Phosphate Control of Oxytetracycline Production by *Streptomyces rimosus* Is at the Level of Transcription from Promoters Overlapped by Tandem Repeats Similar to Those of the DNA-Binding Sites of the OmpR Family

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Physiological studies have shown that *Streptomyces rimosus* produces the polyketide antibiotic oxytetracycline abundantly when its mycelial growth is limited by phosphate starvation. We show here that transcripts originating from the promoter for one of the biosynthetic genes, *otcC* (encoding anhydrotetracycline oxygenase), and from a promoter for the divergent *otcX* genes peak in abundance at the onset of antibiotic production induced by phosphate starvation, indicating that the synthesis of oxytetracycline is controlled, at least in part, at the level of transcription. Furthermore, analysis of the sequences of the promoters for *otcC*, *otcX*, and the polyketide synthase (*otcY*) genes revealed tandem repeats having significant similarity to the DNA-binding sites of ActII-Orf4 and DnrI, which are *Streptomyces* antibiotic regulatory proteins (SARPs) related to the OmpR family of transcription activators. Together, the above results suggest that oxytetracycline production by *S. rimosus* requires a SARP-like transcription factor that is either produced or activated or both under conditions of low phosphate concentrations. We also provide evidence consistent with the *otrA* resistance gene being cotranscribed with *otcC* as part of a polycistronic message, suggesting a simple mechanism of coordinate regulation which ensures that resistance to the antibiotic increases in proportion to production.

Oxytetracycline (5′-hydroxytetracycline; OTC), a polyketide antibiotic (48) effective against a broad spectrum of microorganisms, is produced by strains of *Streptomyces rimosus* (for reviews, see references 5 and 27). Environmental factors, such as carbon and nutrient sources, temperature, and method of cultivation can affect the timing and extent of production of this antibiotic, as has been found for many other secondary metabolites (30, 35). However, OTC production is particularly sensitive to phosphate; i.e., OTC can be produced abundantly only in media containing levels of phosphate lower than that required for rapid mycelial growth (3, 4). Moreover, by starving cultures of only phosphate, it is possible to induce abundant OTC production by *S. rimosus*, including the Pfizer production lineage derived from strain 4018 (42). To date, the molecular basis of phosphate regulation of OTC production by *S. rimosus* has not been investigated beyond showing that the activity of one of the biosynthetic enzymes, anhydrotetracycline oxygenase, is lower in mycelia grown in excess phosphate (2, 8).

The cellular activity of enzymes associated with the production of other antibiotics has also been found to be regulated by phosphate; these include *p*-aminobenzoate (PABA) synthase from the candicidin producer *S. griseus* and deacetoxycephalosporin C synthase from *S. clavuligerus* and *Cephalosporium acremonium* (for a review, see reference 31). The structural gene (*pabS*) and promoter for PABA synthase have been cloned and characterized (18, 19, 41). Comparison of the cellular levels of transcripts originating from the *pabS* promoter has revealed that they are higher when phosphate is limiting, suggesting that the cellular activity of PABA synthase is controlled, at least in part, at the level of transcription (1). For deacetoxycephalosporin C synthase from *S. clavuligerus* and *C. acremonium*, there is evidence that the catalytic activity of this enzyme is inhibited by growth in medium containing high levels of phosphate (55), indicating that transcription may not be the only level at which regulation occurs.

The *otc* biosynthetic genes are clustered and are flanked by two resistance genes in a 34-kb segment of the *S. rimosus* chromosome (9) that is sufficient to confer OTC production when introduced on a plasmid into the heterologous hosts *S. albus* and *S. lividans* (6). Much of the *otc* cluster, including a 6.6-kb region (Fig. 1) that contains the gene encoding anhydrotetracycline oxygenase (*otcC*), has been sequenced (27). Upstream and reading in the direction opposite that of *otcC* are two genes (*otcX-orf1* and *orf2*) with unknown functions. Given this genetic organization, we considered it likely that any elements controlling the transcriptional initiation of *otcC* would be located in the intergenic region between *otcC* and *otcX-orf1*. Here we report the mapping of promoters within this region and provide evidence that they are activated, when mycelial growth is limited by phosphate starvation, by a transcriptional factor related to ActII-Orf4 and DnrI of the actinorhodin and daunorubicin clusters, respectively (16, 46). This switch may also regulate OTC resistance, as the *otcC* transcript appears to be polycistronic and to include the *otrA* gene, whose product, a homologue of elongation factor G (14), protects ribosomes from translational arrest (40). In addition, we show

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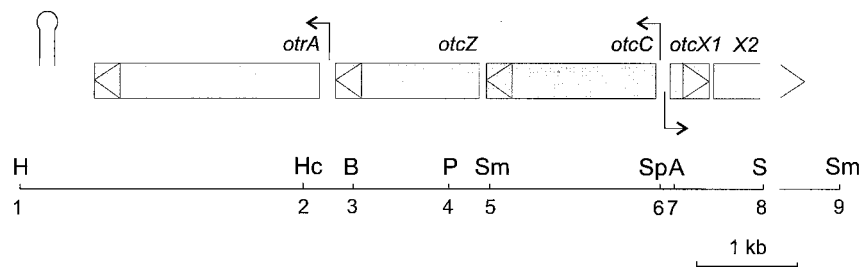
FIG. 1. Genetic organization at the *otrA* end of the *otc* cluster. Shaded boxes indicate the positions of identified genes. The triangles within each box show the direction of translation. The stem-loop structure indicates the position of a rho-independent terminator (10), and the arrows (→) indicate the positions of the *otrAp*$_1$ promoter (14) and the *otcCp*$_1$ and *otcXp*$_1$ promoters identified here. Only the restriction sites mentioned in the text are shown: H, *Hin*dIII; Hc, *Hin*cII; B, *Bam*HI; P, *Pst*I; Sm, *Sma*I; Sp, *Sph*I; A, *Ava*I; and S, *Sst*I.

that the three major promoters of the polyketide synthase complex (*otcY*) share common regulatory motifs with *otcC*.

