::*neo-2 albABC*::pMUPE1 (Erm^r)	This study
ORB3154	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::pTK-lac (Erm^r Cm^r)	This study
ORB3158	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::pTK-sboΔEB (Erm^r Cm^r)	This study
ORB3159	*trpC2 pheA1 sbo*::*neo-1* SPβ*c2del2*::Tn*917*::pTK-sboΔEB (Erm^r Cm^r)	This study
ORB3160	*trpC2 pheA1 sbo*::*neo-2* SPβ*c2del2*::Tn*917*::pTK-sboΔEB (Erm^r Cm^r)	This study
ORB3161	*trpC2 pheA1 alb*::pE1 SPβ*c2del2*::Tn*917*::pTK-sboΔEB (Erm^r Cm^r)	This study
ORB3162	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::pTK-sboΔBH (Erm^r Cm^r)	This study
ORB3163	*trpC2 pheA1 sbo*::*neo-1* SPβ*c2del2*::Tn*917*::pTK-sboΔBH (Erm^r Cm^r)	This study
ORB3164	*trpC2 pheA1 sbo*::*neo-2* SPβ*c2del2*::Tn*917*::pTK-sboΔBH (Erm^r Cm^r)	This study
ORB3165	*trpC2 pheA1 alb*::pE1 SPβ*c2del2*::Tn*917*::pTK-sboΔBH (Erm^r Cm^r)	This study
ORB3166	*trpC2 pheA1* SPβ*c2del2*::Tn*917*::pTK-sboEH (Erm^r Cm^r)	This study
ORB3167	*trpC2 pheA1 sbo*::*neo-1* SPβ*c2del2*::Tn*917*::pTK-sboEH (Erm^r Cm^r)	This study
ORB3168	*trpC2 pheA1 sbo*::*neo-2* SPβ*c2del2*::Tn*917*::pTK-sboEH (Erm^r Cm^r)	This study
ORB3169	*trpC2 pheA1 alb*::pE1 SPβ*c2del2*::Tn*917*::pTK-sboEH (Erm^r Cm^r)	This study
ORB3230	*trpC2 pheA1 sbo*::*neo-2 alb*::pMUALBG (Erm^r)	This study
ORB3284	*trpC2 pheA1 sbo*::*neo-1 alb*::pMUALBG (Erm^r)	This study
ORB3231	*trpC2 pheA1 alb*::pMUALBG (Erm^r)	This study
ORB3237	*trpC2 pheA1 abrB*::*neo*	This study
ORB3238	*trpC2 pheA1 abrB*::*neo alb*::pE1 (Cm^r)	This study

a linear gradient, starting with 20 mM Tris (pH 7.5) and ending with 1 M NaCl in 20 mM Tris (pH 7.5), over 50 min. Fractions were collected, and those showing inhibitory activity against *L. monocytogenes* F4244 (between 100 and 200 mM NaCl) were pooled. The pooled fractions were extracted with one-fourth volume of butanol at room temperature for 1 h and allowed to stand overnight at room temperature. The butanol layer was dried by evaporation at 55°C. The residue was dissolved in 1 ml of methanol and subjected to chromatography on a Sephadex LH-20 column (1.5 by 25 cm). Elution was performed with methanol at a rate of 3 ml/min. Fractions (3 ml each) of the first absorption peak (at 280 nm) were pooled, dried by evaporation, and resuspended in 400 μl of 20 mM sodium phosphate buffer (pH 7.0).

Subtilosin A activity assay and protein concentration determinations. Antilisterial activity of subtilosin A was determined by the critical dilution assay (58). Protein concentrations were determined spectrophotometrically by using the Bio-Rad (Hercules, Calif.) protein assay with bovine serum albumin as a standard.

Bioautography of subtilosin by SDS-PAGE. Samples of Sephadex LH-20 eluates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 16% gels with Tris-Tricine running buffer. Each sample (10 μl) was loaded in duplicate onto the gel. After electrophoresis was conducted at 100 V for 120 min, each lane of the gel was cut vertically. For each sample, one lane was stained with Coomassie blue to visualize the separated protein bands while the other lane was assayed for inhibitory activity against *L. monocytogenes* F4244 according to the method of Zheng and Slavik (58).

Sequencing and primer extension. Total RNA was purified by the method of Nakano et al. (37) from JH642 and ZB449 cells collected at T_2 (i.e., 2 h after the end of the exponential growth phase) from DSM-G cultures. Primer extension was performed with oligonucleotides osboP4 (hybridizing to nucleotides 20 to 55 of the *sbo* coding sequence) and ocalbA-L (hybridizing to a sequence within *albA* from 31 to 61 bp from the TTG start codon). Primer extension was carried out according to published protocols (37). DNA sequencing was conducted by using a Sequenase version 2.0 kit (U.S. Biochemical Corp.), [α-^{35}S]dATP (ICN), and plasmid pTK-sboEH (as a template).

RESULTS

Studies of the nature of the antilisterial activity produced by the wild strain of *B. subtilis* known as 22a, isolated from Oriental fermented food (58), and the standard genetic strain JH642 were conducted. The *sbo* gene, encoding the bacteriocin subtilosin (Fig. 1) (1), and the genes of the *alb* operon (*albA* to *-G* are, respectively, genes *ywiA*, *ywhR*, *ywhQ*, *ywhP*, *ywhO*, *ywhN*, and *ywhM*, according to the *B. subtilis* genome sequencing consortium [30]), which function in the production of an-

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence
osboP1	CCTCATGACCAGGACTTCGCCTTCGCTTACTTT
osboP2	CGGTGCCGAGCGCTTCAGGTAAGCTTTCCAAA
osboP3	TGCTGGATCCGAGCCGCTTGTCTAGTGGACGGTCCTAT
osboP4	CTCGGATCCAGCATGTTGCACAACCTTTGTTTTCTA
oywhm-U	GCACTGTCTTTAAGCTTTTATTGCTGCTTA
oywhm-L	GGATCAGTAGGATCCCAAGTCCCATTGAAA
ocalbA-L	CCCTCAGGAAGCTGGTGAACTCTTACACTTT

