Identification of the Promoter for a Peptide Antibiotic Biosynthesis Gene from *Bacillus brevis* and Its Regulation in *Bacillus subtilis*

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Tyrocidine is a cyclic decapeptide antibiotic which is produced and secreted by stationary-phase cells of the sporeforming bacterium *Bacillus brevis*. We identified the promoter for the *B. brevis* structural gene (*tycA*) for tyrocidine synthetase I, the enzyme catalyzing the first step in tyrocidine biosynthesis, and studied its regulation in cells of *B. brevis* and *Bacillus subtilis*. Transcription from the *tycA* promoter was induced at the end of the exponential phase of the growth cycle in *B. brevis* cells growing in sporulation medium. To study the regulation of *tycA* in *B. subtilis*, we constructed a derivative of the *B. subtilis* bacteriophage SP β containing a transcriptional fusion of the *tycA* promoter to the *lacZ* gene of *Escherichia coli* and introduced the *tycA-lacZ* operon fusion by means of specialized transduction into sporulation mutants known to be blocked in sporulation-associated antibiotic production. Our principal finding was that *tycA*-directed *lacZ* expression was impaired in the stage-0 mutants with mutations *spo0A*, *spo0B*, and *spo0E* but not in *spo0C*, *spo0F*, *spo0H*, or *spo*⁺ bacteria. The dependence on the *spo0A* gene product could be entirely bypassed by an *abrB* suppressor mutation, which caused *tycA-lacZ* to be transcribed constitutively at all stages of growth. A simple model is proposed for the mechanism of *tycA* induction based on the Spo0A-dependent inactivation of AbrB protein, which is proposed to be a negative regulator of *tycA* transcription.

Bacteria of the genus *Bacillus* produce and secrete a wide variety of small molecules, such as antibiotics and other secondary metabolites, as they enter the stationary phase (9, 10). Often, the appearance of these molecules is closely associated with the onset of sporulation, and for this reason the idea has developed that the genes for antibiotic production in *Bacillus* species (and in other microorganisms as well) are subject to some of the same regulatory mechanisms that trigger spore formation (28). Although much has been learned about the enzymatic pathways that govern the production of various kinds of antibiotics, little is known about the mechanisms that control the expression of antibiotic biosynthesis genes or the way in which and the extent to which the expression of these genes is coupled to regulatory events occurring at the onset of sporulation (12, 13).

The recent availability of cloned *Bacillus* genes encoding biosynthetic enzymes for peptide antibiotics such as bacitracin (14), gramicidin S (15), and tyrocidine (19) has provided an opportunity to investigate the regulation of antibiotic biosynthesis genes directly at the level of gene transcription. Here we investigate the transcriptional control of a gene (*tycA*; formerly *tyl*) from *Bacillus brevis* that encodes tyrocidine synthetase I, the enzyme that catalyzes the first step in the synthesis of the cyclic decapeptide antibiotic tyrocidine. We report the identification of the promoter for the *tycA* gene and demonstrate that the synthesis of tyrocidine synthetase mRNA in *B. brevis* cells growing in medium supporting antibiotic production is deferred until the end of the exponential phase of the growth cycle. We also report the construction of a derivative of the *Bacillus subtilis* bacteriophage SP β bearing a fusion of *tycA* to the *lacZ* gene of *Escherichia coli* and the use of this transcriptional fusion to study the genetic control of the *tycA* gene in *B. subtilis*, a

Bacillus species in which highly developed tools for studying the control of gene expression are available. The experiments described here show that the transcription of *tycA* is dependent on the products of certain stage-0 sporulation regulatory genes. The dependence on the *spo0A* gene product could be bypassed by a mutation at a suppressor locus called *abrB*.

