JOURNAL OF BACTERIOLOGY, Nov. 1999, p. 6958–6968 Vol. 181, No. 22
0021-9193/99/$04.00+0

Characterization of the Pathway-Specific Positive Transcriptional Regulator for Actinorhodin Biosynthesis in *Streptomyces coelicolor* A3(2) as a DNA-Binding Protein

PALOMA ARIAS, MIGUEL A. FERNÁNDEZ-MORENO,† AND FRANCISCO MALPARTIDA*

Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas, Campus Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain

Received 24 June 1999/Accepted 7 September 1999

The ActII-ORF4 protein has been characterized as a DNA-binding protein that positively regulates the transcription of the actinorhodin biosynthetic genes. The target regions for the ActII-ORF4 protein were located within the *act* cluster. These regions, at high copy number, generate a nonproducer strain by in vivo titration of the regulator. The mutant phenotype could be made to revert with extra copies of the wild-type *act*II-ORF4 gene but not with the *act*II-ORF4-177 mutant. His-tagged recombinant wild-type ActII-ORF4 and mutant ActII-ORF4-177 proteins were purified from *Escherichia coli* cultures; both showed specific DNA-binding activity for the *act*VI-ORF1–ORFA and *act*III-*act*I intergenic regions. DNase I footprinting assays clearly located the DNA-binding sites within the −35 regions of the corresponding promoters, showing the consensus sequence 5′-TCGAG-3′. Although both gene products (wild-type and mutant ActII-ORF4) showed DNA-binding activity, only the wild-type gene was capable of activating transcription of the *act* genes; thus, two basic functions can be differentiated within the regulatory protein: a specific DNA-binding activity and a transcriptional activation of the *act* biosynthetic genes.

Members of the genus *Streptomyces* are filamentous soil bacteria, and they produce about half of all known microbial antibiotics (4, 47). This genus also possess a complex life cycle, which includes morphological and physiological differentiation (11, 27). Antibiotic production usually begins at the transition phase between vegetative growth and the development of spore-bearing aerial mycelium, suggesting that there is a close relationship between the two processes antibiotic production and morphological differentiation (11).

Streptomyces coelicolor is an excellent model to study these process; it is the genetically most intensively studied *Streptomyces* species and produces four chemically different antibiotics, whose biosynthetic genes have been isolated: the blue-pigmented polyketide actinorhodin (65), undecylprodigiosin (42, 55), methylenomycin (12, 66), and the calcium-dependent antibiotic (14, 35, 37). There are extensive studies showing a close correlation between antibiotic production and other cellular processes, suggesting that control of antibiotic production might be operating on several levels. The first level includes genes which are implicated in antibiotic production and morphological differentiation, such as the *bld* genes (10, 25, 38, 46, 52), or those acting on the stringent response, such as *relC* (50) and *relA* (8, 44). A second level is represented by genes with pleiotropic effects on one or more antibiotic biosynthetic pathways, such as *absA*, *absB* (1, 6, 9), *afsB* (24), *afsR* (30), *afsS* (45), *afsQ1* and *afsQ2* (32), and *ptpA* (62). Finally the last level of this presumptive cascade of regulators is represented by the so called pathway-specific regulators, because mutations in these genes specifically affect only one antibiotic.

Several specific regulators for different biosynthetic pathways such as those for streptomycin (16), bialaphos (2), undecylprodigiosin (42, 49), cephamycin (51), and daunorubicin (59), have been identified in *Streptomyces*. For actinorhodin, the specific regulator, which was shown to be a positive regulator, was located in the central part of the *act* cluster (18) and assigned to the *act*II-ORF4 gene.

The amino acid sequence of ActII-ORF4 protein is very similar to the deduced products of the *dnrI* (59), *redD* (49), and *ccaR* (51) transcriptional activators and the N terminus of the AfsR protein (31) (37, 33, 25.9, and 34% identity, respectively). All of these proteins belong to an expanding family of regulatory proteins (SARPs) that possibly have a similar mechanism of transcriptional activation throughout DNA binding to specific nucleotides sequences (64). The *dnr*I gene can complement an *act*II-ORF4 mutation (59), but *redD* and *ccaR* cannot. Recent studies (60) have demonstrated that DnrI protein binds specifically to the promoter regions in the daunorubicin cluster and in this way controls the expression of many of the DNR biosynthetic genes. Nevertheless, the role of ActII-ORF4 protein as transcriptional regulator of the biosynthetic *act* genes is supported by indirect evidence (7, 22, 23, 44).

For further characterization of the *act*II-ORF4 gene product as transcriptional regulator, we overexpressed and purified the wild-type and mutant 177 (17) ActII-ORF4 proteins in *Escherichia coli*. This paper demonstrate that the positive regulator *act*II-ORF4 product is a DNA-binding protein that recognize specific regions of DNA within the *act* promoters.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. The *E. coli* strains used were JM101 (67) and BL21(DE3)pLysS (58). The *Streptomyces coelicolor* A3(2) strains used were J1501 (*hisA1 uraA1 strA1 pgl* SCP1$^-$ SCP2$^-$) (13) and JF1 (*argA1 guaA1 redD42 act*II-177 SCP1$^-$ SCP2$^-$) (17). The *Streptomyces lividans* strain used was TK21 (*str-6* SLP2$^-$ SLP3$^-$) (29). *E. coli* and *Streptomyces* vectors are summarized in Table 1.

Media, culture conditions, and microbiological procedures. *Streptomyces* manipulations were as described previously (28). Thiostrepton (Sigma no. T-8902) was used at 50 μg/ml in agar medium and 10 μg/ml in broth cultures. Hygromycin

* Corresponding author. Mailing address: Centro Nacional de Biotecnología, CSIC, Campus Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain. Phone: 34-915854548. Fax: 34-915854506. E-mail: fmalpart@cnb.uam.es.

† Present address: Departamento de Bioquímica, Facultad de Medicina de la UAM e Instituto de Investigaciones Biomédicas, 28029 Madrid, Spain.