MATERIALS AND METHODS

***Streptomyces* strains, growth conditions, and bacteriophage M13-based constructs.** *S. rimosus* 4018 (42) and 15883 were derived by Pfizer Ltd. (Sandwich, Kent, United Kingdom) during a strain improvement program. 15883S was derived from 15883 by spontaneous deletion of the OTC gene cluster. To analyze *otc* transcription during antibiotic fermentation, 15883 was grown in Trusoya Medium 1 (TSM1), a proprietary medium from Pfizer which contains soya flour, starch, vegetable oil, and inorganic salts (7), while 4018 was grown in liquid complete medium (LCM) (42). Control RNA was isolated from 15883S grown in tryptone soya broth (TSB) (25). Cultures (50 ml) were grown in 250-ml Erlenmeyer flasks at 28°C with shaking (250 rpm). All of the constructs used to generate single-stranded DNA (ssDNA) probes for the analysis of transcription were derived from M13mp18 (54). mL6A has, in the *Sma*I site, a 2.1-kb *Sma*I fragment containing the intergenic region between *otcC* and *otcX*; the insert is oriented such that the *otcC* gene is nearest the *Hin*dIII site in the polylinker. In mL6C, the *Sma*I insert is in the opposite orientation. mAT12a and mAT12b differ only in the orientation of a 1.4-kb *Sph*I insert that encodes the *otcY2* intergenic region, which in mAT12a is oriented such that the *orf1* gene is nearest the *Hin*dIII site in the polylinker. mKM803 has a 250-bp *Pst*I/*Sma*I fragment containing the 5′ end of the *otcZ* gene in the corresponding sites of the polylinker. Similarly, the insert in mKM804 is a 387-bp *Hin*cII/*Bam*HI fragment containing the 5′ end of the *otrA* gene.

Measurement of OTC in cultures. Culture samples (1 ml) were extracted in 9 ml of HCl (pH 1.7) for 30 min and filtered through Whatman no. 1 papers. Samples (1 ml) were then analyzed for OTC by isocratic, ion-paired, reverse-phase high-performance liquid chromatography with a C_{18} µBondabank column (Waters) and a mobile phase of acetonitrile-water (3:7) that contained 5% (wt/vol) 1-hexanesulfonic acid and that had a pH adjusted to 1.7 with sulfuric acid.

Dry weight determinations. Whatman GF/C filters (4.7-cm diameter) were dried for 15 min in a microwave oven set at 150 W and allowed to cool to room temperature in a desiccator for 30 min. The weight of each filter was determined to three decimal places. Mycelia were harvested by passing 5 ml of culture through a dried filter held in a sintered-glass vacuum unit (Millipore). The filter was washed with 15 ml of distilled H_2O, dried, and weighed as described above, and the net weight was recorded. At least three independent measurements were used to produce each dry weight value, which had a standard error of the mean of less than 5%.

Isolation of total RNA. At the appropriate stage of growth, 50 ml of culture was decanted into a 250-ml flask containing 20 g of 0.5-cm^3 ice pellets prepared by freezing double-distilled water at −20°C and was mixed by swirling. This step rapidly lowered the temperature of the culture to ca. 0°C. Using the protocol described by Hopwood et al. (25), we harvested the mycelia by centrifugation; the cells were lysed by a modification of the method of Kirby et al. (29), and the lysate was extracted with phenol-chloroform. The aqueous phase of the lysate (3.5 ml) was carefully added to the top of 1.5 ml of 5.7 M CsCl–0.1 M EDTA (density, 1.71 g ml^{-1}) in a 5-ml Polyallomer centrifuge tube (Beckman) and centrifuged at 35,000 rpm in an SW40 rotor for 16 h at 20°C. The RNA in the pellet was redissolved in 400 µl of 0.1 M NaCl, extracted with 0.5 volumes of phenol-chloroform and chloroform, precipitated with 2 volumes of ethanol, reharvested by centrifugation in a Microfuge (12,000 × g for 10 min at 4°C), washed twice with 70% (vol/vol) ethanol, dried in open Microfuge tubes at room temperature, and redissolved in 100 µl of H_2O. The concentration of the RNA was determined by measuring the absorbance at 260 nm, the integrity of the RNA was checked by testing in 0.8% (wt/vol) agarose–Tris-borate-EDTA gels, and 10-µg aliquots were stored as ethanol precipitates at −70°C.

Primer extension mapping of RNA 5′ ends. Oligonucleotide primers were labelled at the 5′ ends as described by Sambrook et al. (43), and 1-ng aliquots were mixed with 10 µg of RNA precipitate. The nucleic acid mixture was har-

vested by centrifugation, washed with 70% (vol/vol) ethanol, and dried in an open Microfuge tube at room temperature. The oligonucleotide primer was annealed to the RNA and extended with avian myeloblastosis virus reverse transcriptase (Pharmacia) as described previously (17), and the reaction was terminated by the addition of an appropriate amount of sequencing gel loading buffer (43); after heating to 85°C, samples were run in an 8% (wt/vol) polyacrylamide sequencing gel. The sizes of the extension products were determined by comparing their migration with that of sequencing ladders generated from single-stranded M13-derived templates by use of a Sequenase kit as described by the vendor (Amersham). Single-stranded M13 templates were prepared as described by Sambrook et al. (43).