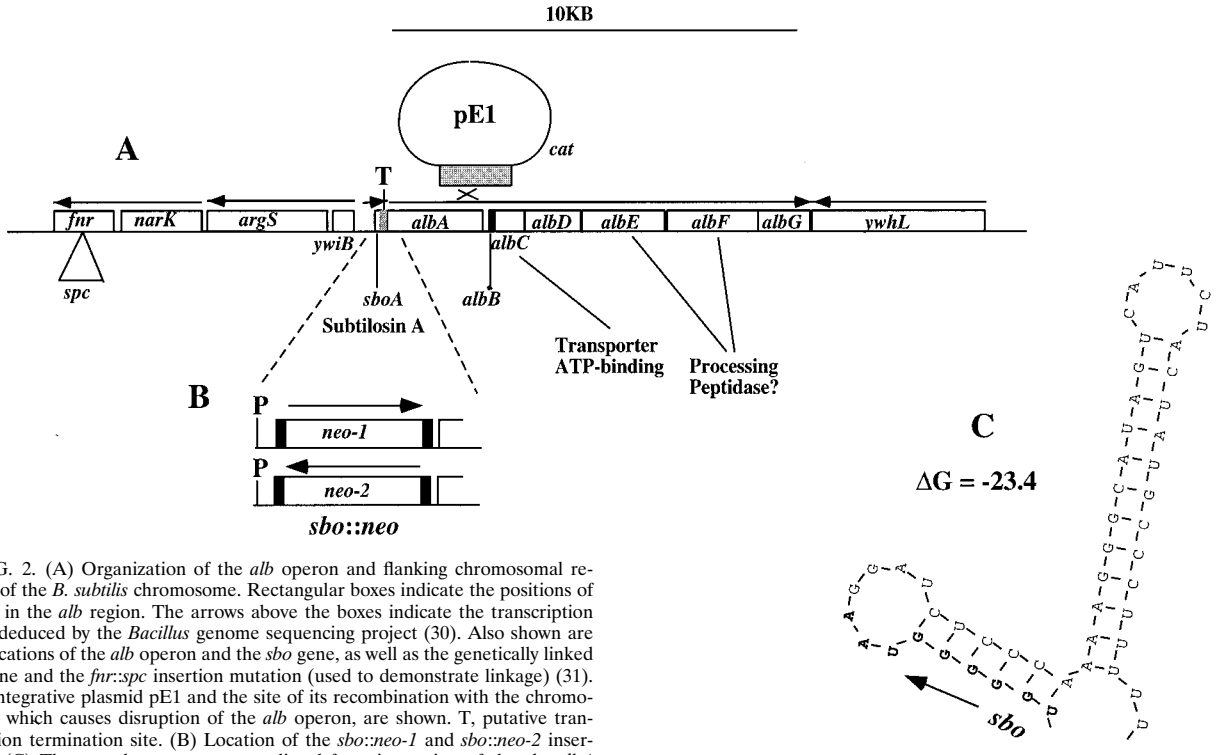


FIG. 2. (A) Organization of the *alb* operon and flanking chromosomal regions of the *B. subtilis* chromosome. Rectangular boxes indicate the positions of genes in the *alb* region. The arrows above the boxes indicate the transcription units deduced by the *Bacillus* genome sequencing project (30). Also shown are the locations of the *alb* operon and the *sbo* gene, as well as the genetically linked *fnr* gene and the *fnr*::*spc* insertion mutation (used to demonstrate linkage) (31). The integrative plasmid pE1 and the site of its recombination with the chromosome, which causes disruption of the *alb* operon, are shown. T, putative transcription termination site. (B) Location of the *sbo*::*neo-1* and *sbo*::*neo-2* insertions. (C) The stem-loop structure predicted from inspection of the *sbo-albA* intergenic-region sequence that overlaps with the 3′ end of the *sbo* gene (indicated in boldface). Also shown is the free energy of secondary-structure formation, ΔG.

tilisterial activity, were identified in a search for mutants defective in production of the antilisterial activity.

Nucleotide sequence analysis of the *sbo-alb* locus. Mutants of *B. subtilis* 22a (58) and JH642 that were defective in production of the antilisterial activity were identified by using a plasmid insertion library constructed by Serror and Sonenshein (47). The plasmid insertion clone pE1 contains an insert corresponding to the region from *albA* (*ywiA*) to *albC* (*ywhQ*) (30) and linked by transformation to an *fnr*::*spc* marker (Fig. 2). Sequence analysis revealed that the insertion took place within a cluster of seven genes putatively constituting an operon (Fig. 2) that we called *alb* (for antilisterial bacteriocin).

Immediately upstream of the *alb* operon is the *sbo* gene, encoding the 43-amino-acid precursor of the bacteriocin subtilosin (30), which was originally characterized by Kurahashi and coworkers (1) (Fig. 1). The subtilosin precursor contains no obvious leader peptide sequence, which is normally required for peptide export, nor are there the typical motifs associated with the processing of prelantibiotics. The peptide appears to undergo some unique modifications during maturation. Asn9 is linked to the C-terminal glycine, and Cys21 has been proposed to be linked to Glu31 (1) (Fig. 1A). Although codons specifying phenylalanine at positions 30 and 39 and Thr at position 36 are present in the nucleotide sequence (Fig. 1B), the corresponding amino acids are not found in the mature subtilosin peptide (1); this was confirmed by amino acid analysis (described below). It is possible that these residues play some role in intrachain cross-linking. As described further below, mutations in either the *alb* operon or *sbo* eliminated the antilisterial activity.

Overlapping with the C-terminal coding end of *sbo* and residing 93 bp upstream of the *albA* TTG start codon is a 55-bp sequence that potentially codes for a region of RNA secondary structure (location in the *sbo-alb* region denoted as T in Fig. 2A). The stem-loop depicted in Fig. 2C has a ΔG of −23.4 and could impede transcriptional readthrough from upstream. A similar structure, found between the *mutA* gene, encoding mutacin II, and the remainder of the *mut* operon, is thought to reduce transcriptional readthrough from the upstream *mut* operon promoter (42).

The putative coding sequences of the *alb* operon encode proteins that potentially function in the processing and export of peptides, such as an ATP-binding cassette transport complex (*albC*) and two processing peptidases. Interestingly, one of these peptidases, the product of *albF* (*ywhN*), shows significant sequence similarity to mitochondrial zinc-endoproteinase (3, 20, 39, 41) and to PqqF, a protein required for the synthesis of the cofactor pyrroloquinoline quinone (PQQ). PqqF is thought to cleave the PqqA peptide, thereby releasing glutamate and tyrosine, which are precursors of PQQ (34, 52, 53, 56). AlbE and the putative peptidase encoded by the gene *albF* (30) could function in the processing of subtilosin or a protein required for subtilosin maturation. The first gene of the *alb* operon, *albA* (*ywiA*) (30), encodes a protein with significant homology to those that function in cofactor heme, PQQ, and molybopterin cofactor synthesis (9, 34, 43, 52). The *albA* product is thought to function in the association of a metal ion with an enzyme-bound cofactor. It is possible that the product of *albA* (*ywiA*) activates a metalloenzyme that catalyzes modification of prosubtilosin.

A mutation in *sbo* confers loss of antilisterial activity. We sought to determine if the *sbo* gene was required for the observed antilisterial activity. A *Bam*HI-*Bgl*II fragment bearing a neomycin resistance cassette was inserted into the *sbo* gene at the *Bam*HI site, thereby bisecting the *sbo* gene (see Materials and Methods). The plasmid was used to transform competent cells of JH642 and strain 22a, resulting in the *sbo*::*neo* insertion

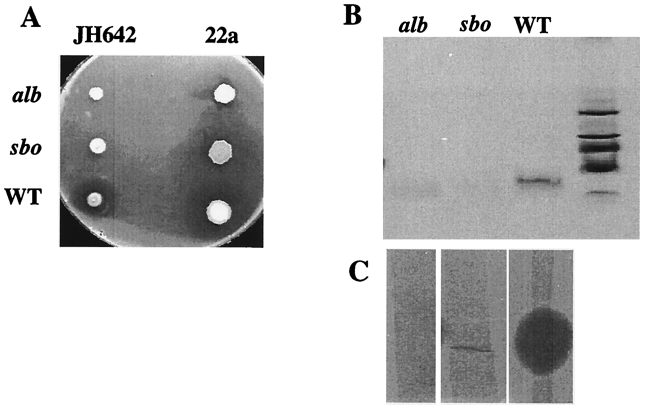


FIG. 3. Antilisterial substance produced by *B. subtilis* JH642 and 22a. (A) The strains are shown as colonies that were overlayed with soft agar containing a suspension of *L. monocytogenes* cells. Growth of *Listeria* is inhibited by wild-type (WT) JH642 and by 22a but not by the *sbo* and *alb* mutants of these organisms. Some slight inhibition is observed around the colonies of the 22a *sbo* and *alb* mutant cells. (B) A Tricine-SDS-PAGE gel that is stained with Coomassie blue shows the antilisterial peptide produced in WT JH642 but not present in cultures of strains ORB3148 (*sbo*) and ORB3146 (*alb*). (C) A bioautograph (see Materials and Methods) of the gel in panel B, showing the antilisterial activity of the peptide and the absence of activity in the lanes containing the *sbo* and *alb* mutant culture extracts.