TABLE 1. Vectors and recombinant clones used in this study

Vector	Relevant characteristics[a]	Reference or source
E. coli		
Plasmids		
pUC18	pBR322-derived *E. coli* vector; *bla*	67
pBR329	*E. coli* general-purpose vector	15
pIJ2921	pUC-derived *E. coli* vector with a modified polylinker; *bla*	33
pIJ2925	pUC-derived *E. coli* vector with a modified polylinker; *bla*	33
pSU19	pACYC184-derived *E. coli* vector; *cat*	3
pET-19b	pBR322-derived *E. coli* T7 polymerase expresion vector	(Novagen, Madison, Wis.)
pMV303	*Kpn*I-*Pst*I fragment of *act*VI-ORF1–ORFA (nt 1406–2632) cloned in pSU19; *cat*	20
pMF1013	*Sal*I(11.8–13.3) fragment cloned in pIJ2925; carries the wild-type *act*II-ORF4 gene; *bla*	18
pMF2001.2	*Nae*I-*Sma*I fragment (nt 1914–2231) of the *act*VI intergenic region, cloned in pIJ2925 (*Hin*cII site); *bla*	20
pMF2065	447-bp *Mbo*II fragment carrying the *act*III-*act*I intergenic region, cloned in pIJ2925, *bla*	19, 23
pPAC2	*Sph*I-*Sac*I fragment (positions 13.4–14.1) of the *act*III-*act*I intergenic region, cloned in pIJ2925; *bla*	19
pPAC6	Same as pMF1013; carries the *act*II-ORF4-177 gene from *S. coelicolor* JF1; *bla*	This work
pPAC10	*Nde*I-*Bgl*II fragment carrying the recombinant wild-type *act*II-ORF4 gene cloned in pET-19b (*Nde*I-*Bam*HI sites); *bla*	This work
pPAC14	Same as pPAC10 but with the *act*II-ORF4-177 gene; *bla*	This work
Phage		
M13mp18	*E. coli* phage vector for DNA sequencing	67
Streptomyces		
Plasmids		
pIJ486	High-copy-number pIJ101 derivative vector, *tsr*	63
pIJ80	Unstable SCP2* derivative plasmid; *neo*	60a
pIJ702	High-copy-number vector; *mel tsr*	34
pIJ941	Low-copy-number SCP2* derivative plasmid; *tsr hyg*	40
pMF1100	*Sal*I fragment (positions 11.8–13.3) cloned in pIJ941; carries the wild-type *act*II-ORF4 gene; *hyg tsr*	18
pMF1125	*Sal*I fragment (positions 11.8–13.3) cloned in pIJ702; carries the wild-type *act*II-ORF4 gene; *tsr*	This work
pMF1123	*Eco*RI-*Hin*dIII fragment of pMF2001.2 cloned in pIJ486; *tsr*	This work
pMF1135	*Eco*RI-*Hin*dIII fragment of pMF2065 cloned in pIJ486; *tsr*	This work
pPAS1	*Bgl*II fragment of pPAC6 cloned in pPAS3; carries the *act*II-ORF4-177 gene; *hyg*	This work
pPAS3	pIJ941-derivd vector with a frameshit within the thiostrepton resistance gene; *hyg*	44
pPAS4	*Sal*I fragment (positions 11.8–13.3) cloned in pPAS3; carries the wild-type *act*II-ORF4 gene; *hyg*	44
Phages		
PM1	φC31-derived vector; *att*$^-$ *tsr hyg*	41
φAB9	*Bgl*II fragment from pMF1013 cloned in the *Bam*HI-*Bgl*II sites of PM1; *hyg*	This work

[a] Abbreviations: *bla*, ampicillin resistance gene; *cat*, chloramphenicol acetyltransferase gene; *hyg*, hygromicin resistsance gene; *neo*, neomycin resistance gene; *tsr*, thiostrepton resistance gene; *mel*, tyrosinase gene; nt, nucleotides.

B (Sigma no. H-2638) was used at 200 and 50 μg/ml in solid and liquid media, respectively. Neomycin (Sigma no. N-1876) was used at 12 μg/ml in solid media. *E. coli* was grown on Luria agar or in Luria broth (43), and transformants were selected with 50 μg of ampicillin per ml and 25 μg of chloramphenicol per ml.

DNA and RNA manipulations. For isolation, cloning, and manipulation of nucleic acids, the methods used were those previously described for *Streptomyces* (28) and *E. coli* (43).

For high-resolution S1 assays, 0.08 pmol of $^{32}P_i$-labeled probe denatured at 65°C for 15 min was hybridized to 50 μg of total RNA as previously described (48). RNA was extracted from 3-day-old mycelium (28) grown on the surface of cellophane discs on R5 agar plates as previously described (39). For *act*I-ORF1, a 798-bp *Sph*I-*Sac*I fragment (positions 13.4 to 14.1 [19]) labeled at the 5′ end of the *Sac*I site was used as a probe. For *act*VI-ORF1, the *Kpn*I-*Bss*HII fragment (nucleotides 1406 to 2252 [20]) was labeled at the 5′ end of the *Bss*HII site. For *act*VI-ORFA, the *Cel*II-*Sma*I fragment (nucleotides 1757 to 2231 [20]) was labeled at the 5′ end of the *Cel*II site.