Synthesis of ssDNA probes and nuclease protection assays. 5′-end-labelled or continuously labelled probes were synthesized and used as described by Sambrook et al. (43). The procedure involved extension of oligonucleotide primers annealed to single-stranded templates derived from recombinant bacteriophage M13, digestion of the newly synthesized double-stranded DNA with a suitable restriction enzyme, and separation of the radiolabelled probe from the linearized ssDNA template by electrophoresis through a 6% (wt/vol) polyacrylamide sequencing gel. The position of the probe was determined by autoradiography, and the probe was eluted from a slab of gel by overnight incubation in 0.5 ml of 0.5 M ammonium acetate (pH 8.0)–10 mM magnesium acetate–1 mM EDTA–10% (wt/vol) sodium dodecyl sulfate. The eluate was extracted twice with phenol-chloroform and twice with chloroform, and the probe was precipitated by the addition of 2.5 volumes of ethanol. The precipitate was harvested by centrifugation in a microcentrifuge (12,000 × g for 30 min at 4°C), washed with 70% (vol/vol) ethanol, dried as described above, and redissolved in 50 µl of H_2O. The probe (0.2 pmol) was mixed with 10 µg of total RNA (harvested, washed, dried as described above, and redissolved in 20 µl of hybridization buffer (43). The hybridization mixture was denatured at 85°C for 10 min, allowed to anneal at 55 to 65°C overnight, and treated with S1 nuclease (Sigma) or exonuclease VII (Life Technologies). The S1 nuclease reaction mixture (300 µl) contained 375 U of enzyme, and the reaction was carried out at 37°C for 30 min. The hybridization mixture was treated with exonuclease VII by the addition of 280 µl of 50 mM Tris-Cl (pH 7.8)–50 mM KCl–10 mM EDTA containing 10 U of enzyme and incubation at 37°C for 45 min. The reaction was terminated by extraction with phenol-chloroform and chloroform and processed as described for the S1 nuclease digestion products (43).

Densitometry scanning. The intensity of autoradiographic images was determined with a *PDI* scanner and software.

RESULTS

Mapping of promoters within the intergenic region between *otcC* and *otcX-orf1*. To identify promoters within the intergenic region between the 5′ end of *otcC* and the 5′ end of *otcX-orf1*, we first used a primer extension assay to analyze total RNA isolated from *S. rimosus* 15883 grown under conditions that produce OTC and, as a negative control, from 15883S, which has a deletion of the entire *otc* cluster. Single extension products were obtained for both *otcC* and *otcX* mRNAs (Fig. 2A and B, respectively). Confirmation that both the *otcC* and the *otcX* extension products terminated at RNA 5′ ends and not at internal secondary structures, which occur frequently in streptomycete transcripts due to their high (73%) G+C content (15), was obtained by use of a high-resolution exonuclease VII protection assay (Fig. 2C and D, respectively). The protected products for *otcC* and *otcX* mRNAs were 5 and 7 nucleotides (nt) longer, respectively, than the corresponding primer exten-
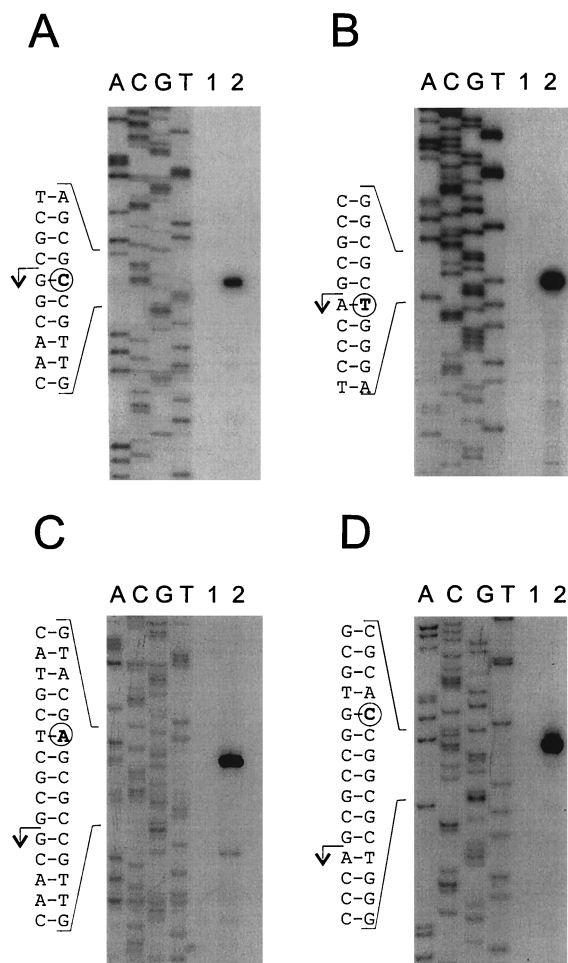
FIG. 2. Primer extension and nuclease protection analyses of the *otcC* and *otcX* transcripts. (A) Lanes 1 and 2 contain cDNA products extended from oligonucleotide C68 hybridized to 10 μg of total RNA from *S. rimosus* 15883S grown in TSB and from *S. rimosus* 15883 grown in TSM1 for 46 h (see Fig. 6A), respectively. (B) Like panel A, except that the cDNA products were extended from oligonucleotide X84. (C) Lanes 1 and 2 contain the products of exonuclease VII digestion of *otcC* transcripts in 10 μg of total RNA from *S. rimosus* 15883S and 15883, respectively. (D) Like panel C, except that *otcX* transcripts were analyzed. The probe for *otcC* transcripts was generated by extending 5′-labelled oligonucleotide C64 annealed to template mL6A and then cutting with *Ava*I, while the *otcX* transcript probe was generated by extending oligonucleotide X84 annealed to template mL6C and then cutting with *Sph*I. Both probes were hybridized with *S. rimosus* RNA at 37°C. See Fig. 3 for the sequences of the primers and the locations of the restriction sites. The sequencing ladders (lanes A, C, G, and T) for analysis of the *otcC* and *otcX* transcripts were extended from oligonucleotides C68 and X84 annealed to templates mL6A and mL6C, respectively. Circles indicate the 3′ ends of the products of either primer extension (A and B) or nuclease protection (C and D) analyses. The arrows show the deduced 5′ ends of the *otcC* and *otcX* transcripts.

sion products (Fig. 2A and B) because exonuclease VII is only able to digest single-stranded probes to within a few nucleotides of protected regions (11). No primer extension or nuclease protection was obtained with RNA from 15883S, indicating that our assays were specific for *otc* transcripts.