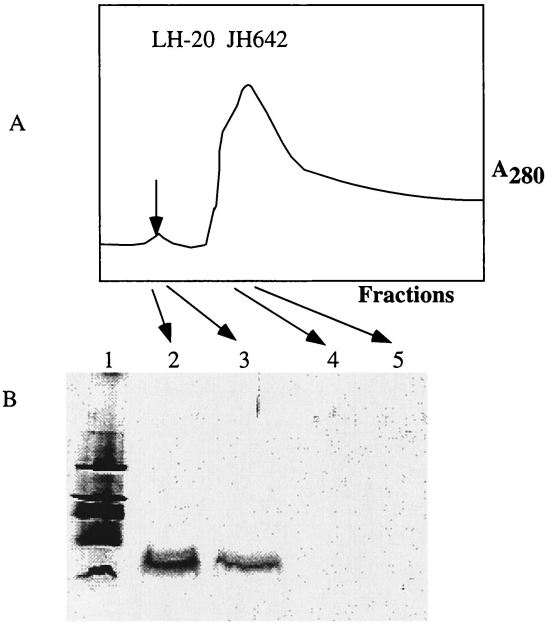


FIG. 4. (A) Profile of fractions collected from an LH-20 size exclusion column onto which a methanol extract of concentrated JH642 culture supernatant was applied. A small absorbance peak ($\lambda = 280$) (arrow) contains the putative subtilosin peptide, as shown by Tricine-SDS-PAGE (B).

mutation. The colonies of the resulting *sbo* mutant derivative of JH642 did not exhibit the antilisterial phenotype on YG plates (Fig. 3). The antilisterial activity of strain 22a was reduced but not eliminated. This was due to the presence of other antilisterial activities produced by 22a. These other, low-level activities could be detected in supernatant fluid from liquid cultures of strain 22a (data not shown).

Purification of antilisterial activity and evidence that the bacteriocin subtilosin is the antilisterial agent. Further purification of subtilosin was carried out from supernatant fluid collected from JH642 cultures, since the fluid from strain 22a cultures contained other antilisterial activities that might interfere with subtilosin purification. Supernatant fluid from YG cultures of JH642 was precipitated with 65% $(NH_4)_2SO_4$. The precipitate was extracted twice with methanol, evaporated, and subjected to Tricine-SDS-PAGE analysis (46). A single band migrating at approximately 4,000 Da, which was absent from the *sbo*::*neo-1* mutant and plasmid *alb*::pE1 insertion mutant cultures (Fig. 3), was detected. Bioautography was performed by overlaying the proteins of the Tricine-SDS gel with molten soft brain-heart infusion agar in which cells of an overnight culture of *L. monocytogenes* were suspended. A zone of lysis was observed over the area of the gel where the subtilosin band was located (Fig. 3C). At this same position in the lanes containing the supernatant of the mutant cultures, no band was evident in the Tricine-SDS gel and no zone of lysis occurred in the bioautograph.

Culture fluid was also subjected to ion-exchange chromatography, and fractions exhibiting antilisterial activity were collected. This material was extracted with butanol, evaporated, dissolved in methanol, and resolved further by Sephadex LH-20 gel filtration column chromatography. The stepwise purification (7.1-fold) is outlined in Table 3. A single peak running in the early fractions of the void volume possessed the antilisterial activity and contained the 4,000-Da band evident on Tricine-SDS gels (Fig. 4). The purified substance, found to be greater than 90% pure by high-performance liquid chromatography, was subjected to amino acid analysis, which revealed that its composition was the same as that previously published for subtilosin (data not shown). The identification of the substance as subtilosin was confirmed by Edman degradation sequence analysis (data not shown) of peptide fragments generated by partial acid hydrolysis. Again, no Phe residues were detected despite the fact that there are two Phe codons in the *sbo* coding sequence. Additionally, only one Thr residue was detected, confirming previously published data (1).

Expression of *alb-lacZ* is observed in DSM-G and in *sbo*::*neo* mutants. To begin to understand the organization and regula-

TABLE 3. Purification of subtilosin A

Purification step	Vol (ml)	Total protein (μg)	AU[a]		Recovery (%)	Fold purification
			Total	Specific (AU/μg)		
Culture supernatant	250	13,000	5,000	0.38	100	1
Fractions from High Q column	60	ND[b]	1,600	ND	32	ND
Butanol extraction	15	400	1,024	2.56	20.5	6.7
Suspension after LH-20 chromatography	0.4	240	640	2.7	12.8	7.1

[a] AU, arbitrary units.
[b] ND, not done.