MATERIALS AND METHODS

Bacterial strains and plasmids. All *Bacillus* strains are listed in Table 1. All *B. subtilis* strains are derivatives of JH642 (*spo*⁺ and *spo0* strains were obtained from J. Hoch). Tyrocidine-producing strain *B. brevis* ATCC 8185 was described previously (24). Plasmid pBT2 (19) contains the *tycA* gene on a 5.2-kilobase (kb) *EcoRI* fragment in pBR322. Plasmid pUM18, a derivative of the *Staphylococcus aureus* plasmid pUB110 which is functional in *B. subtilis*, carries the entire *tycA* gene on a 5.2-kb *EcoRI* fragment. The plasmid pZ Δ 327 (34) is a pBR322 derivative that bears a promoterless *lacZ* gene and a chloramphenicol resistance determinant which is functional in *B. subtilis*. pZ Δ 327 was used in the construction of the *tycA-lacZ* fusion plasmid pMZ12 (see Fig. 7). Plasmids pGEM3-1, pGEM3-38, and pGEM3-20 (Fig. 1) were derived from pGEM3 (Promega Biotec, Madison, Wis.) and were used to generate radiolabeled cRNA probes for the identification of *tycA* transcripts.

Transformation. Competent cells of *B. subtilis* were prepared and transformed by the method of Gryczan and Dubnau (6), whereas *B. subtilis* protoplast transformation was performed by the method of Chang and Cohen (4). *E. coli* cells were transformed by the method of Dagert and Ehrlich (5).

RNA preparation and transcriptional mapping. Cells of *B. brevis* ATCC 8185 or *B. subtilis* were grown in 500 ml of liquid DS medium (29) at 37°C. At appropriate times during growth, cells from a 50-ml sample were collected by centrifugation at 10 krpm for 10 min in a Sorvall SS34 rotor. RNA

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TABLE 1. Bacterial strains

Strain	Genotype
<i>Bacillus subtilis</i>	
JH642	...trpC2 pheA1
JH646	...trpC2 pheA1 spo0A12
JH648	...trpC2 pheA1 spo0B136
JH647	...trpC2 pheA1 spo0E11
JH649	...trpC2 pheA1 spo0F221
JH651	...trpC2 pheA1 spo0H81
JH693	...trpC2 pheA1 spo0J93
JH695	...trpC2 pheA1 spo0C9V
ZB369	...trpC2 pheA1 spo0AΔ204 abrB703
ZB449	...trpC2 pheA1 abrB703
ZB493	...trpC2 pheA1 abrB703 SPβ c2 del2::Tn917::pSK10Δ6
<i>Bacillus brevis</i>	
ATCC 8185	...spo ⁺

was prepared from each sample by the procedure of Penn et al. (26) as modified by Igo and Losick (10a).

³²P-labeled RNA probes were synthesized from pGEM3 plasmid derivatives with SP6 RNA polymerase (Promega Biotec) under the conditions recommended by the supplier. The ³²P-labeled RNA was combined with 20 to 100 μg of total cellular RNA, and the mixture was precipitated with ethanol in the presence of 0.2 M LiCl at -20°C. In the time course experiments equal amounts of RNA were added to each hybridization reaction, as normalized by examination of 16S and 23S rRNAs on ethidium bromide-stained gels and by A₂₆₀. The precipitate was collected by centrifugation, and the RNA was dissolved in 25 μl of hybridization buffer (80% formamide, 40 mM PIPES [piperazine-N,N'-bis(2-ethanesulfonic acid)], 400 mM NaCl, 1 mM EDTA). The mixture was heated at 85°C for 5 min and incubated for 3 h at 45 to 50°C. RNase A and RNase T₁ were added to final concentrations of 0.4 and 0.02 mg/ml, respectively, in a buffer containing 10 mM Tris hydrochloride (pH 7.4), 300 mM NaCl, and 5 mM EDTA and incubated at 30°C for 1 h. The reaction was terminated by adding 5 μl of 10-mg/ml proteinase K (BioRad Laboratories, Richmond, Calif.) and 20 μl of 10% sodium dodecyl sulfate at 37°C for 15 min. The RNase-resistant RNA-RNA duplexes were denatured and resolved on a 3.5 or 5% polyacrylamide-7 M urea gel.

Construction of *tycA-lacZ* fusion. The plasmid pBT2 was cleaved with *Pvu*II and *Xmn*I to liberate the fragment bearing the putative *tycA* promoter sequence. The fragment was purified electrophoretically from an agarose gel and

combined with plasmid pZΔ327 which had been linearized with *Hind*III and treated with the Klenow fragment of DNA polymerase I to render flush each end of the linear plasmid molecule. The mixture of linear pZΔ327 and *Pvu*II-*Xmn*I fragment was treated with T4 DNA ligase and introduced into *E. coli* HB101 by transformation. A plasmid of the proper construction was identified, and its structure was confirmed by restriction analysis.