For DNA-binding assays with the *act*VI-ORF1–ORFA intergenic region, pMF2001.2 was digested with *Bam*HI and end labeled with polynucleotide kinase and 50 pmol of [γ-^{32}P]ATP (5,000 Ci mmol^{-1}; Amersham). Then the DNA was digested with *Hin*dIII, and a 329-bp fragment was purified. For the *act*III-*act*I intergenic region, pMF2065 was digested with *Hin*dIII and end-labeled with polynucleotide kinase as described above; a second digestion with *Xba*I gave the 450-bp fragment used as a probe. Shorter DNA fragments within these intergenic regions were generated by PCR (56). PCR mixtures consisted of 5 ng of pMF2001.2 or pMF2065 for the *act*VI-ORF1–ORFA or *act*III-*act*I region, respectively, as templates, 20 pmol of primers G1 (5′-GGAAGCGCGGTGATG GTCAT-3′) and G39 (5′-GTGAACAGGTTGATCCATCCCAGG-3′) for the *act*VI-ORF1–ORFA region and primers G10 (5′-TTTGGGCGCCCGGCTCG AGC-3′) and G31 (5′-ATGACGACTCTGCGCTTCAATCCG-3′) for the *act*III-*act*I region, 200 μM deoxynucleoside triphosphate, and 1 U of *Taq* polymerase in a final volume of 50 μl, in the commercial buffer (Boehringer). The cycling conditions were 2 min of denaturation at 94°C; 30 cycles of denaturation (2 min at 90°C), annealing (1 min at 55°C), and extension (2 min at 72°C); and one cycle of 10 min at 72°C. The PCR-generated products, called F139 and F1031 for *act*VI-ORF1–ORFA and *act*III-*act*I respectively, were purified from low-melting-temperature agarose. To prepare radiolabeled DNA fragments for gel mobility shift analysis and footprinting assays, primers were previously end labeled with [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase and then subjected to PCR amplification as described above.

Construction of *S. coelicolor* PM218. For a further characterization of the role of the *act*II-ORF4 gene product as a transcriptional regulator, a strain with most of the *act* cluster deleted (including the regulatory region but leaving some *act* cluster deleted (including the regulatory region but leaving some *act* biosynthetic genes intact) was generated. As an additional advantage, this strain would not contain intermediates of the *act* pathway, which might affect the transcription of the *act*II-ORF4 or *act*II-ORF4-dependent genes. To construct this strain, the

4.4-kb *Bam*HI (positions 1 to 3) and the 4.0-kb *Bam*HI-*Bgl*II (positions 18 to 21) (41) fragments of the *act* cluster were ligated (in the same relative positions as they are located in the *act* cluster) within the *Bam*HI site of pBR329. The resulting pBR329 derivative plasmid was linearized with *Eco*RI and ligated into the unique *Eco*RI site of pIJ941, and the ligation mixture was used to transform *S. lividans* TK21. The pIJ941 derivative carrying the recombinant pBR329 was selected by its hygromycin sensitivity. The resulting plasmid was finally introduced by transformation into *S. coelicolor* J1501, in which we expected that the deletion of the *act* cluster, cloned in the recombinant plasmid, would be transferred by homologous recombination into the chromosome. Colonies derived from a single crossover would carry an extra copy of *act*II-ORF4 and so might have an overproducing phenotype (18). A single colony with the predicted phenotype was isolated. The strain was cured for the pIJ941 derivative by introduction of pIJ80 (Table 1) after selection for its neomycin resistance marker. pIJ80 was then easily lost after growing the strain on R5 plates without selective pressure. The deletion in the *act* cluster was confirmed by Southern hybridization experiments (data not shown), and the strain was named *S. coelicolor* PM218. Few *act* genes are still present in this mutant: the *act*VI-ORFA, 1 and 2, a non-in-frame fusion between the truncated 5′ end of *act*VI-ORF3 and the 3′ end of *act*VII genes, and the complete *act*IV and *act*VB genes.

Isolation of *act*II-ORF4-177. To isolate the *act*II-ORF4-177 gene, primers *Nde*I (5′-GGGGGCGCATATGAGATTC-3′) and *Bam*HI (5′-CGCTGGATTT CCGGCGTCGGATCC-3′) were used in PCR amplifications of a 735-bp DNA fragment from the *S. coelicolor* JF1 chromosome under the conditions described above. The amplified fragment was cloned in an *E. coli* vector, and several clones were sequenced. The wild type *Pst*I-*Bam*HI fragment, internal to the *act*II-ORF4 gene (18) (from pMF1013), was replaced with that from the amplified fragment carrying the *act*II-177 mutation, yielding pPAC6 (see Table 1).

DNA sequencing. DNA sequencing was carried out by the dideoxy-chain termination method (57) with the 7-deaza-dGTP reagent kit from U.S. Biochemical Corp. (no. 70750).

Cloning of *act*II-ORF4 in *E. coli* expression plasmids. A *Nde*I site at translational start codon was generated by PCR with specific primers. The amplified fragment was cloned in the pIJ2921 vector, and the *Nde*I-*Bgl*II fragment carrying the whole gene was finally ligated into the *Nde*I-*Bam*HI sites of pET-19b vector (Novagen), to give pPAC10. In this construction, the *Pst*I(12)-*Bam*HI(13) internal fragment (18) was replaced with the corresponding fragment from pPAC6, giving plasmid pPAC14, which carries the recombinant *act*II-ORF4-177 gene (Table 1).

Protein analysis. Protein concentrations were determined by the method of Bradford (5). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Laemmli (36). Western blotting was carried out by transferring proteins to polyvinylidine difluoride (Immobilon-P; Millipore) membranes in 10mM CAPS buffer (pH 11) as is described previously (61). The immunodetection assay was performed with a monoclonal antibody raised against the His tag (Dianova) at a 1:1,000 dilution. A goat anti-mouse immunoglobulin G peroxidase conjugate at a 1:1,000 dilution was used as the secondary antibody (Dako); the immunodetection assay mixture was processed with 4-chloro-1-naphthol and H_2O_2 as substrates.

Preparation of crude extracts from *Streptomyces*. *S. coelicolor* PM218 containing pIJ702 vector or pMF1125 was grown in YEME medium (28) with 5 μg of thiostrepton per ml at 30°C with stirring at 250 rpm for 48 h. The cells were harvested, washed, and resuspended in TEG buffer (50 mM Tris-HCl [pH 7.5], 0.5 mM EDTA, 5% glycerol, 50mM NaCl) plus 1 mM β-mercaptoethanol and 0.5 mM phenylmethylsulfonyl fluoride. Cells were disrupted by sonication, and cell debris was removed by centrifugation to yield the cell extract. Proteins in the cell extract were fractionated between 20 and 60% saturated ammonium sulfate, prepared in TEG buffer, and collected by centrifugation. The protein samples were dialyzed overnight at 4°C against TEG buffer and stored at −80°C until use.