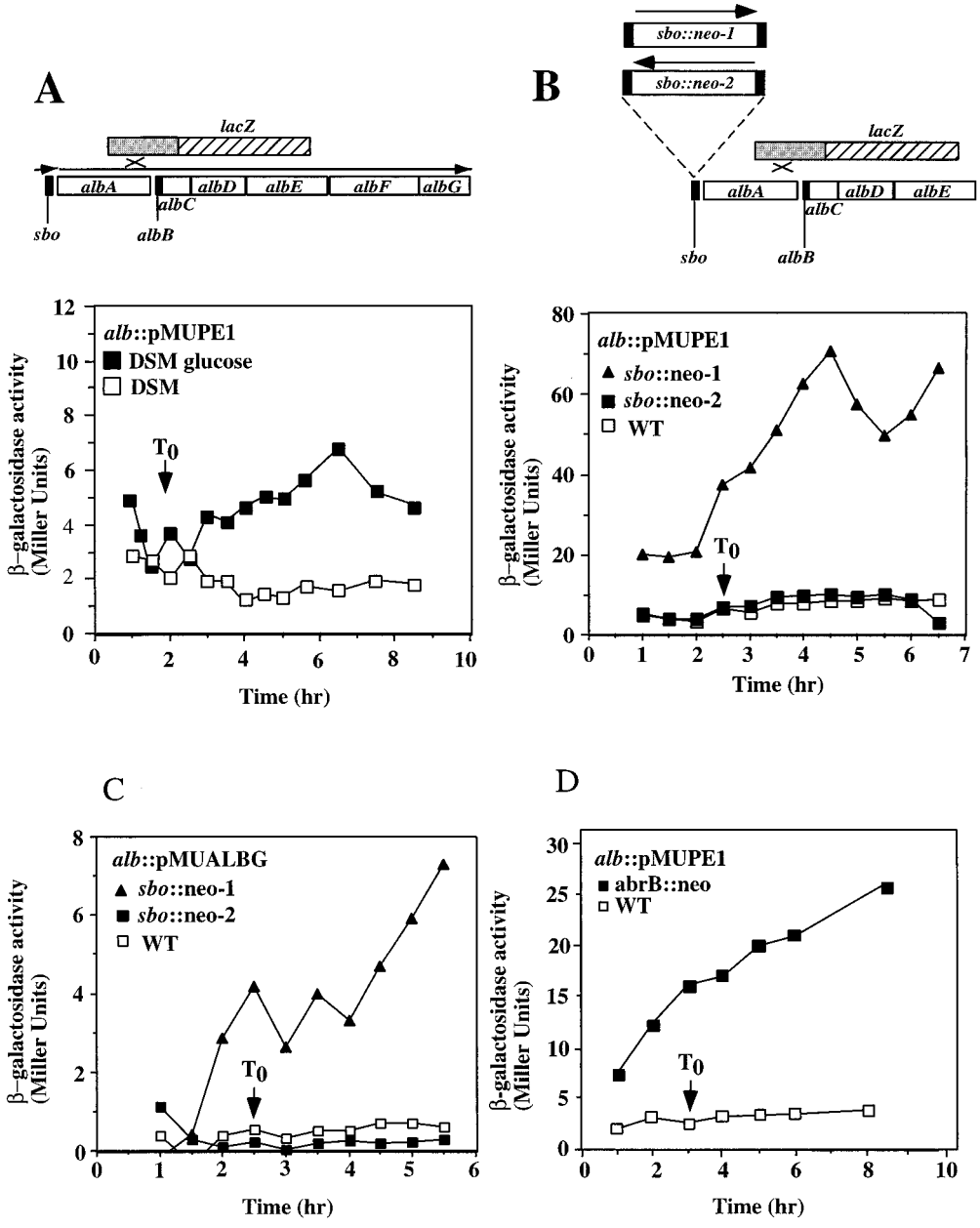


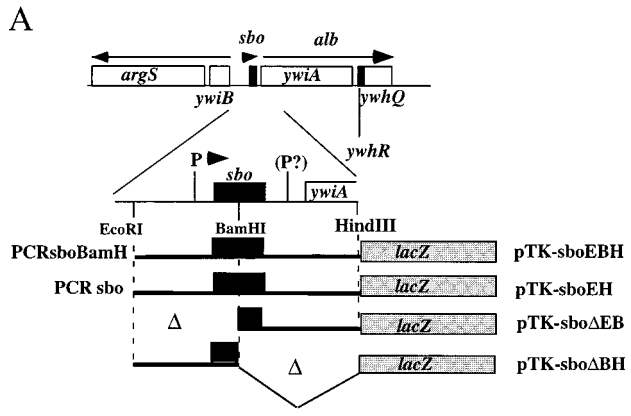
FIG. 5. Expression of an *alb-lacZ* fusion constructed by creation of the *alb*::pMUPE1 insertion. Cultures were grown in DSM or DSM-G, and 1-ml samples were collected at either 30-min or 1-h intervals. β-Galactosidase activity was determined and plotted versus time. T_0 indicates the end of the exponential growth phase. (A) Map of the *alb* operon and location of the *lacZ* fusion generated by recombination between the DNA of the *alb* locus and pMUPE1. Below the map is the expression profile of *alb-lacZ* in cells of strain ORB3147 grown in DSM and DSM-G. (B) Location of the *sbo*::*neo-1* and *sbo*::*neo-2* insertions with respect to the *alb*::pMUPE1 *lacZ* fusion. Also shown below the map is the expression profile of *alb-lacZ* in strains ORB3152 (*sbo*::*neo-2*) and ORB3153 (*sbo*::*neo-1*). (C) Expression profile of the *albG-lacZ* of strain ORB3231 (*alb*::pMUALBG) and its *sbo*::*neo-1* (ORB3284) and *sbo*::*neo-2* (ORB3230) mutant derivatives. (D) Effect of an *abrB*::*neo* insertion on the expression profile of *alb-lacZ* (*alb*::pMUPE1).

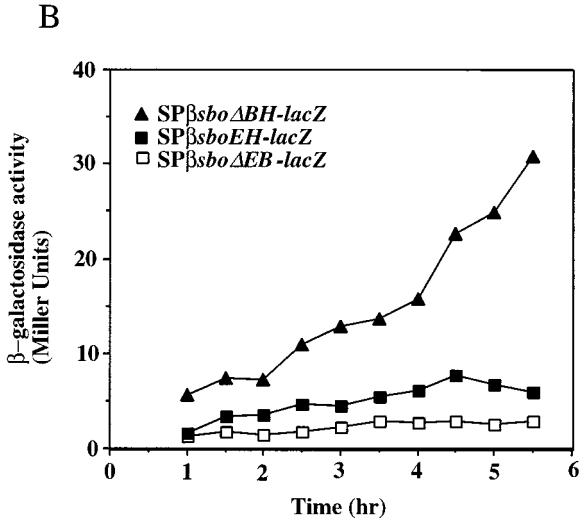
tion of the *sbo-alb* genes, the expression of *sbo-* and *alb-lacZ* gene fusions was analyzed. The insert of plasmid pE1 was cloned into the gene disruption vector pMUTIN2 (55). The resulting plasmid (pMUPE1) was introduced by transformation into cells of JH642, thus creating a strain containing a disruption of the *alb* operon and a transcriptional fusion of the 5′ end of the operon with the vector-borne *lacZ* gene. Expression of the fusion was very near background levels in TSS minimal medium with either ammonium or glutamate as the nitrogen source (data not shown). Expression was also at background levels in DSM medium (Fig. 5A) but was observed to be higher in DSM-G, with activity accumulating in stationary phase. The introduction of the *sbo*::*neo* mutation (*sbo*::*neo-1*) resulted in high-level expression of *alb-lacZ* throughout growth (Fig. 5B), but this only occurred when the neomycin resistance gene was oriented in the same direction as the *alb* transcription unit. In the reverse orientation, the *sbo*::*neo* insertion (*sbo*::*neo-2*) showed much-reduced activity.