Analytical procedures. The β-galactosidase specific activity in cells of *B. subtilis* containing the *tycA-lacZ* fusion was determined by a previously described procedure (22, 35). The cells were grown in DS medium to the mid-log phase and were diluted 100-fold into 50 ml of fresh DS medium. After the cells had entered exponential growth, usually around an optical density at 595 nm of 0.2, 1-ml samples were collected every 30 min. The cell samples were assayed for β-galactosidase activity. To identify proteins that are encoded by plasmid-borne *tycA* in *B. subtilis* and *E. coli* cells, we analyzed total cell protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Laemmli (17) followed by the Western blot immunodetection technique (30). Proteins reacting with anti-TycA antiserum were identified in a reaction sequence with primary antibody, biotinylated protein A, and streptavidin-biotinylated peroxidase complex as described by Marahiel et al. (19).

DNA preparation, restriction endonuclease digestion, ligation, and other standard methods used for DNA manipulation were as described by Maniatis et al. (18). Isolation and purification of SPβ transducing phage from *B. subtilis* lysogens and transduction methods were as previously described (33, 36). DNA sequencing was carried out as described by Sangar et al. (27).

High-resolution S1 nuclease mapping. Total cellular RNA prepared either from the antibiotic producer strain *B. brevis* ATCC 8185 or from strain ZB369 bearing the *tycA-lacZ* fusion was combined with 0.1 to 0.5 μg of the *Hinc*II-*Pvu*II *tycA* promoter fragment that was ³²P, 5' labeled at the *Hinc*II end (2). Hybridization and S1 nuclease digestion were performed as previously described (1). The S1-resistant hybrids were denatured and resolved on an 8% polyacrylamide-7 M urea gel alongside the ³²P-end-labeled *Hinc*II-*Pvu*II fragment DNA which had been subjected to the nucleotide-specific cleavage reactions of Maxam and Gilbert (20). The ³²P-labeled DNA was visualized by autoradiography with Kodak XAR-5 X-ray film.

RESULTS

Localizing the *tycA* transcription start site by nuclease mapping. As a background to our efforts to localize the *tycA*

FIG. 1. Endonuclease restriction map of DNA in the vicinity of the *tycA* gene. The figure shows the 5.2-kb *Eco*RI insert of DNA in plasmid pBT2 containing the *B. brevis* *tycA* gene. The location and orientation of the *tycA* coding sequence are indicated by the heavy black arrow. The transcriptional start site (P) is indicated by the small arrow. Plasmid subclones containing segments of pBT2 insert DNA are listed below the restriction map.

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-100      -80      -60      -40
CCAAGCAAACTTGGATTACGTAAAAAGATGTGAAAAAACTGGTGAATTTTTCGAAAAATATCCCTATTTTT

-20      +1----->      +20      +40
TAATCGACTTCGAATTTTCTCTGCTATAATGAGTTTCAGCGTCAGTAACCTAGTGTCTTCAGCCTGTCAGCCGA
      ^
      +60      +80      +100
TCGGGAACCTCCTGTGATTGTTTTCATGCAAATCAGTTTCCTTCGTCGAGCGTAAGCAGGCGTATCCGGCAGC

+120      +140      +160      +180
GGAATACCAGCACCCATTTTCGGCTACAGCATGAGCAAAATATGACTTTAGTATGAAGGGAATTTCCGAGAAACA

+200      +220      +240      +260
GGAAATTTTCTGTGTTAAATACCCAAATGATGGAAATGGGAAATGGAATGGAACGTTGACCTTGCCTGT
      ^HincII

+280      +300      +320
CTCTGTGTGGCAACCAATTTCGTACACTTCATGATAAGCAGAAGTAATTCATTATCGGAGGGGACAT ATG
      ^XmnI      met

+340      +360      +380
TTA GCA AAT CAG GCC AAT CTC ATC GAC AAC AAG CGG GAA CTG GAG CAG CAT CGC CTA
leu ala asn gln ala asn leu ile asp asn lys arg glu leu glu gln his arg leu