Analysis of the DNA sequence upstream of the positions mapped for the 5′ ends of the *otcC* and *otcX* mRNAs revealed hexanucleotide sequences that are centered around positions −10 and −36 (Fig. 3) and that are similar to the consensus sequences for the −10 and −35 regions of the major class of promoters in eubacteria (22) and in streptomycetes (26, 45), suggesting that these 5′ ends are the starts of primary tran-

scripts rather than the 5′ ends of RNA decay intermediates. Accordingly, we designated these conserved hexanucleotide sequences to be the −10 and −35 regions of promoters *otcCp*$_1$ and *otcXp*$_1$. Further sequence analysis revealed a perfect tandem repeat between positions −41 and −23 of *otcXp*$_1$ (Fig. 3). Similarly, a related, but imperfect, tandem repeat was located at precisely the same positions in *otcCp*$_1$.

Tandem repeats also overlap the *otcY* promoters. To determine whether tandem repeats overlapping −35 regions are a general feature of the promoters of *otc* biosynthetic genes, we also mapped and analyzed the sequences of promoters in the intergenic region between the polyketide synthase genes *otcY2-orf1* and *otcY2-orf2*. This is the only other segment where biosynthetic genes diverge in the *otc* cluster (27). Single major primer extension products were obtained for both of the *otcY2* transcripts (Fig. 4); moreover, as was found for *otcCp*$_1$ and *otcXp*$_1$, the promoters in the *otcY2* intergenic region were overlapped by tandem repeats (Fig. 5). Interestingly, the 11-nt separation (one turn of supercoiled B-form DNA) between the centers of these repeats and the location of the first repeat 10 nt upstream of the −10 regions of the *otc* promoters is typical of the DNA-binding sites of members of the OmpR family of transcription factors (32–34, 44, 51).

Transcription from *otcCp*$_1$ and *otcXp*$_1$ is regulated. We investigated whether transcription from *otc* promoters overlapped by tandem repeats is temporally regulated. Primer extension assays were used to compare the levels of transcripts extending from *otcCp*$_1$ and *otcXp*$_1$ at different stages during a strain 15883 fermentation that produced significant levels of OTC (ca. 6 mg ml^{-1}) when phosphate became limiting. As shown in Fig. 6, after 18 h, when OTC was barely detectable in the culture (<50 μg ml^{-1}), transcripts originating from *otcCp*$_1$ and *otcXp*$_1$ were present at low levels; however by 46 h, when antibiotic production had started, the amount of each transcript had increased 10- to 20-fold. Despite the fact that the amount of OTC in the medium continued to increase at later times (69 and 93 h), the abundance of the *otcC* transcripts declined steadily. The coincidence of the peak in the abundance of transcripts originating from *otcCp*$_1$ and *otcXp*$_1$ with the onset of OTC production suggests that the biosynthesis of this antibiotic is controlled, at least in part, at the level of transcription.