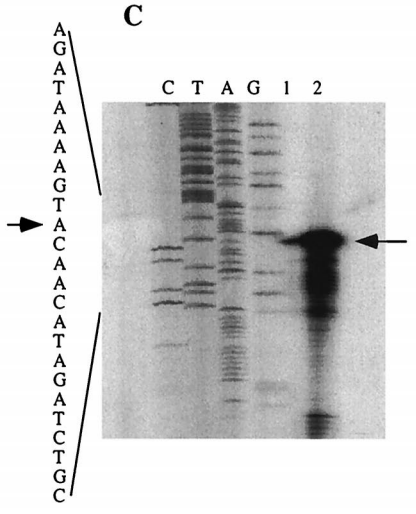
A pMUTIN disruption of the last gene of the operon, *albG*, using plasmid pMUALBG resulted in reduced antilisterial activity, as judged by the size of the zone of inhibition of *albG*::pMUALBG colonies on lawns of *L. monocytogenes*. The

level of expression of the *albG*::pMUALBG fusion was low in DSM-G but was elevated when the *sbo*::neo-1 mutation was introduced (Fig. 5C). The *sbo*::*neo-2* mutation did not elevate the level of expression of *albG*::pMUALBG, indicating that the derepression observed in the *sbo*::*neo-1* mutant was not due to the *sbo* mutation per se but was likely due to transcription from the *neo* promoter. The similar responses to the presence of the *sbo*::*neo-1* insertion observed for *albG*::pMUALBG and *alb*::pMUPE1 indicate that the two fusions reside in the same transcription unit. The observed transcriptional activity could originate from a promoter residing between *sbo* and *alb*, as well as from within the *neo* gene. The putative transcription termination sequence downstream of the *sbo* gene would limit transcription from a promoter upstream of *sbo*, but the fact that transcription from the *neo* gene is observed to traverse the termination sequence suggests that *alb* expression in wild-type cells could also be the result of transcription initiation upstream of *sbo*.

The expression of *alb-lacZ* is regulated by *abrB*. The production of and resistance to antibiotics observed for *B. subtilis* are known to be under the control of the *abrB* gene (13, 14, 19), encoding the transition state regulator of late-growth gene transcription (40, 48). The effect of *abrB* mutations on the expression of *alb*::pMUPE1 was examined. The *abrB*::*neo* insertion mutation was introduced into the *alb*::pMUPE1 mutant by transforming competent cells of strain ORB3147 with DNA from strain TT71. The level of expression of *alb*::pMUPE1 was observed to be nearly 10-fold higher in the *abrB* mutant (Fig.








```
1 ACCTTTAGGA AATGTTACAT TTTTCCGAAA

31 TCTATCATTT CCTTTTCACA TTTTTTTCAA

                  -35
61 AATATATGTA TTGAATTAGT AATTTGATAG

       -10
91 TTTTAAGATA AAAGTACAAC ATAGATCTGC
                    *
                        SD          sbo
121 TAGAAAAACA AAAAAAGGGA GGATTCAATT ATG
```

FIG. 6. (A) A fragment containing the *sbo* gene and the 5′ end of the *alb* operon was obtained by PCR and inserted into the promoter probe plasmid pTKlac (24). The *Eco*RI-*Bam*HI fragment bearing the putative *sbo* promoter region was deleted to create an *alb-lacZ* (*sboΔEB-lacZ*) fusion, and the *Bam*HI-*Hin*dIII fragment was deleted to create an *sbo-lacZ* (*sboΔBH-lacZ*) fusion. Additionally, the *Eco*RI-*Hin*dIII fragment containing the entire *sbo* gene and flanking DNA was inserted into pTKlac to create *sboEH-lacZ*, while the fragment containing the PCR-generated *sboEBH* allele was inserted into pTKlac to create the *sboEBH-lacZ* construct. The fusions thus constructed were inserted into the SPβ prophage of *B. subtilis*, using a published protocol. (B) Expression of the phage-borne fusions in cells of strains ORB3158 (SPβ*sboΔEB-lacZ*), ORB3162 (SPβ–*sboΔBH-lacZ*), and ORB3166 (SPβ*sboEH-lacZ*) grown in DSM-G. T_0, the time at which exponential growth ceases, is indicated by an arrow. (C) Primer extension analysis of RNA from JH642 and ZB449 (*abrB703*) cells. RNA was purified from cells of cultures grown to T_2 in DSM-G. On the left is the autoradiograph showing the sequence pattern of a dideoxynucleotide sequencing reaction containing primer osboP4 and pTK-sboEH DNA. Lane 1, primer extension product from a reaction containing JH642 RNA and the osboP4 oligonucleotide; lane 2, primer extension product of the reaction of ZB449 RNA and osboP4 primer. The arrow indicates the primer extension products and the proposed start site of transcription in the sequence at the left. On the right is the nucleotide sequence of the *sbo* promoter region, with the ATG start codon and Shine-Delgarno (SD) sequence shown. The putative −10 and −35 regions of the *sbo* promoter are underlined. An asterisk marks the transcriptional start site.

5D). These data and data presented below indicate that transcription of the *sbo-alb* genes is under the negative control of the *abrB* gene product.

Identification of the *sbo* promoter region. The *Bam*HI site in the *sbo* coding sequence of plasmid pUC-sboEBH was used to isolate two fragments, one containing the 5′ end of *sbo* and the putative *sbo* promoter region and the other containing the 3′ end of *sbo* and the *sbo-albA* intergenic region. Both were inserted into plasmid pTKlac (24), yielding pTK-sboΔBH and pTK-sboΔEB, respectively (Fig. 6A). The *Eco*RI-*Hin*dIII fragment of pUC-sboEH containing the entire wild-type *sbo* gene and flanking DNA was also inserted into pTKlac, as was the *Eco*RI-*Hin*dIII fragment of plasmid pUC-sboEBH. The resulting plasmids were introduced into the SPβ prophage of strain ZB307 (59). Transducing phages carrying the fusions were generated and used to lysogenize cells of strain JH642. The level of expression of the SPβ–*sboEH-lacZ* fusion peaked at 6 Miller units in stationary phase (Fig. 6B), while the level of expression of SPβ–*sbo*Δ*EB-lacZ* was the same as that of the SPβ-pTKlac negative control. The level of expression of the SPβ–*sboEBH-lacZ* fusion was similar to that observed for the SPβ–*sboEH-lacZ* construct. The level of expression of the SPβ–*sbo*Δ*BH-lacZ* fusion began at 10 Miller units and increased to 30 Miller units in stationary phase. The results suggest that the major promoter of the *sbo* and *alb* genes resides upstream of the *sbo* gene.

This conclusion was supported by the identification of the transcriptional start site of the *sbo* gene by primer extension analysis. RNA was purified from JH642 cells and from cells of the ZB449 (*abrB703*) (59) mutant strain collected from cultures grown to T_2 (2 h after the end of the exponential growth phase). Two oligonucleotides were used to generate primer extension products, one (osboP4) hybridizing with the *sbo* coding sequence beginning at bp 57 and the other, ocalbA-L, hybridizing to bp 35 to 65 of the *albA* coding region. Using osboP4, a primer extension product was generated whose length indicated the presence of a transcriptional start site residing 45 bp upstream of the *sbo* ATG (Fig. 6C). No primer extension product was detected with the ocalbA-L oligonucleotide. The RNA from *abrB* mutant cells yielded a primer extension product of the same size as that obtained from a reaction containing JH642 RNA and the osboP4 primer, but the product was much more abundant. This result is further evidence that *sbo-alb* transcription is under the negative control of *abrB*.