+400      +420      +440
GTT CAT ATG CAC AGG GCA AGT CGA TCC ATC AAT TGT TCG AGG AAC AAG CAG AGG CTT
val his met his arg ala ser arg ser ile asn cys ser arg asn thr gln arg leu

+460      +480      +500
TTC CAG ACC GCG TTG CCA TCG TTT TTG AAA ACA GGC GGC TTT CGT AAT CAG GAG TTA
phe gln thr ala leu pro ser phe leu lys thr gly gly phe arg asn gln glu leu

AGC AGG AAA GCC
ser arg lys ala

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FIG. 2. Nucleotide sequence of nontranscribed strand of the *tycA* promoter region. +1 is the 5' terminus of the *tycA* mRNA. Underlined nucleotides indicate the putative -10 and -35 regions of the promoter and the putative ribosome-binding site of the *tycA* mRNA open reading frame.

transcription start site, Fig. 1 shows the location and orientation of the tyrocidine synthetase I gene in cloned DNA as determined in work to be reported elsewhere (G. Czekay, Diplomarbeit, Technische Universität Berlin). In brief, physical mapping of the tyrocidine synthetase-coding sequence was based on the use of anti-tyrocidine synthetase I antibodies to detect the synthetase polypeptide in *E. coli* cells bearing plasmids that contained all or part of the *tycA* gene. The principal findings were first the detection of the full-length, 120-kilodalton polypeptide product of the *tycA* gene in *E. coli* cells bearing plasmid pGC22 (Fig. 1). This finding established that the entire *tycA* coding sequence was contained within the 3.3-kb *HincII* insert of *B. brevis* DNA in pGC22. Because this insert was only marginally larger than the predicted coding sequence for the 120-kilodalton synthetase polypeptide (roughly 3 kb), the 5' end of the *tycA* open reading frame must lie near one end of the 3.3-kb *HincII* fragment of *B. brevis* DNA. Second, the use of anti-tyrocidine synthetase antibodies revealed that the plasmid subclone pGCΔ22-1 representing a deletion of the DNA insert in pGC22 directed the synthesis in *E. coli* of a truncated synthetase polypeptide of 70 kilodaltons. This finding established that the orientation of the *tycA* coding sequence was from right to left as shown in Fig. 1. Finally, nucleotide sequence analysis extending to the right (Fig. 1 and 2) of the *HincII* site located just upstream to the region that we deduced contained the putative coding sequence of the *tycA* gene revealed the presence of a putative ribosome-binding site, an initiation codon, and an open reading frame extending for at least 250 base pairs (bp) (the extent of our sequencing analysis; Fig. 2).

Knowing the location and orientation of the synthetase-coding sequence, we devised a nuclease hybridization ex-

periment to determine the position of the 5' end of the *tycA* mRNA. ³²P-labeled probe RNAs were generated by runoff transcription from the DNA inserts in the Riboprobe (Promega Biotec) plasmids pGEM3-1, pGEM3-38, and pGEM3-20 with phage SP6 RNA polymerase (21). The DNA inserts in these plasmids covered the region upstream to the 5' end of the *tycA* coding sequence, and the RNAs that were synthesized from the inserts were complementary to the *tycA* mRNA. The radioactive runoff RNAs were incubated under hybridization conditions with total cellular RNA purified from *B. brevis* cells that had been grown to the early stationary phase in DS medium. After hybridization and treatment with RNase, the resulting nuclease-resistant RNA-RNA hybrids were denatured and subjected to electrophoresis. The experiment of Fig. 3 shows that hybridization of radioactive probes copied from pGEM3-38 and pGEM3-1 to total cellular RNA generated nuclease-resistant RNAs of about 300 and 250 bases in length, respectively. We conclude on this basis that the 5' terminus of *tycA* mRNA originated at the site indicated in Fig. 1 and 3. That transcription from this site proceeded into the *tycA* coding sequence was verified by the demonstration that hybridization of cellular RNA to probe RNA transcribed from pGEM3-20 protected a radioactive RNA of 1,500 bases from the action of RNase (data not shown). The time course of appearance of *tycA* mRNA as detected by the nuclease hybridization assay (Fig. 4) was in excellent agreement with previous studies demonstrating that the synthesis of tyrocidine synthetase I is induced after the end of the exponential phase of the *B. brevis* growth cycle (11, 16).