Purification of the His-tagged ActII-ORF4 proteins from *E. coli*. BL21(DE3)pLysS harboring plasmid pPAC10 or pPAC14 was grown at 37°C to an optical density at 600 nm of 0.6; isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 100 μM and incubated for 3.5 h at 28°C. Cells were harvested, washed with binding buffer (50 mM Tris-HCl [pH 8], 1 M NaCl, 5 mM $MgCl_2$, 10% glycerol, 0.1% Brij 36T, 100 mM imidazole) plus 1 mM β-mercaptoethanol and 0.5 mM phenylmethylsulfonyl fluoride, resuspended in the same buffer, and disrupted by sonication. The cell lysate was centrifuged at 13,000 rpm for 15 min at 4°C in a Sorvall SS 34 rotor to remove the insoluble fraction. The supernatant containing the soluble His-tagged ActII-ORF4 protein was loaded on a Ni^{2+} chelating column (Invitrogen) and eluted with 20 mM Tris-HCl (pH 8)–100 mM NaCl–5 mM $MgCl_2$–20% glycerol–0.1% Brij 36T–1 M imidazole–1 mM β-mercaptoethanol–0.5 mM phenylmethylsulfonyl fluoride. The GroEL protein, which copurified with the recombinant ActII-ORF4 proteins, was removed by an additional wash, before the elution step, with binding buffer supplemented with 5 mM ATP. The eluted fractions containing the protein were pooled and dialyzed overnight against TEG buffer. Aliquots of the protein sample were stored at −80°C for DNA-binding assays. The purified ActII-ORF4 proteins (wild type and mutant) were unstable, and they lost most of the DNA-binding activity when stored at −80°C for 2 months. The wild-type and mutant ActII-ORF4 proteins behaved identically on the chromatography columns and showed the same purification pattern.

DNA-protein-binding assays. For the gel mobility shift assays 0.6 to 0.9 μg of purified His-tagged protein was allow to stand for 10 min at 4°C in TEG buffer plus 70 mM KCl (final volume, 35 μl). Between 1 and 3 ng of end-labeled DNA fragments was finally added and incubated for 30 min at room temperature. For the supershift assays, the monoclonal antibody (0.3 μg) was preincubated with the proteins for 15 min at room temperature before the probe was added. Protein-bound and free DNA were applied on the loading buffer (10% glycerol, 0.5× TBE buffer [89 mM Tris (pH 8.3), 89 mM boric acid 2 mM EDTA], 0.05% bromphenol blue) to 4% nondenaturing polyacrylamide gels and run in 0.5× TBE buffer. The gels were dried and exposed to Kodak X-ray film at −80°C. DNase I footprinting assays were performed essentially as described previously (21). These assays were carried out in 100 μl of the same reaction mixture described above for gel mobility shift assays. After incubation for 30 min at room temperature, 50 ng of DNase I (prepared in a solution containing 100 mM $MgCl_2$ and 5 mM $CaCl_2$) was added to each reaction mixture, and the mixtures were incubated for 3 min at room temperature, extracted with phenol, and then precipitated. The resulting pellet was resuspended in the sequencing loading buffer (U.S. Biochemical Corporation) and applied to an acrylamide-bisacrylamide (38:2)–40% urea sequencing gel along with dideoxy DNA sequencing ladders made with primers G1 and G39 for the *act*VI-ORF1–ORFA region and primers G10 and G31 for *act*III-*act*I. After electrophoresis, the gels were dried and analyzed by autoradiography. In all DNA-protein-binding assays, the DNA-binding specificity was tested with 1 μg of poly(dI-dC)-(dI-dC) as a nonspecific competitor.

Computer analysis of sequences. Protein secondary-structure prediction was made with the PHD program (53).

RESULTS

Cloning the *act*II-ORF4-177 gene. The region of the *act* cluster which was genetically postulated as a regulatory region (*act*II) (54) for actinorhodin biosynthesis is physically located in the middle of the *act* cluster (41). One particular mutation (*act*II-177) was mapped on the 515-bp *Pst*I-*Bam*HI fragment (positions 12 to 13) which lies within the coding sequence of the *act*II-ORF4 gene (18). To gain insight into the structure-function relationship of the positive regulator, it was of interest to isolate this mutation for further characterization of the biological activity of its gene product. Thus, the *Pst*I-*Bam*HI fragment from *S. coelicolor* JF1 was cloned from the chromosome as described in Materials and Methods. The fragment showed that the *act*II-177 mutation carries a single change (C instead of T) at nucleotide 257 of the coding sequence of the *act*II-ORF4 gene (18), giving rise to a translated product with a change of a single amino acid (serine instead of leucine) at position 86 of the protein. No complementation was observed when this gene was introduced into the *act*II mutants (pPAS1), suggesting that the *act*II-ORF4-177 gene had lost the ability to induce expression of the *act* genes, and thus it would be useful in attempts to understand its role as a transcriptional regulator of the *act* biosynthetic genes.

The *act*II-ORF4 gene as transcriptional activator. From genetics and DNA sequence analysis, it was suggested that transcription of the *act* genes might take place in a small number of polycistronic units and that it is presumably controlled by promoters located at the *act* intergenic regions (19, 20, 23, 41). To gain insight into the role of *act*II-ORF4 gene, we focused on one of these regions as a possible target for the ActII-ORF4 protein: the *act*VI-ORF1–ORFA (20) intergenic region. This region contains divergently arranged promoters for transcription of the *act*VI-ORFA and *act*VI-ORF1 genes. *S. coelicolor* PM218 was used for transcription determination of *act*VI-ORFA or *act*VI-ORF1, in either the presence or absence of the *act*II-ORF4 gene. Thus, *S. coelicolor* J1501 and PM218 (with most of the *act* cluster deleted, as indicated in Materials and Methods) were transformed with pPAS3 (control vector), pPAS4, or pPAS1 (carrying the *act*II-ORF4 and *act*II-ORF4-177 genes, respectively) (Table 1). Total RNA from all of them was extracted and hybridized with the corresponding probes. As shown in Fig. 1, S1 digestion-resistant fragments were observed only in the strains carrying the wild-type *act*II-ORF4