Expression of SPβ–*sbo-lacZ* is not regulated by subtilosin but is stimulated by *alb* operon expression. We next investigated the possibility that the *sbo* gene and *alb* operon are autoregulated via the subtilosin peptide. DSM-G cultures of the SPβ–*sbo*Δ*BH-lacZ* lysogen ORB3162 were treated with subtilosin at a concentration of 7 μg/ml before the end of the exponential growth phase. No increase in *sbo-lacZ* expression was observed other than the normal post-exponential-phase induction of expression (data not shown). The existence of autoregulation was tested again by examining the expression of *sbo-lacZ* in *sbo* and *alb* mutant strains. A heat-induced lysate of SPβ–*sbo*Δ*BH-lacZ* was used to lysogenize cells of strains ORB3146 (*alb*::pE1), ORB3148 (*sbo*::*neo-1*), and ORB3149 (*sbo*::*neo-2*). *sbo*-directed β-galactosidase activity was measured in cells collected throughout growth and stationary phase in DSM-G. Post-exponential-phase induction of *lacZ* expression was observed in the ORB3162 (SPβ–*sbo*Δ*BH-lacZ*) cultures (Fig. 7B). The *sbo*::*neo-1* mutant ORB3163, which cannot produce subtilosin but constitutively expresses *alb*, shows high-level constitutive expression of SPβ–*sbo*Δ*BH-lacZ*. The *sbo:neo-2* mutant ORB3164 does not show high-level constitutive expression but exhibits a low level of expression that increases in stationary phase. Disruption of the *alb* operon with pE1 did not consistently affect SPβ–*sbo*Δ*BH-lacZ* expression, suggesting that the defect conferred by the insertion, while eliminating subtilosin production, may not be severe enough to affect *sbo* transcription. It is also possible that the *albA* and/or *albB* gene, the disposition of which is not affected by the pE1 insertion, is responsible for stimulating the expression of SPβ–*sbo*Δ*BH-lacZ*. The constitutive expression of phage-borne *sbo-lacZ* in the *sbo*::*neo-1* mutant suggests that the transcription of *sbo-alb* is positively regulated by one or more of the *alb* operon products, which constitute an autoregulatory loop controlling *sbo-alb* transcription.

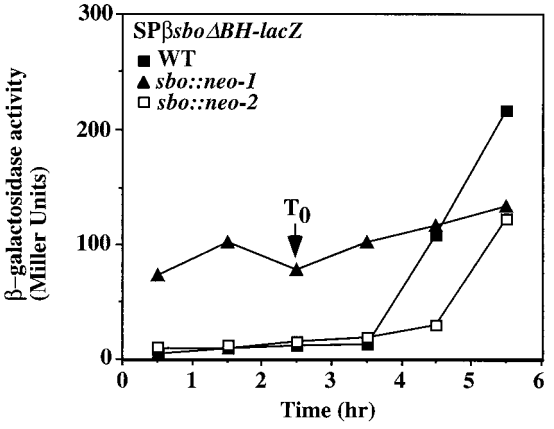


FIG. 7. *alb*-dependent stimulation of *sbo* transcription. Strains ORB3162 (SPβ–*sbo*Δ*BH-lacZ*), ORB3163 (*sbo*::*neo-1* SPβ–*sbo*Δ*BH-lacZ*), and ORB3164 (*sbo*::*neo-2* SPβ–*sbo*Δ*BH-lacZ*) were grown in DSM-G. The profiles of *sbo*Δ*BH-lacZ* expression in the three strains over the exponential and stationary phases of growth are shown. WT, wild type.

DISCUSSION

The production of an antilisterial peptide by *B. subtilis* is dependent on the *sbo* and *alb* genes, as judged by the phenotypes of three mutant strains, the *alb*::pE1 operon disruption mutant, the *sbo*::*neo* mutant, and the *albG*::p*MUALBG* insertion mutant. The *albABCDEFG* genes are believed to constitute an operon that encodes the proteins that function in presubtilosin processing and subtilosin export. The *sbo* gene encodes presubtilosin, which is a 43-amino-acid peptide that likely undergoes processing steps that include proteolytic cleavage at the Asn9 residue, modification at the two Phe residues at positions 30 and 39, and modification of Thr36. A cross-link of unusual composition is thought to connect Cys21 and Glu31 (adjacent to the modified Phe residue). Finally, the cyclization of the presubtilosin peptide involves covalent linking of the C-terminal Gly with the N-terminal Asn. The involvement of modified Phe and Glu, along with the similarities revealed by aligning the amino acid sequences of the AlbA and AlbE products with those of proteins that function in PQQ synthesis (which is initiated by a condensation of Glu and Tyr [10]), suggests that PQQ synthesis and subtilosin processing may proceed through some common reaction mechanisms.

The *sbo* gene is followed by a sequence resembling a factor-independent transcriptional termination sequence. Yet, transcription can proceed through the sequence, as shown by the introduction of the *neo* gene upstream of the terminator that drives constitutive expression of the *alb*::pMUPE1 *lacZ* fusion. Other bacteriocin biosynthesis operons have a similar organi-

zation (21, 42). The positioning of the terminator (Fig. 2C) between the bacteriocin structural gene and the genes required for processing and export is thought to ensure that the proteins that carry out peptide modification and processing are present in small, catalytic amounts while the peptide substrate is produced in larger quantities. Transcription from the *neo* fragment can drive expression of the *alb* genes, including the last gene of the operon, *albG*. No promoter activity could be detected in the *sbo-alb* intergenic region by measuring *lacZ* activity of the SPβ–*sboΔEB-lacZ* cells or by primer extension analysis. However, we cannot rule out the possibility that within this region there is a weak promoter that is utilized under specific growth conditions. The major transcriptional start site for *sbo-alb* lies upstream of the *sbo* gene and is associated with a sequence resembling promoters utilized by the σ^A form of *B. subtilis* RNA polymerase (−35 TTGAAT [17bp]−10 TAAGAT [Fig. 6C]). Strong evidence that *sbo-alb* is under the negative control of the global transition state transcriptional regulatory protein AbrB is presented here. In support of this conclusion is the observation that *spo0A* mutant cells, in which the AbrB protein is overproduced, do not exhibit antilisterial activity (data not shown). As with other AbrB-controlled genes, we would expect to find that AbrB protein directly interacts with the *sbo* promoter, thereby preventing RNA polymerase from establishing contacts with the −35 and −10 sequences.

Another form of regulation was revealed by examining the expression of SPβ–*sboΔBH-lacZ* in *sbo* and *alb* mutant strains. The *sbo*::*neo-1* insertion was observed to drive the high-level constitutive expression of *alb-lacZ* (*alb*::pMUPE1). This mutation also caused constitutive expression of the *sboΔBH-lacZ* fusion positioned in the SPβ prophage, while the *sbo*::*neo-2* insertion did not. Disruption of the *sbo* gene did not cause a decrease in the level of *sbo-lacZ* expression, and addition of subtilosin to cultures of low cell density did not stimulate expression. From these observations, we conclude that unlike the situation in the case of the *nis* or *spa* operon, the exogenous presence of the bacteriocin encoded by *sbo* does not stimulate expression of *sbo*. However, a form of positive autoregulation appears to exist, and it involves one or more of the *alb* gene products. The *alb*::pMUPE1 *lacZ* fusion has very low activity, which is likely due to the fact that the *alb* operon is disrupted by the fusion and there is no positive autoregulation. This can be suppressed by introduction of an *abrB* mutation (Fig. 5D), suggesting a link between the regulatory function of *alb* and AbrB activity.