Evidence for an *otcC-otcC-otrA* polycistronic message. Previously, it had been shown that the *otrA* resistance gene is transcribed in part by read-through from *otcZ*, the methyltransferase biosynthetic gene (38, 39) that lies between *otrA* and *otcC* (Fig. 1); therefore, if transcription extends from *otcC* into *otcZ*, then *otcCp*$_1$ could participate in the regulation of antibiotic resistance as well as biosynthesis. This possibility was investigated with an S1 nuclease protection assay (Fig. 7). Two protected species of 252 and 297 nt were detected with total RNA isolated from strains 4018 and 15883 (Fig. 7, lanes 2 and 3 and lane 4, respectively); however, the 297-nt species was also detected with total RNA isolated from strain 15883S (lane 1), indicating that only the 252-nt fragment was protected by *otc* transcripts. The length of the *otc*-specific fragment corresponded to protection of the probe by transcripts covering the entire *otcC-otcZ* intergenic region between the *Pst*I and *Sma*I sites (Fig. 1, positions 4 and 5), indicating that *otcZ* and thus *otrA* could be transcribed from *otcCp*$_1$. Consistent with the results of the protection assay, we were unable to detect any sequence between *otcC* and *otcZ* that was predicted to form a rho-independent terminator. The 297-nt species detected in all the samples in Fig. 7 corresponds to the full-length probe protected from S1 nuclease digestion by a small amount of
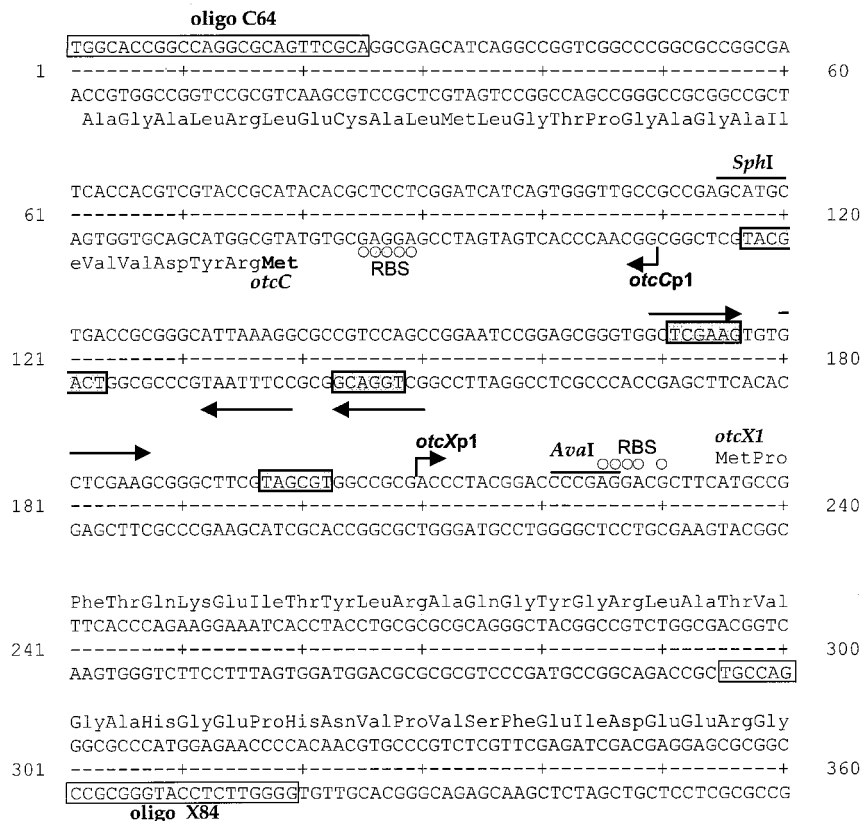
FIG. 3. Annotated sequences of *otcCp*$_1$ and *otcXp*$_1$. The positions of the transcription start sites (bent arrows) are indicated, together with the putative −10 and −35 regions of the promoters (shaded boxes), the direct repeats which have sequence similarity to the DNA-binding sites of *E. coli* PhoB (straight arrows), the primer sequences (unshaded boxes), and restriction enzyme sites used in mapping the 5′ ends of the *otcC* and *otcX* transcripts (see Fig. 2). Potential ribosome-binding sites (RBS) are shown by shaded circles.

contaminating template DNA in the probe preparation (see Materials and Methods).

Attempts to confirm the presence of the 6-kb *otcC-otcZ-otrA* polycistronic message by Northern blot analysis were unsuccessful. Although *otc*-specific RNA species were detected, they were heterogeneous in size, from 1.5 to 3.0 kb (data not shown). A possible explanation for this result is that decay of the *otcC-otcZ-otrA* polycistronic message commences before transcription has terminated. In any case, further evidence supporting the notion that *otrA* is transcribed from *otcCp*$_1$ was obtained by use of a high-resolution S1 nuclease protection assay to determine the relative levels of transcripts for the segment between *otcZ* and *otrA* (Fig. 8) in the RNA samples used to analyze transcription from *otcCp*$_1$ at different stages during OTC production (Fig. 6). The probe used was complementary to the sense strand between the *Hinc*II and *Bam*HI sites (Fig. 1, positions 2 and 3), and total RNA isolated from strain 15883S served as a negative control (Fig. 8, lane 1). The 449-nt species in the 15883S RNA sample (Fig. 8, lane 1) corresponds to the full-length probe, while the 387-nt protected fragment in the RNA samples isolated from 15883 at the stages analyzed during the production of OTC (lanes 3 to 6) corresponds to transcriptional read-through from the *otcZ* gene. Consistent with *otrA* being transcribed from *otcCp*$_1$, the abundance of transcripts encoding the segment between the 3′ end of *otcZ* and the 5′ end of *otrA* peaked early in OTC production, at 48 h (compare with Fig. 6). Interestingly, in strain 15883, no transcription was detected from *otrAp*$_1$ (14), which lies in the intergenic region between *otcZ* and *otrA*;
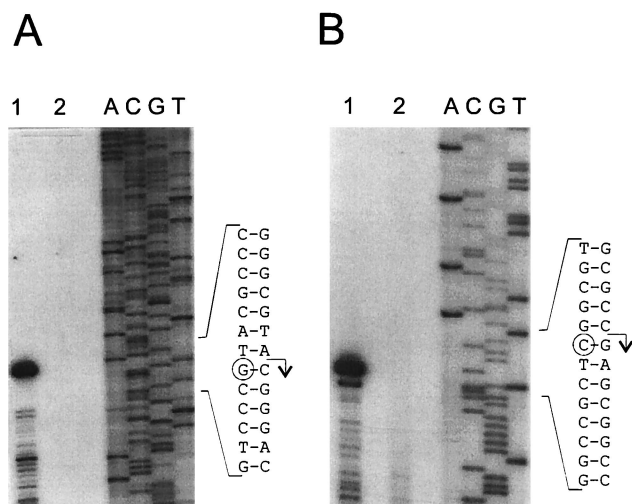
FIG. 4. Primer extension analysis of transcription from divergent promoters in the intergenic region between *otcY2-orf1* and *orf2*. (A) Lanes 1 and 2 contain cDNA products extended from primer L12A3 (5′-TCACCACGGCGAGCCCG ATG) hybridized to 10 μg of total RNA from *S. rimosus* 4018 grown in TSM1 for 2 days and from 15883S grown in TSB, respectively. (B) Like panel A, except that the cDNA products were extended from primer L12B1 (5′-AGCGCGGTGTC CACGGGGACGC). The sequencing ladders (lanes A, C, G, and T) for analysis of the *otcY2-orf1* and *orf2* transcripts were extended from L12A3 and L12B1 annealed to templates mAT12a and mAT12b, respectively. As in Fig. 2, circles indicate the 3′ ends of primer extension products, and arrows show the deduced 5′ ends of the transcripts.