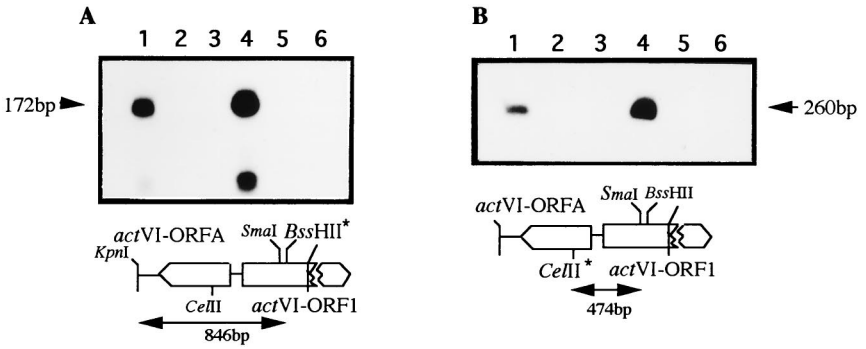

FIG. 1. High-resolution S1 mapping within the *act*VI-ORF1–ORFA intergenic region in *S. coelicolor* PM218. Transcriptional analysis of the *act*VI-ORF1 (A) and *act*VI-ORFA (B) genes is shown. The RNAs were extracted from the *S. coelicolor* strains: J1501 (lane 1), PM218 (lane 2), PM218 carrying pPAS3 as a control (lane 3), PM218 carrying the wild-type *act*II-ORF4 gene in pPAS4 (lane 4), PM218 carrying the *act*II-ORF4-177 gene in pPAS1 (lane 5), and *E. coli* tRNA as a control (lane 6). The probes, indicated by double-headed arrows, were the 846-bp *Kpn*I-*Bss*HII fragment (A) and the 474-bp *Cel*II-*Sma*I fragment (B). The fragments were labeled at the positions indicated by the asterisks. Protected fragments of the expected size are indicated by arrows. As size marker, the *Hin*fI-digested pBR329 was used.

gene but not in *S. coelicolor* PM218 or in this strain carrying the *act*II-ORF4-177 gene, which gave similar results to the strain carrying the vector alone. When extra copies of the *act*II-ORF4 gene (strains carrying pPAS4) were present, the detected level of the S1-resistant fragments increased in relation to that obtained with the wild-type strain, suggesting that transcription of the *act* genes was enhanced by the *act*II-ORF4 gene. The *act*VI-ORF1 transcript was increased nearly twofold above the level of the wild-type strain, whereas that of *act*VI-ORFA was increased up to fivefold (Fig. 1). All these results strongly suggest that transcription of these *act* genes are clearly dependent on both the presence of the *act*II-ORF4 gene and its gene dose within the range of our experimental conditions.

The *act* intergenic regions as targets for the *act*II-ORF4 gene product. (i) In vivo titration of the *act*II-ORF4 gene product. To clarify the possible role of the *act* intergenic regions as targets for the ActII-ORF4 protein, the *act*VI-ORF1–ORFA (20) and *act*III-*act*I (19, 23) intergenic regions were cloned in the high-copy-number vector pIJ486. Thus, the 447-bp *Mbo*II fragment (19, 23), carrying the *act*III and *act*I-ORF1 intergenic region, was blunt ended and cloned in the *Hin*cII site of pIJ2925 to yield pMF2065; the insert was recovered with *Eco*RI-*Hin*dIII double digestion and recloned into the *Eco*RI-*Hin*dIII sites of pIJ486, yielding pMF1135. In the same way, the 317-bp *Nae*I-*Sma*I fragment (nucleotides 1914 to 2231 [20]), carrying the *act*VI-ORF1–ORFA intergenic region, was cloned in the *Hin*cII site of *E. coli* vector pIJ2925 to yield pMF2001.2; then the insert was recloned into the *Eco*RI-*Hin*dIII sites of pIJ486 to give plasmid pMF1123. *S. coelicolor* J1501 was transformed with either pMF1135 or pMF1123; the transformants showed an actinorhodin-nonproducing phenotype, suggesting that a *trans*-acting element might be titrated in vivo by the cloned DNA. No changes were observed when the *act*II-ORF4 promoter region was present in high copy number (data not shown). When one extra copy of the *act*II-ORF4 gene was introduced in *cis* by using the att$^-$ recombinant phage φAB9 (Table 1), actinorhodin production was partially restored in transformants carrying pMF1135 but not in those carrying pMF1123. The wild-type phenotype was completely recovered when either strain was transformed with pPAS4 but not with the plasmid carrying the mutant *act*II-ORF4-177 gene (pPAS1), suggesting that the functional *act*II-ORF4 gene is needed for complete restoration of the wild-type phenotype.

All these results strongly suggest that (i) the *act*VI-ORF1–ORFA and *act*III-*act*I intergenic regions in high copy number might titrate, in vivo, most of the free regulatory protein, thus preventing its interaction with the corresponding chromosomal regions, and (ii) this regulatory element may well be the *act*II-ORF4 gene product. If these assumptions are correct, a strong reduction in the transcription rate within both chromosomal intergenic regions, irrespective of which intercistron is present in high copy number, without affecting the *act*II-ORF4 transcription levels, might be expected.