To map the 5' terminus of *tycA* mRNA at high resolution (at the nucleotide sequence level), we used as a hybridization probe pGEM3-1 DNA that had been radioactively

FIG. 3. Nuclease mapping of the 5' end of the *tycA* transcript. The 5' end of the *tycA* transcript was mapped with radiolabeled RNAs synthesized from the indicated plasmids. (A) Inserts of plasmid subclones pGEM3-1 and pGEM3-38 which were used as templates for the synthesis of [^{32} P]RNA probes. (B) Autoradiograph of a polyacrylamide gel displaying the following RNAs: lane a, [^{32} P]RNA synthesized from the plasmid template pGEM3-38; lane b, nuclease-resistant RNAs generated by the hybridization of *B. brevis* cellular RNA with [^{32} P]RNA synthesized from plasmid pGEM3-38; and lane c, [^{32} P]RNA synthesized from pGEM3-1. Numbers on the right show size in bases.

labeled with $^{32}\text{PO}_4$ at the 5' terminus of the *HincII* site located approximately 250 bp downstream from the *tycA* start site. The end-labeled DNA was denatured, hybridized to total cellular RNA, and then treated with nuclease S1. Next, the resulting nuclease-resistant RNA-DNA hybrids were denatured and subjected to electrophoresis alongside ^{32}P , *HincII*, end-labeled DNA that had been subjected to the nucleotide-specific cleavage reactions of Maxam and Gilbert (20). From the position of the S1-protected DNAs relative to the Maxam and Gilbert sequencing ladder shown in Fig. 5, we deduced that the *tycA* start site was about 252 bp upstream from the *HincII* site (330 bp upstream from the open reading frame for the synthetase-coding sequence; Fig. 2) and 10 and 35 bp downstream from hexanucleotide sequences (TATAAT and TCGACT, separated by a spacing of 16 bp) that are in good conformity to the canonical recognition sequences for promoters used by the primary holoenzyme form $\text{E}\sigma^{43}$ ($\text{E}\sigma^A$) in *Bacillus* sp. (25).

tycA-directed transcription in *B. subtilis*. We wished to

FIG. 4. (Left) Time course of appearance of *tycA* RNA in *B. brevis*. (Right) Autoradiograph of a polyacrylamide gel displaying the following: [^{32} P]RNA synthesized from plasmid pGEM3-1 (probe), nuclease-resistant RNAs generated by hybridization of the radioactive probe to *B. brevis* RNAs purified from cells harvested at the times indicated on the growth curve (lanes a to f), and the result of the hybridization of probe RNA to control RNA purified from *B. subtilis* cells (B.s.). O.D.600, Optical density at 600 nm.

FIG. 5. High-resolution S1 nuclease mapping of the 5' terminus of *tycA* RNA. The *HincII*-*PvuII* fragment of pGEM3-1 was ^{32}P labeled at its *HincII* end. The radiolabeled DNA was combined with total cellular RNA of *B. brevis* under hybridization conditions (Materials and Methods). After S1 treatment, the nuclease-resistant DNA-RNA hybrids were denatured and resolved by electrophoresis on a 6% polyacrylamide-7 M urea gel. The radiolabeled DNA fragment was also subjected to the base-specific cleavage reactions of Maxam and Gilbert (20). Arrowheads indicate the position of the bands corresponding to the *tycA* transcript.

FIG. 6. Time course of appearance of TycA protein in *B. subtilis* cells containing the plasmid pUM18. (A) Growth curve of pUM18-containing *B. subtilis* cells grown in DS medium. Arrows indicate the points during growth when samples were collected for immunodetection (Western blot) analysis. (B) Protein extracts of cells collected from the *B. subtilis*(pUM18) culture were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to Western blot immunodetection analysis (Materials and Methods) (19). Lane GC22 shows the band of the TycA protein that was synthesized in *E. coli* cells which contain the plasmid pGC22.

study the regulation of the promoter for the *tycA* gene in *B. subtilis* to take advantage of the availability of well-characterized mutations affecting sporulation-associated antibiotic production and the highly developed system for genetic manipulation in this bacterium. Preliminary evidence that the *B. brevis tycA* gene would be expressed and regulated appropriately in the heterologous *B. subtilis* host was the demonstration that the *tycA*-bearing plasmid pUM18

(Fig. 1) directed the synthesis of the 120-kilodalton TycA polypeptide in *B. subtilis* cells and that the appearance of this polypeptide was delayed until the stationary phase in plasmid-bearing cells growing in Difco sporulation medium (Fig. 6).