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                -50              Repeat 2      Repeat 1                              +1
                 |              --------->    --------->                              |
   otcCp1        CTCCGGATTCCGGCTGGACGGCGCCTTTAATGCCCGCGGTCAGCATGCTCGGCGGCAAC--24nt--ATG
                              (GCCTTTAAT)
   otcXp1        CCGGAGCGGGTGGCTCGAAGTGTGCTCGAAGCGGGCTTCGTAGCGTGGCCGCGACCCTA--19nt--ATG
                              (TGCTCGAAG)
   Y2-orf1p1     CGCCCCAACCGCGGTCGGTCCAGCCTCGAACGGGCCTTGCAGGCTGGGGCGTACGGGAC--86nt--ATG
                              (GCCTCGAAC)
   Y2-orf2p1     GGACCGACCACGGCTCGAAGAACGGTCGAAGACCGGCTGCCACGCTCACGCCCGAGCGG--35nt--ATG
                              (CGGTCGAAG)

   Consensus              gGCTCGAAg

                 --------->    --------->    --------->
   otcY1p1       GCGTCGAATCCGCCTCCAAACCGCCTTGAGGTCCGAAGGTCACTCTGTGCCTCGACGCG--43nt--GTG
                              (GCCTTGAGG)
                              (GCGTCGAAT)
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FIG. 5. Alignment of the nucleotide sequences of *otc* promoter regions. The transcription start sites (designated +1) were determined as described in the legends to Fig. 2 and 4. Underlined sequences are hexanucleotide segments having similarity to consensus sequences TAG[Pu][Pu]T and TTGAC[Pu], which correspond to the −10 and −35 regions, respectively, of the major class of streptomycete promoters (22, 49). The putative −35 regions of the *otc* promoters are overlapped by tandem repeats indicated by arrows. To allow a consensus sequence to be derived for the repeated sequences, the repeat closest to the transcriptional start site of each promoter was copied, placed within parentheses, and aligned under its partner. In the consensus sequence for the repeated sequences, lowercase, uppercase, bold, and underlined characters represent nucleotides conserved at the same positions in five, six, seven, and eight of the repeats, respectively. The putative sequence of the promoter for the *otcY1* genes (28) is also shown but was not used to derive the consensus sequence for the repeated sequences. The *otcY1* promoter is overlapped by three related repeats whose centers are separated by 11 nt.

however, this strain produced detectable levels of transcripts during growth on TSB (protection of 166 to 167 nt in Fig. 8, lane 2), a medium that does not support antibiotic production.

The *otc* genes are transcribed as mycelial growth slows. The fermentation conditions for strain 15883 (Fig. 6) made it impossible to correlate the transcription of the *otc* genes with the phase of mycelial growth or to show that *otrA* is transcribed from $otrAp_1$ before antibiotic production, because TSM1, the production medium developed for 15883, contains insoluble components that interfered with both the measurement of mycelial growth and the isolation of RNA at early times. We therefore analyzed transcription during the early stages of OTC production by strain 4018 grown in LCM, a completely soluble medium. As shown in Fig. 9, the combined results from the 4018 fermentation indicate that the onset of OTC production coincides not only with increased transcription of *otrA* by read-through from *otcZ* but also with the slowing of mycelial growth and decreased transcription of *otrA* from its own promoter ($otrAp_1$). The transition between rapid growth and the stationary phase is also the point at which increased transcription of the actinorhodin biosynthetic genes in *S. coelicolor* occurs (20).

DISCUSSION

Our findings that (i) promoters of *otc* biosynthetic genes are overlapped by tandem repeats (Fig. 5), whose spacing resembles that of the activation sites of the OmpR family of transcriptional factors, and (ii) transcripts originating from these promoters reach a peak at the onset of antibiotic production that is triggered by limiting phosphate (Fig. 6) together suggest that the expression of some, and possibly all, of the *otc* biosynthetic genes is regulated, at least in part, at the level of transcription by an activation mechanism. Furthermore, this notion provides a possible explanation for the observation that three nonoverlapping segments of the *otc* cluster prevent the production of OTC by *S. rimosus* when cloned with high-copy-number but not low-copy-number plasmids (the switch-off phenomenon [9]). We now know that two of these segments correlate with the intergenic region between *otcC* and *otcX-orf1*