The regulation of *sbo-alb* is more complex, however, since it is subject to control exerted by factors that regulate anaerobic gene expression, including ResDE and Fnr (37a). How these factors interact with those functioning in autoregulation and AbrB-dependent control presents an interesting problem for further investigation.

ACKNOWLEDGMENTS

Research reported herein was supported by grant GM45898 from the National Institutes of Health, a grant from the Oregon Medical Research Foundation, and funds from the Natural Sciences and Engineering Council of Canada.

REFERENCES

1. **Babasaki, K., T. Takao, Y. Shimonishi, and K. Kurahashi.** 1985. Subtilosin A, a new antibiotic peptide produced by *Bacillus subtilis* 168: isolation, structural analysis, and biogenesis. J. Biochem. **98:**583–603.
2. **Burbulys, D., K. A. Trach, and J. A. Hoch.** 1991. Initiation of sporulation in *B. subtilis* is controlled by a multicomponent phosphorelay. Cell **64:**545–552.
3. **Clary, D. O., J. A. Wahleithner, and D. R. Wolstenholme.** 1984. Sequence and arrangement of the genes for cytochrome *b*, URF1, URF4L, URF4, URF5, URF6 and five tRNAs in *Drosophila* mitochondrial DNA. Nucleic Acids Res. **12:**3747–3762.
4. **de Vos, W. M., O. P. Kuipers, J. Roelof van der Meer, and R. J. Siezen.** 1995. Maturation pathway of nisin and other lantibiotics: post-translationally modified antimicrobial peptides exported by Gram-positive bacteria. Mol. Microbiol. **17:**427–437.
5. **Dubnau, D., and R. Davidoff-Abelson.** 1971. Fate of transforming DNA following uptake by competent *Bacillus subtilis*. I. Formation and properties of the donor-recipient complex. J. Mol. Biol. **56:**209–221.
6. **Entian, K. D., and W. M. de Vos.** 1996. Genetics of subtilin and nisin biosyntheses: biosynthesis of lantibiotics. Antonie Leeuwenhoek **69:**109–117.
7. **Fouet, A., and A. L. Sonenshein.** 1990. A target for carbon source-dependent negative regulation of the *citB* promoter of *Bacillus subtilis*. J. Bacteriol. **172:**835–844.
8. **Furbaß, R., M. Gocht, P. Zuber, and M. A. Marahiel.** 1991. Interaction of AbrB, a transcriptional regulator from *Bacillus subtilis*, with the promoters of the transition state-activated genes *tycA* and *spoVG*. Mol. Gen. Genet. **225:** 347–354.
9. **Glaser, P., A. Danchin, F. Kunst, P. Zuber, and M. M. Nakano.** 1995. Identification and isolation of a gene required for nitrate assimilation and anaerobic growth of *Bacillus subtilis*. J. Bacteriol. **177:**1112–1115.
10. **Goodwin, P. M., and C. Anthony.** 1998. The biochemistry, physiology and genetics of PQQ and PQQ-containing enzymes. Adv. Microb. Physiol. **40:**1–80.
11. **Grossman, A. D.** 1995. Genetic networks controlling the initiation of sporulation and the development of genetic competence in *Bacillus subtilis*. Annu. Rev. Genet. **29:**477–508.
12. **Guerout-Fleury, A. M., K. Shazand, N. Frandsen, and P. Stragier.** 1995. Antibiotic-resistance cassettes for *Bacillus subtilis*. Gene **167:**335–336.
13. **Guespin-Michel, J. F.** 1971. Phenotypic reversion in some early blocked sporulation mutants of *Bacillus subtilis*: isolation and phenotype identification of partial revertants. J. Bacteriol. **108:**241–247.
14. **Guespin-Michel, J. F.** 1971. Phenotypic reversion in some early blocked sporulation mutants of *Bacillus subtilis*. Genetic study of polymyxin resistant partial revertants. Mol. Gen. Genet. **112:**243–254.
15. **Harwood, C. R., and S. M. Cutting.** 1990. Molecular biological methods for *Bacillus*. John Wiley & Sons, Chichester, United Kingdom.
16. **Havarstein, L. S., D. B. Diep, and I. F. Nes.** 1995. A family of bacteriocin ABC transporters carry out proteolytic processing of their substrates concomitant with export. Mol. Microbiol. **16:**229–240.
17. **Hoch, J. A.** 1995. Control of cellular development in sporulating bacteria by the phosphorelay two-component signal transduction system, p. 129–144. *In* J. A. Hoch and T. J. Silhavy (ed.), Two-component signal transduction. ASM Press, Washington, D.C.
18. **Ireton, K., D. Z. Rudner, K. J. Siranosian, and A. D. Grossman.** 1993. Integration of multiple developmental signals in *Bacillus subtilis* through the Spo0A transcription factor. Genes Dev. **7:**283–294.
19. **Ito, J., G. Mildner, and J. Spizizen.** 1971. Early blocked asporogenous mutants of *Bacillus subtilis* 168. I. Isolation and characterization of mutants resistant to antibiotic(s) produced by sporulating *Bacillus subtilis* 168. Mol. Gen. Genet. **112:**104–109.
20. **Iwata, S., J. W. Lee, K. Okada, J. K. Lee, M. Iwata, B. Rasmussen, T. A. Link, S. Ramaswamy, and B. K. Jap.** 1998. Complete structure of the 11-subunit bovine mitochondrial cytochrome bc1 complex. Science **281:**64–71.
21. **Jack, R., B. Bierbaum, C. Heidrich, and H.-G. Sahl.** 1995. The genetics of lantibiotic biosynthesis. Bioessays **17:**793–802.
22. **Jack, R. W., F. R. Tagg, and B. Ray.** 1995. Bacteriocins of gram-positive bacteria. Microbiol. Rev. **59:**171–200.
23. **Katz, E., and A. L. Demain.** 1977. The peptide antibiotics of *Bacillus*: chemistry, biogenesis, and possible functions. Bacteriol. Rev. **41:**449–474.
24. **Kenney, T. J., and C. P. Moran, Jr.** 1991. Genetic evidence for interaction of σ^A with two promoters in *Bacillus subtilis*. J. Bacteriol. **173:**3282–3290.
25. **Klaenhammer, T. R.** 1988. Bacteriocins of lactic acid bacteria. Biochemie **70:**337–349.
26. **Klein, C., C. Kaletta, and K.-D. Entian.** 1993. Biosynthesis of the lantibiotic subtilin is regulated by a histidine kinase/response regulator system. Appl. Environ. Microbiol. **59:**296–303.
27. **Kleinkauf, H., and H. von Dohren.** 1984. Peptide antibiotics, p. 284–307. *In* H. Pape and H.-J. Rehm (ed.), Biotechnology, vol. 4. Microbiol products II. VCH Berlagsgesellschaft GmbH, Weinheim, Germany.
28. **Kolter, R., and F. Moreno.** 1992. Genetics of ribosomally synthesized peptide antibiotics. Annu. Rev. Microbiol. **46:**141–164.
29. **Kuipers, O. P., M. M. Beerthuyzen, P. G. G. A. de Ruyter, E. J. Luesink, and W. M. de Vos.** 1995. Autoregulation of nisin biosynthesis in *Lactococcus lactis* by signal transduction. J. Biol. Chem. **270:**27299–27304.
30. **Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V. Azevedo, M. G. Bertero, P. Bessieres, A. Bolotin, S. Borchert, R. Borriss, L. Boursier, A. Brans, M. Braun, S. C. Brignell, S. Bron, S. Brouillet, C. V. Bruschi, B. Caldwell, V. Capuano, N. M. Carter, S. K. Choi, J. J. Codani, I. F. Connerton, A. Danchin, et al.** 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature **390:**249–256.
31. **LaCelle, M., M. Kumano, K. Kurita, K. Yamane, P. Zuber, and M. M.**