To measure transcription from the *tycA* promoter in *B. subtilis*, we constructed a transcriptional fusion of the putative *tycA* promoter region contained in a *XmnI*-*PvuII* fragment of approximately 900 bp to a promoterless *E. coli lacZ* gene that had been substituted with a *B. subtilis* ribosome-binding site (Fig. 7) (Materials and Methods). Next, the *tycA-lacZ* fusion was introduced into the prophage of the thermoinducible *B. subtilis* phage SP β *c2 del2::Tn917::pSK10A6* by the marker replacement (double) recombination event depicted in Fig. 8 (35, 36). This procedure resulted in the creation of phage SP β *tycA-lacZ*, making it possible to transfer the SP β -borne fusion into Spo⁺ cells and various Spo⁻ mutants simply by specialized transduction.

We monitored utilization of the *tycA* promoter in the gene fusion in two ways. First, we showed that JH642 cells bearing the SP β *tycA-lacZ* transducing phage produced blue colonies on solid sporulation medium containing the chromogenic substrate X-gal (5-bromo-4-chloro-3-indoyl- β -galactoside). As controls, colonies of cells lacking the gene fusion or bearing a promoterless *lacZ* gene were completely white on indicator plates. Second, we measured the synthesis of β -galactosidase by assaying hydrolysis of O-nitrophenyl- β -D-galactopyranoside (ONPG) in permeabilized fusion-bearing bacteria that had been grown in liquid sporulation medium. Because the level of β -galactosidase synthesis observed in Spo⁺ cells containing the SP β *tycA-lacZ* transducing phage was low (less than 5 Miller units), it was important to take into account the known background of ONPG-hydrolyzing activity that appears during sporulation in *B. subtilis*. (Interestingly, this indigenous ONPG-hydrolyzing activity did not cause colonies of sporulating cells that lack *E. coli lacZ* to turn blue on X-gal plates.) For this purpose we used as a control Spo⁺ cells that had been lysogenized with SP β bearing a promoterless *lacZ* gene, and as indicated in the legend to Fig. 9, we subtracted the background activity at each time point from that observed in the *tycA-lacZ*-bearing cells. It can be seen that the *XmnI*-

FIG. 7. Construction of *tycA-lacZ* fusion plasmid pMZ12. Details of the construction are provided in Materials and Methods.

FIG. 8. Construction of the specialized transducing phage SP β *tycA-lacZ*. The figure depicts the recombination event between pMZ12 and the prophage SP β *c2 del2::Tn917::SK10A6* of *B. subtilis* ZB493, by which the transducing phage was generated.

*Pvu*II fragment caused β -galactosidase synthesis to be induced postexponentially. The nuclease mapping experiment shown in Fig. 10B confirms that *tycA-lacZ* transcription originated from the same start site within the *Xmn*I-*Pvu*II fragment as that used in *B. brevis*.

***spo0*-dependent utilization of *tycA* promoter.** To study genetic control of the *tycA* promoter in *B. subtilis*, we introduced the *tycA-lacZ* gene fusion in various early-blocked sporulation mutants by means of specialized transduction. As assayed either by colony color on sporulation indicator plates (Table 2) or by ONPG-hydrolyzing activity in liquid-grown cells (Fig. 9), *tycA*-directed β -galactosidase synthesis was significantly impaired in *spo0A*, *spo0B*, and *spo0E* mutants but not in *spo0C*, *spo0F*, *spo0H*, or *spo0J* cells. This pattern of *spo0*-dependent transcription is in substantial conformity with the genetic requirements for antibiotic production in *B. subtilis* (3, 8, 32).