FIG. 6. Quantification of levels of transcripts originating from $otcCp_1$ and $otcXp_1$ at different stages during the production of OTC by *S. rimosus* 15883 grown in TSM1. (A) Open circles indicate the amount of OTC in the cultures. The values were obtained from samples taken from 14 cultures (50 ml) that were inoculated and incubated at 28°C on a rotary platform at the same time. Closed circles indicate the concentration of OTC in cultures used to analyze *otc* transcripts. Crosses and triangles indicate the levels of transcripts originating from $otcCp_1$ and $otcXp_1$, respectively, as measured by densitometry scanning of the autoradiographs of the primer extension products shown in panel B. (B) Primer extension assays were done as described in the legend to Fig. 2 with total RNA isolated from *S. rimosus* 15883 grown in TSM1 for 18, 46, 69, and 93 h. TSM1 contains a colloidal suspension of starch and soya flour, which prevented the isolation of RNA and prevented the measurement of mycelial growth before 18 h.
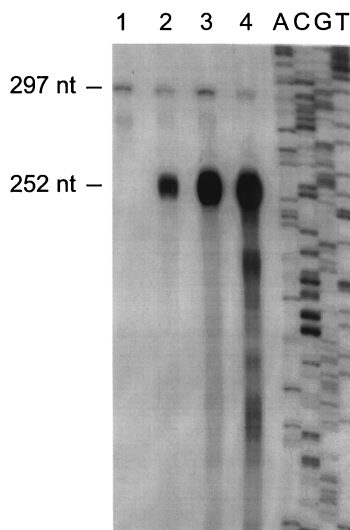
FIG. 7. Analysis of transcription in the intergenic region between *otcC* and *otcZ*. High-resolution S1 nuclease protection assays were performed with 10 μg of total RNA from *S. rimosus* 15883S grown in TSB until the late logarithmic phase, 4018 grown in the same manner as 15883S, 4018 producing OTC (ca. 60 μg ml^{-1}) after 56 h in LCM (see Fig. 9), and 15883 producing OTC (ca. 700 μg ml^{-1}) after growth in TSM1 (see Fig. 6) for 46 h (lanes 1, 2, 3, and 4, respectively). A continuously labelled ssDNA probe was generated from template mKM803 by extension of the −20 universal primer and *Eco*RI digestion. The sequencing ladder (lanes A, C, G, and T) was produced by extension of the −40 universal primer annealed to template mKM803. The numbers on the left indicate the sizes of the protected probe fragments, as determined by comparison with the sequencing ladder.
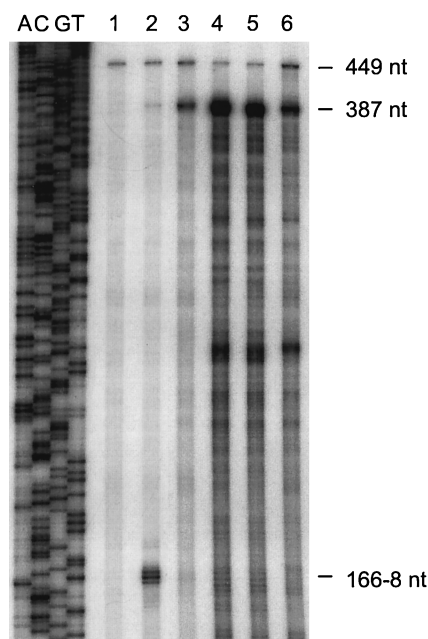

FIG. 8. High-resolution S1 nuclease protection analysis of *otrA* transcription at the onset of OTC production by *S. rimosus* 15883 grown in TSM1. Lanes 1 and 2 contain probe fragments protected by 10 μg of total RNA from *S. rimosus* 15883 and 4018 grown in TSB, respectively, while lanes 3, 4, 5, and 6 correspond to 15883 grown in TSM1 for 18, 46, 69, and 93 h, respectively (see Fig. 6). A continuously labelled ssDNA probe was generated by extension of the −20 universal primer annealed to template mKM804 and digestion with *Eco*RI. The sequencing ladder (lanes A, C, G, and T) was produced by extension of the −40 universal primer annealed to template mKM804 (36). The numbers on the right indicate the sizes of the protected probe fragments. Species shorter that 387 nt in lanes 3, 4, 5, and 6 may represent RNA processing or degradation or an artifact of the S1 nuclease mapping procedure.

and the intergenic region between *otcY2-orf1* and *otcY2-orf2*, which have been shown here to contain divergent promoters overlapped by tandem repeats (Fig. 5). The third corresponds to the likely location of the promoter for the transcription of the *otcY1* genes, which encode the minimal polyketide synthase (27). Although the transcription start site of the *otcY1* promoter has not yet been determined, tandem repeats resembling those overlapping the *otcC-otcX* and *otcY* promoters have been identified (Fig. 5). Given the above correlation, we suggest that the basis of the switch-off phenomenon (9) is the sequestration of a transcriptional activator by an excessive number of plasmid-borne copies of the *otc* tandem repeats.

Transcriptional activators are also known to regulate the expression of antibiotic gene clusters of other streptomycetes, e.g., ActII-Orf4 and DnrI of the actinorhodin and daunorubicin clusters, respectively (16, 46). Recently, it has been noted (53) that these streptomycete antibiotic regulatory proteins (SARPs) are similar to the DNA-binding domains of the OmpR family (37). Furthermore, an analysis of the promoter regions of the *act* and *dnr* biosynthetic genes (53) has revealed tandem repeats which, like the *otc* repeats identified in this study, are characteristic of the DNA-binding sites of all members of the OmpR family (34). Combined with the knowledge that the *dnr* tandem repeats were located within footprints of bound DnrI, Wietzorrek and Bibb (53) suggested that the tandem repeats identified in the *act* and *dnr* promoter regions are the binding sites of ActII-Orf4 and DnrI, respectively. We find that in addition to having similar spacing, the most highly conserved nucleotide positions (underlined) of the *otc* tandem repeats (consensus sequence, 5′-GC<u>TCGA</u>A [Fig. 5]) are also the most highly conserved positions in the proposed DNA-binding sites of ActII-Orf4 and DnrI [repeat consensus sequence, 5′-<u>TCGA</u>GC(G/C) (53)]. In the case of the *dnr* re-

peats, the most conserved nucleotide positions have been shown to be protected by DnrI from digestion by DNase I (47). On the basis of the above similarity, we suggest that the activator of the *otc* genes may be a member of the OmpR family, most likely a member of the SARP group. Experiments are currently under way to identify the factor that activates transcription from the *otc* promoters as, unlike the situation for actinorhodin and daunorubicin, no *sarp*-like gene has been identified within the *otc* cluster (28).