(ii) Transcriptional analysis of *act* biosynthetic genes in the recombinant mutants. To confirm that the mutant phenotype was due to a reduced transcription of the *act* structural genes rather than a reduction of the *act*II-ORF4 transcription, two sets of S1-mapping experiments were carried out with RNA extracted from those mutants, with or without extra copies of either wild-type or mutant *act*II-ORF4 genes (Fig. 2 and 3). In the first set, transcription of the *act* structural genes was determined in those mutants generated with the *act*VI-ORF1–ORFA intergenic region in high copy number (Fig. 2). The detected transcription levels for *act*I-ORF1 (Fig. 2A) and *act*VI-ORFA (Fig. 2B) were only 25% of that of the control strains (*S. coelicolor* J1501 with or without pIJ486), while for the *act*VI-ORF1 gene (Fig. 2C), the detected transcription level was nearly 40% of that of the wild-type strains. When extra copies of the wild-type *act*II-ORF4 gene, but not the mutant gene, were introduced in these strains, the observed levels of *act*I-ORF1 and *act*VI-ORFA increased 3-fold whereas that of *act*VI-ORF1 was 1.5-fold higher than that of the control strain. In the second set of experiments, transcription levels were determined in mutants carrying the *act*III-*act*I region in high copy number (Fig. 3). In these mutants, the transcription of the structural genes showed a different pattern: the levels of the *act*I-ORF1 (Fig. 3A) and *act*VI-ORFA (Fig. 3B) genes were similar to those in the previous mutants, whereas that of the *act*VI-ORF1 gene (Fig. 3C) showed no differences in any of these mutants. The levels of the *act*II-ORF4 transcripts were also determined on both recombinant mutants (data not shown) and proved to be unaffected in any of them. These results strongly suggest that the actinorhodin-nonproducing phenotype might well be due to a reduction of the amount of the free ActII-ORF4 protein (below the levels needed for activation of the *act* structural genes) rather than to a reduction of its transcription levels. With these data, it can be concluded that the ActII-ORF4 protein would probably be a DNA-binding protein whose targets could be located within the *act* intergenic regions.

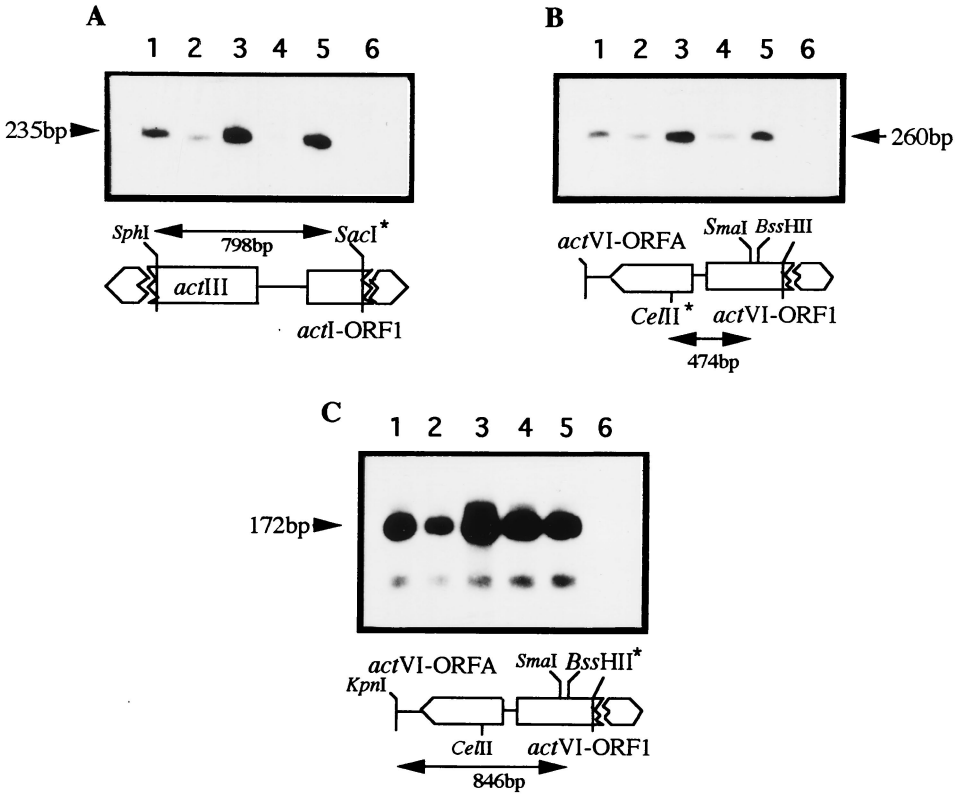

FIG. 2. Transcriptional analysis of some *act* genes in the recombinant strains carrying the *act*VI-ORF1–ORFA region in high copy number. Transcription was determined for the *act*I-ORF1 (A), *act*VI-ORFA (B), and *act*VI-ORF1 (C) genes. The RNAs were extracted from the following *S. coelicolor* strains: J1501 carrying pIJ486 as a control (lane 1); J1501 containing pMF1123, the *act*VI-ORF1–ORFA intergenic region, in high copy number (lane 2); the same strain carrying, in addition to pMF1123, the wild-type *act*II-ORF4 gene (lane 3) or the *act*II-ORF4-177 gene (lane 4) respectively, in the compatible plasmids pPAS4 or pPAS1; J1501 (lane 5); and *E. coli* tRNA as a control (lane 6). The probes and their respective sizes are indicated accordingly. The labeled positions are indicated by asterisks. The respective S1-protected fragments are indicated by arrows. The size markers are as in Fig. 1.

Overexpression of *act*II-ORF4 gene in *S. coelicolor*. To test a possible DNA-binding activity related to the presence of the *act*II-ORF4 gene, *S. coelicolor* PM218 was transformed with a high copy number of *act*II-ORF4 gene (pMF1125) and with the control plasmid (pIJ702). In this strain we could overexpress the regulatory gene without overproduction of intermediates of the *act* biosynthetic pathway, thus avoiding a putative lethality. Cell extracts and fractions precipitated with ammonium sulfate were prepared from *S. coelicolor* PM218 harboring pMF1125 or pIJ702; these samples were used in gel mobility shift analysis with the *act*VI-ORF1–ORFA (20) (Fig. 4) and *act*III-*act*I (19) (data not shown) intergenic regions as probes. DNA-binding activity was detected only with samples from *S. coelicolor* PM218 containing the *act*II-ORF4 gene (Fig. 4). Because the only difference between both strains is the presence of the regulator, we can concluded that the observed band shift might be due to the ActII-ORF4 protein.