Nakano. 1996. Oxygen-controlled regulation of the flavohemoglobin gene in *Bacillus subtilis*. J. Bacteriol. **178:**3803–3808.
32. **Li, Y. M., J. C. Milne, L. L. Madison, R. Kolter, and C. T. Walsh.** 1996. From peptide precursors to oxazole- and thiazole-containing peptide antibiotics: microcin B17 synthase. Science **274:**1188–1193.
33. **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.
34. **Meulenberg, J. J., E. Sellink, N. H. Riegman, and P. W. Postma.** 1992. Nucleotide sequence and structure of the *Klebsiella pneumoniae pqq* operon. Mol. Gen. Genet. **232:**284–294.
35. **Montville, T. J., and K. Winkowski.** 1997. Biologically based preservation systems and probiotic bacteria, p. 557–577. *In* M. P. Doyle, L. R. Beuchat, and T. J. Montville (ed.), Food microbiology: fundamentals and frontiers. ASM Press, Washington, D.C.
36. **Nakano, M. M., M. A. Marahiel, and P. Zuber.** 1988. Identification of a genetic locus required for biosynthesis of the lipopeptide antibiotic surfactin in *Bacillus subtilis*. J. Bacteriol. **170:**5662–5668.
37. **Nakano, M. M., L. Xia, and P. Zuber.** 1991. Transcription initiation region of the *srfA* operon, which is controlled by the *comP-comA* signal transduction system in *Bacillus subtilis*. J. Bacteriol. **173:**5487–5493.
37a. **Nakano, M. M., G. Zheng, and P. Zuber.** Unpublished results.
38. **Nes, I. F., D. B. Diep, L. S. Havarstein, M. B. Brurberg, V. Eijsink, and H. Holo.** 1996. Biosynthesis of bacteriocins in lactic acid bacteria. Antonie Leeuwenhoek **70:**113–128.
39. **Paces, V., L. E. Rosenberg, W. A. Fenton, and F. Kalousek.** 1993. The beta subunit of the mitochondrial processing peptidase from rat liver: cloning and sequencing of a cDNA and comparison with a proposed family of metallopeptidases. Proc. Natl. Acad. Sci. USA **90:**5355–5358.
40. **Perego, M., G. B. Spiegelman, and J. A. Hoch.** 1988. Structure of the gene for the transition state regulator *abrB*: regulator synthesis is controlled by the Spo0A sporulation gene in *Bacillus subtilis*. Mol. Microbiol. **2:**689–699.
41. **Pollock, R. A., F. U. Hartl, M. Y. Cheng, J. Ostermann, A. Horwich, and W. Neupert.** 1988. The processing peptidase of yeast mitochondria: the two co-operating components MPP and PEP are structurally related. EMBO J. **7:**3493–3500.
42. **Qi, F., P. Chen, and P. W. Caufield.** 1999. Functional analyses of the promoters in the lantibiotic mutacin II biosynthetic locus in *Streptococcus mutans*. Appl. Environ. Microbiol. **65:**652–658.
43. **Rivers, S. L., E. McNairn, F. Blasco, G. Giordano, and D. H. Boxer.** 1993. Molecular genetic analysis of the *moa* operon of *Escherichia coli* K-12 required for molybdenum cofactor biosynthesis. Mol. Microbiol. **8:**1071–1081.
44. **Robertson, J. R., M. Gocht, M. A. Marahiel, and P. Zuber.** 1989. AbrB, a regulator of gene expression in *Bacillus*, interacts with the transcription initiation regions of a sporulation and an antibiotic biosynthesis gene. Proc. Natl. Acad. Sci. USA **86:**8457–8461.
45. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
46. **Schagger, H., and G. von Jagow.** 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. **166:**368–379.
47. **Serror, P., and A. L. Sonenshein.** Unpublished data.
48. **Strauch, M. A.** 1993. AbrB, a transition state regulator, p. 757–764. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology, Washington, D.C.
49. **Strauch, M. A.** 1995. Delineation of AbrB-binding sites on the *Bacillus subtilis spo0H*, *kinB*, *ftsAZ*, and *pbpE* promoters and use of a derived homology to identify a previously unsuspected binding site in the *bsuB1* methylase promoter. J. Bacteriol. **177:**6999–7002.
50. **Strauch, M. A., G. B. Spiegelman, M. Perego, W. C. Johnson, D. Burbulys, and J. A. Hoch.** 1989. The transition state transcription regulator AbrB of *Bacillus subtilis* is a DNA binding protein. EMBO J. **8:**1615–1621.
51. **Strauch, M. A., V. Webb, B. Spiegelman, and J. A. Hoch.** 1990. The Spo0A protein of *Bacillus subtilis* is a repressor of the *abrB* gene. Proc. Natl. Acad. Sci. USA **87:**1801–1805.
52. **Toyama, H., L. Chistoserdova, and M. E. Lidstrom.** 1997. Sequence analysis of *pqq* genes required for biosynthesis of pyrroloquinoline quinone in *Methylobacterium extorquens* AMI and the purification of a biosynthetic intermediate. Microbiology **143:**595–602.
53. **Toyama, H., and M. E. Lidstrom.** 1998. *pqqA* is not required for biosynthesis of pyrroloquinoline quinone in *Methylobacterium extorquens* AM1. Microbiology **144:**183–191.
54. **Trowsdale, J., S. M. 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 chromosome region near the origin of replication. Mol. Gen. Genet. **173:**61–70.
55. **Vagner, V., E. Dervyn, and S. D. Ehrlich.** 1998. A vector for systematic gene inactivation in *Bacillus subtilis*. Microbiology **144:**3097–3104.
56. **Velterop, J. S., E. Sellink, J. J. M. Meulenberg, S. David, I. Bulder, and P. W. Postma.** 1995. Synthesis of pyrroloquinoline quinone in vivo and in vitro and detection of an intermediate in the biosynthetic pathway. J. Bacteriol. **177:**5088–5098.
57. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
57a. **Zheng, G.** 1997. Ph.D. thesis. University of Arkansas, Fayetteville.
58. **Zheng, G., and M. F. Slavik.** 1999. Isolation, partial purification and characterization of a bacteriocin produced by a newly isolated *Bacillus subtilis* strain. Lett. Appl. Microbiol. **28:**363–367.
59. **Zuber, P., and R. Losick.** 1987. Role of AbrB in the Spo0A- and Spo0B-dependent utilization of a sporulation promoter in *Bacillus subtilis*. J. Bacteriol. **169:**2223–2230.
60. **Zuber, P., M. M. Nakano, and M. A. Marahiel.** 1993. Peptide antibiotics, p. 897–916. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology, Washington, D.C.