The experimental system used to investigate OTC production (shown best in Fig. 9) used limitation by phosphate to arrest the growth of the culture, at which time antibiotic biosynthesis was induced. Thus, transcription from the *otc* promoters occurred in response to the environmental stress of phosphate limitation. ActII-Orf4 and DnrI, together with their cognate DNA-binding sites, may also be involved in mediating phosphate control of the production of actinorhodin (23) and daunorubicin (12, 47). Consistent with this notion, detailed physiological studies have shown that phosphate starvation can induce actinorhodin production at least in part at the level of transcription of *actIII* (23, 24), whose promoter (21) is now known to be overlapped by a proposed DNA-binding site of ActII-Orf4 (53).

At present, the best-characterized phosphate-controlled system is the *pho* regulon of *Escherichia coli*, which encodes a set of genes required for the uptake of phosphate (for reviews, see references 44 and 51). Under conditions of low phosphate, PhoB, a transcriptional activator that (like SARPs) is a member of the OmpR family, is phosphorylated by PhoR (a sensory
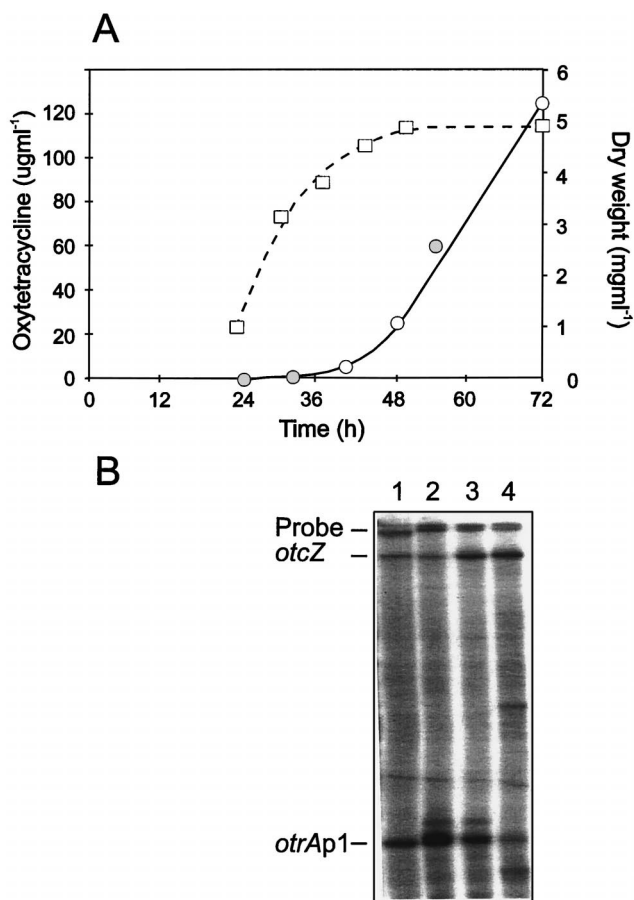
FIG. 9. Analysis of *otrA* transcription and mycelial growth at the onset of OTC production by *S. rimosus* 4018 grown in LCM. (A) Open circles indicate the amount of OTC in samples taken from six cultures (50 ml) that were inoculated at the same time and incubated at 28°C on the same rotary platform. Closed circles indicate the concentration of OTC in cultures used to analyze *otc* transcripts. Squares indicate the mean dry weight of the mycelium at different stages in the fermentation. The determination of dry weight had a variance of less than 5% error for the mean. (B) S1 nuclease protection analysis of *otrA* transcription. Assay conditions were as described in the legend to Fig. 8. Lane 1, RNA from 4018 grown in TSB; lanes 2, 3, and 4, RNA from 4018 grown in LCM for 24, 32, and 56 h, respectively.

controlling antibiotic production, it is important for the survival of the producer that it is resistant at the onset of biosynthesis. The combined results of our transcriptional analyses (Fig. 7 to 9) suggest that antibiotic resistance and production may be coordinated in *S. rimosus* at least in part via cotranscription of the *otrA* resistance gene with the *otcC* and *otcZ* biosynthetic genes from the $otcCp_1$ promoter.

OTC resistance is also regulated independently of OTC production: preexposure of *S. rimosus* to sublethal concentrations of OTC during vegetative growth induces higher levels of resistance (40). This response is independent of $otcCp_1$ but probably involves $otrAp_1$, as a DNA segment extending from the 3′ end of *otrA* to the middle of *otcZ* is sufficient to confer inducible resistance (13). At the onset of antibiotic production, when *otrA* is transcribed along with *otcZ* and *otcC*, transcription from $otrAp_1$ appears to be superfluous, as there is a significant (>80%) decrease in the level of transcripts originating from this promoter (Fig. 9). The molecular basis of this observation remains to be determined, but it is conceivable that $otcCp_1$ and $otrAp_1$ are transcribed by different RNA polymerase holoenzymes (10, 52) which are active under different physiological conditions.

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kinase) and then binds to promoters to activate the transcription of genes required for the active uptake of phosphate. Whether covalent modification of SARPs plays a role in the regulation of antibiotic production remains to be determined. Interestingly, however, in *E. coli* cells that lack functional PhoR, the *pho* regulon is no longer under the control of phosphate but is tightly regulated by carbon and energy sources via controls that were not obvious under conditions of phosphate starvation in the presence of functional PhoR (49, 50). Current evidence suggests that PhoB is activated not only by PhoR but also by other sensory kinases, thus allowing the process of phosphate assimilation to be integrated with central pathways for carbon and energy metabolism (for a review, see reference 51). The notion that the control of antibiotic production in *Streptomyces* is subject to cross regulation, similar to that described above for the *E. coli pho* regulon, could explain how factors other than phosphate, for example, carbon and nitrogen sources, can affect the timing and extent of antibiotic production (see references 30 and 36).

Regardless of the actual details of the molecular mechanism

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