The enrichment of the DNA-binding activity observed within the 60% ammonium sulfate fraction could not be correlated with any protein with the expected size of ActII-ORF4, in Coomassie blue-stained gels. This suggest that the expressed level of the ActII-ORF4 might be low, although it is cloned in a high-copy-number vector. To overcome these additional difficulties, we decided to use *E. coli* to express and purify the regulatory protein.

Functional analysis of the ActII-ORF4 protein. (i) Purification of wild-type ActII-ORF4 and ActII-ORF4-177 proteins. To overexpress the ActII-ORF4 protein in *E. coli*, the gene was cloned in the expression vector pET-19b (Novagen) to yield pPAC10 (see Materials and Methods) and used to transform *E. coli* BL21(DE3)pLysS. In the same way, pPAC14 containing the *act*II-ORF4-177 gene from *S. coelicolor* JF1 was constructed. From the induced cultures, the proteins carrying the His tag fused at their N terminus were purified from the soluble fraction by Ni^{2+} affinity chromatography. The steps used in the purification of wild-type His-tagged ActII-ORF4 are shown in Fig. 5; the same purification pattern was obtained for the recombinant mutant protein (data not shown). Western blot analysis with a specific monoclonal antibody against the His tag epitope confirmed that the protein of 30.6 kDa from the eluted fractions was the recombinant His-tagged ActII-ORF4 protein (data not shown). The protein of 60 kDa that copurified with our proteins was end sequenced and shown to be the *E. coli* chaperon GroEL and was removed as in Materials and Methods (Fig. 5, lanes 8 and 9). As shown in lane 9, the ActII-ORF4 protein was purified almost to homogeneity.

To find if the His-tagged fusion proteins were functional, we cloned the recombinant genes from plasmids pPAC10 and pPAC14 in *Streptomyces* vectors. The resulting plasmids were used to transform *S. lividans* TK21 and *S. coelicolor* JF1. Both strains harboring the recombinant wild-type *act*II-ORF4 gene in the low-copy-number vector, but not those harboring the mutant, showed an actinorhodin-producing phenotype, so that the recombinant gene was functional and complemented the mutation *act*II-177 of *S. coelicolor* JF1. Western blot analysis

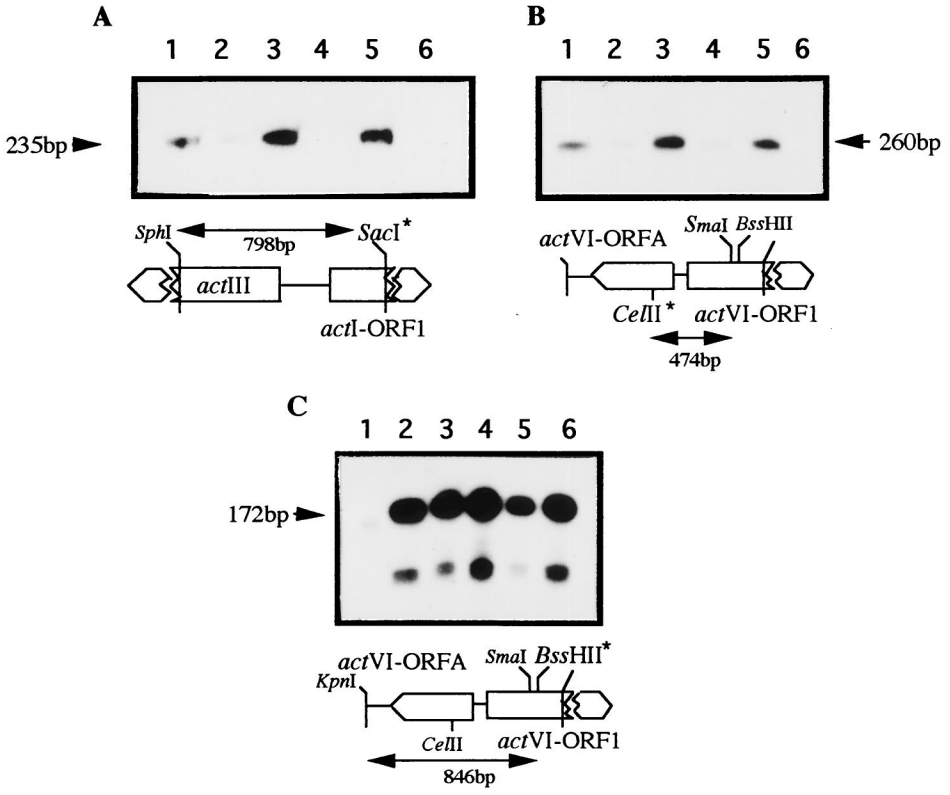

FIG. 3. Transcriptional analysis of some *act* genes in the recombinant strains carrying the *act*III-*act*I region in high copy number. Transcription was determined for the *act*I-ORF1 (A), *act*VI-ORFA (B), and *act*VI-ORF1 (C) genes. The RNAs were extracted from the following *S. coelicolor* strains: J1501 carrying pIJ486 as a control (lane 1); J1501 carrying pMF1135, the *act*III-*act*I intergenic region, in high copy number (lane 2); the same strain carrying, in addition to pMF1135, the wild type *act*II-ORF4 gene (pPAS4) (lane 3) or the *act*II-ORF4-177 gene (pPAS1) (lane 4); J1501 (lane 5); and *E. coli* tRNA (lane 6) as a control. The probes were as in Fig. 2 and were labeled at the restriction site indicated by asterisks. The sizes of the protected fragments are indicated. The size markers are as in Fig. 1.

with the monoclonal anti-His tag antibody was carried out to confirm that the recombinant proteins were present.