The dependence of antibiotic production in *B. subtilis* on the *spo0A* gene product is known to be bypassed by mutations at a locus called *abrB* (7, 31). *abrB* mutations are partial

suppressors that overcome the inability of *spo0A* mutants to produce sporulation-associated antibiotic and protease. *abrB* mutations do not, however, enable stage-0 mutants to form spores. To investigate whether *abrB* would relieve the block in *tycA-lacZ* expression caused by a *spo0A* mutation, we introduced the gene fusion into cells bearing the mutations *spo0A* Δ 204 and *abrB*703 and into cells bearing the suppressor mutation alone. Surprisingly, *abrB*703 caused

FIG. 9. *tycA-lacZ* expression in *B. subtilis* cells grown in DS sporulation medium. The pattern of *tycA-lacZ* expression was determined by assaying β -galactosidase specific activity in 1-ml samples that were collected at hourly intervals. The specific activity was calculated in Miller units. The activity of cells bearing the promoterless *lacZ* fusion *spoVGA308-lacZ* (33) was subtracted from the activity of *tycA-lacZ*-bearing cells at each time point. T₀ represents the end of the exponential phase of growth. Symbols: Δ , JH642 (*spo*⁺); $\blacktriangle$, JH651 (*spo0H81*); $\blacksquare$, JH649 (*spo0F221*); $\blacktriangledown$, JH646 (*spo0A12*).

FIG. 10. Effect of an *abrB* mutation on the expression of *tycA-lacZ*. (A) Pattern of β -galactosidase synthesis in *abrB*⁺ and *abrB*703 *B. subtilis* cells which were lysogenic for SP β *tycA-lacZ*. β -galactosidase specific activity (Miller units) was determined as described in the legend to Fig. 9. Symbols: Δ , *abrB*⁺; $\bullet$, *abrB*703. (B) Autoradiograph of a polyacrylamide gel displaying the following: nucleic-acid-resistant RNA generated by the hybridization of pGEM3-1 with total cellular RNA from *B. brevis* stationary-phase cells (lane a), *B. subtilis* *abrB*⁺ cells collected at T₄ (lane b), and *abrB*703 cells collected at T₋₂ (lane d). Lane c shows the [³²P]RNA probe synthesized from pGEM3-1.

tycA-lacZ to be transcribed at a high level both during growth and during the stationary phase, and this constitutive expression was observed in both the presence (data not shown) and the absence (Fig. 10A) of the *spo0A* mutation. That the high level expression of β -galactosidase synthesis observed in *abrB* cells was due to transcription originating from the *tycA* promoter was verified both by nuclease mapping experiments and by showing that *abrB703* cells containing the promoterless *lacZ* gene were Lac⁻.

DISCUSSION

We identified a promoter governing the transcription of the peptide antibiotic biosynthesis gene *tycA* and studied its regulation in *B. brevis*, the tyrocidine-producing bacterium from which the synthetase I gene was cloned, and in *B. subtilis*, a heterologous host in which it was possible to analyze the requirements for *tycA* promoter utilization genetically. Because the nucleotide sequence of the *tycA* promoter strongly resembled the canonical structure of the principal class of promoters found in *E. coli* and *B. subtilis* (26a), we believe (but have not tested by in vitro transcription experiments) that the tyrocidine synthetase I gene is transcribed by the primary RNA polymerase holoenzyme form of *Bacillus* species, Eo⁴³. Interestingly, however, and in conformity with the known time course of tyrocidine synthetase synthesis, transcription of the *tycA* gene in *B. brevis* and activation of its promoter in *B. subtilis* were deferred until the end of the exponential phase of the growth cycle. We infer from these experiments that the synthesis of tyrocidine synthetase I is regulated, at least in part, at the transcription level from a site at or near the *tycA* promoter by the action of one or more ancillary regulatory proteins and probably not by the appearance of a secondary form of RNA polymerase holoenzyme.

To investigate the mechanisms governing the postexponential-phase induction of *tycA*, we constructed a derivative of phage SP β bearing a *tycA-lacZ* operon fusion. This specialized transducing phage enabled us to study the regulation of the *tycA* promoter in *B. subtilis*, a *Bacillus* species in which an extensive groundwork of genetic information on the regulation of antibiotic production is already available (although structural genes for *B. subtilis* antibiotic biosynthesis genes have not yet been cloned from this bacterium). Our principal finding was that the genetic requirements for transcription from the *tycA* promoter closely mimicked those for antibiotic production in *B. subtilis*. Thus, stage-0 sporulation mutations such as *spo0A12* and *spo0B136*, which are known to block antibiotic production, impaired *tycA* transcription, whereas mutations such as *spo0F221* and *spo0H81*, which do not affect antibiotic production, had little or no effect on *tycA*-directed β -galactosidase synthesis. We were also able to show that the suppressor mutation *abrB*, which is known to relieve the dependence of antibiotic production on the *spo0A* gene product, restored *tycA* transcription in a mutant carrying a stage-0 mutation (preliminary evidence suggests that *abrB703* also restores *tycA* transcription in cells bearing the *spo0B136* mutation).

These findings are consistent with the idea (28) that the mechanisms governing antibiotic production and regulatory events occurring at the onset of differentiation are partially coupled. Thus, like the process of sporulation, antibiotic production and transcription of *tycA* commence at the end of the exponential phase of growth and depend on the stage-0 regulatory proteins Spo0A and Spo0B. The induction of antibiotic production and sporulation differ, however, in the

TABLE 2. Antibiotic production and *tycA-lacZ* expression in *spo*⁺ and *spo0* strains of *B. subtilis*

Strain	Relevant genotype	Sporulation	Antibiotic production ^a	Expression of <i>tycA-lacZ</i> ^b
JH642	<i>spo</i> ⁺	+	+	+
JH646	<i>spo0A12</i>	-	-	-
JH648	<i>spo0B136</i>	-	-	-
JH647	<i>spo0E11</i>	-	-	-
JH649	<i>spo0F221</i>	-	±	+
JH651	<i>spo0H81</i>	-	+	+
JH693	<i>spo0J93</i>	-	+	+
JH695	<i>spo0C9V</i>	-	+	+
ZB369	<i>spo0AΔ204 abrB703</i>	-	+	++
ZB449	<i>spo</i> ⁺ <i>abrB703</i>	+	+	++

^a From Brehm and Hoch (3).

^b LacZ phenotype on solid DS medium containing 40 μ g of X-gal per ml.

following respects. Unlike sporulation, antibiotic production and *tycA* transcription do not depend on certain stage-0 regulatory genes, such as *spo0H* whose product is the principal transcription factor for the activation of *spoVG* and other genes involved in spore formation (35). Also, unlike spore formation, the dependence of antibiotic production and *tycA* expression on Spo0A regulatory protein can be bypassed by mutations at the *abrB* suppressor locus. Finally, we (unpublished data) find that although *tycA-lacZ* is induced in sporulation medium, transcription of *tycA* is not significantly stimulated by treatment of growing cells with decoyinine (23), a drug which is known to induce sporulation and the transcription of the early sporulation gene *spoVG* (36) under conditions of nutritional excess.

The most striking result of our study was that the *abrB703* suppressor mutation not only bypassed the requirement for Spo0A in *tycA* induction but also caused *tycA* to be transcribed at a high level both during growth and during the early stationary phase. The finding that *abrB703* caused constitutive transcription of *tycA* combined with the finding that *abrB703* was epistatic to *spo0A* and the results of the accompanying report (36) suggest a simple model for the function of AbrB and the way in which the *tycA* gene is regulated in *B. subtilis*. We propose that the *abrB* gene product(s) or a protein whose activity is dependent on AbrB is a repressor that blocks transcription of *tycA*. This AbrB-dependent repressor could act directly on the *tycA* gene or indirectly on the synthesis of a regulatory factor required for *tycA* transcription. We further suppose that Spo0A causes the repressor to be inactivated and that this inactivation occurs as a response to nutritional stress as cells enter the stationary phase. For example, Spo0A (and other stage-0 proteins) could be part of a pathway that causes proteolytic cleavage of the repressor. Alternatively, the repressor could be metabolically unstable and Spo0A could act by blocking its synthesis. According to our model, the induction of *tycA* and other antibiotic production genes is a simple consequence of the nutritionally stimulated inactivation of the AbrB-dependent repressor. The recent isolation of a transposon Tn917 insertion (P. Zuber and M. Marahiel, unpublished data) in or very near *abrB* should make it possible to isolate the *abrB* gene and to investigate its role in *B. subtilis* gene expression directly.

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