(ii) Gel mobility shift assays with the recombinant proteins. We next tested the ability of the purified ActII-ORF4 recombinant proteins to interact with specific regions of the *act* promoters. Gel mobility shift assays were initially carried out with probes consisting of those fragments which were previously tested in vivo as possible targets for such interaction. Thus, the *act*VI-ORF1–ORFA and *act*III-*act*I intergenic regions were purified and end labeled (see Materials and Methods). Both proteins showed band shift activity with either fragments (data not shown). These probes were shortened to delimit the interacting regions by using PCR amplified fragments (see Materials and Methods). These smaller *act* regions still retain the band shift activity. Figure 6A shows that both

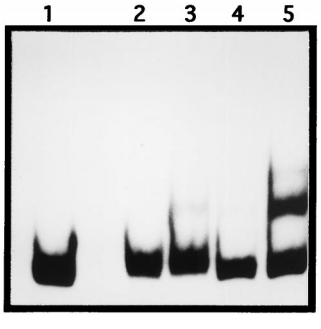

FIG. 4. Gel mobility shift analysis of the *act*VI-ORF1–ORFA intergenic region with crude extracts from *Streptomyces*. Lanes: 1, probe *act*VI-ORF1–ORFA intergenic region (329 bp); 2 and 4, cell extract and 60% ammonium sulfate precipitated from *S. coelicolor* PM218(pIJ702), respectively; 3 and 5, cell extract and 60% ammonium sulfate fraction from *S. coelicolor* PM218(pMF1125), respectively.

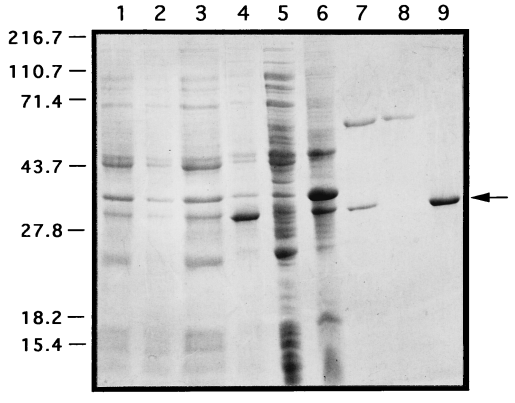

FIG. 5. Purification pattern of the wild-type His-tagged ActII-ORF4 protein. Lanes: 1 and 2, total-cell lysate of *E. coli* BL21(DE3)pLysS/pET-19b not induced and induced, respectively; 3 and 4, total-cell lysate of *E. coli* BL21(DE3)pLysS/pPAC10 not induced and induced, respectively; 5 and 6, soluble and insoluble fractions, respectively, from a cell extract of *E. coli* BL21(DE3)pLysS/pPAC10; 7, fraction from a Ni^{2+} column eluted with 1 M imidazole; 8, fraction eluted with 0.1 M imidazole plus 5 mM ATP; 9, purified His-tagged ActII-ORF4 protein. Molecular masses are indicated in kilodaltons.

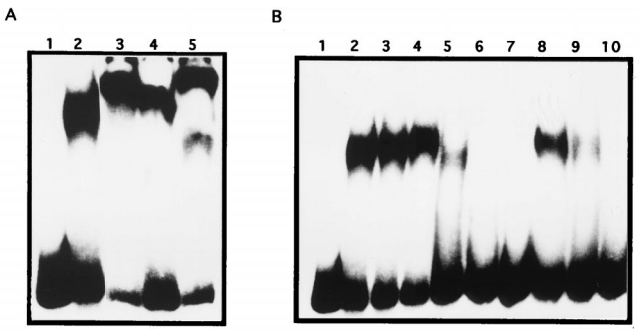

FIG. 6. DNA-binding assays. (A) Gel mobility shift analysis of the *act*VI-ORF1–ORFA intergenic region and supershift assays. Lanes: 1, probe, F139 fragment (154 bp); 2, wild-type His-tagged ActII-ORF4 protein (0.7 μg); 3, wild-type His-tagged ActII-ORF4 protein (0.7 μg) plus anti-His tag antibody; 4, His-tagged ActII-ORF4-177 protein (0.7 μg); 5, His-tagged ActII-ORF4-177 protein (0.7 μg) plus anti-His tag antibody. (B) Cross-competitions in gel mobility shift analysis of the *act*VI-ORF1–ORFA intergenic region. Lanes: 1, probe, F139 fragment; 2, 3, and 4, His-tagged ActII-ORF4 protein (0.7, 0.8, and 0.9 μg, respectively); 5, 6, and 7, wild-type His-tagged ActII-ORF4 protein (0.9 μg) plus cold F139 fragment (50, 100, and 200 ng, respectively; 8, 9, and 10, wild-type His-tagged ActII-ORF4 protein (0.9 μg) plus cold F1031 fragment (50, 100, and 200 ng, respectively).

wild-type and mutant His-tagged ActII-ORF4 proteins were interacting with the F139 fragment of the *act*VI-ORF1–ORFA region. The involvement of the recombinant proteins in the interaction was tested by a supershift assay involving the monoclonal anti-His tag antibody; in this case, a slower-running complex can be detected (Fig. 6A, lanes 3 and 5). The DNA-protein complexes with wild type and mutant proteins seemed to be different, as deduced from the differences in their mobilities. When the probe was the F1031 fragment, similar results were obtained (data not shown).

The relative affinities of ActII-ORF4 protein for either of the *act* regions were tested by gel mobility shift assays with the fragment F139 (Fig. 6B) or F1031 (data not shown) as the probe; cross-competitions for complexes formation were performed with equivalent amounts of the same unlabeled fragments. Providing that the two fragments are similar in size, the results of these assays showed that the wild-type recombinant ActII-ORF4 protein had higher affinity for the *act*VI-ORF1–ORFA than for the *act*III-*act*I intergenic region, because more F1031 fragment was needed to compete the complex generated with the F139 fragment (Fig. 6B, lanes 5 and 8).

(iii) DNase I footprinting analysis. To locate the sites of interaction between ActII-ORF4 and its targets of DNA, DNase I footprinting analysis were carried out (see Materials and Methods). When the fragment F139 (primer G1 5′-labeled) previously incubated in the presence or absence of the two His-tagged ActII-ORF4 recombinant proteins was digested with DNase I, five protected sites of the digestion were observed (Fig. 7A). The protected regions extended from about 4 to 12 bp and are flanked by multiples sites hypersensitive to DNase I digestion. Assays with the same fragment labeled in the complementary strand (Fig. 7B) gave three pro-

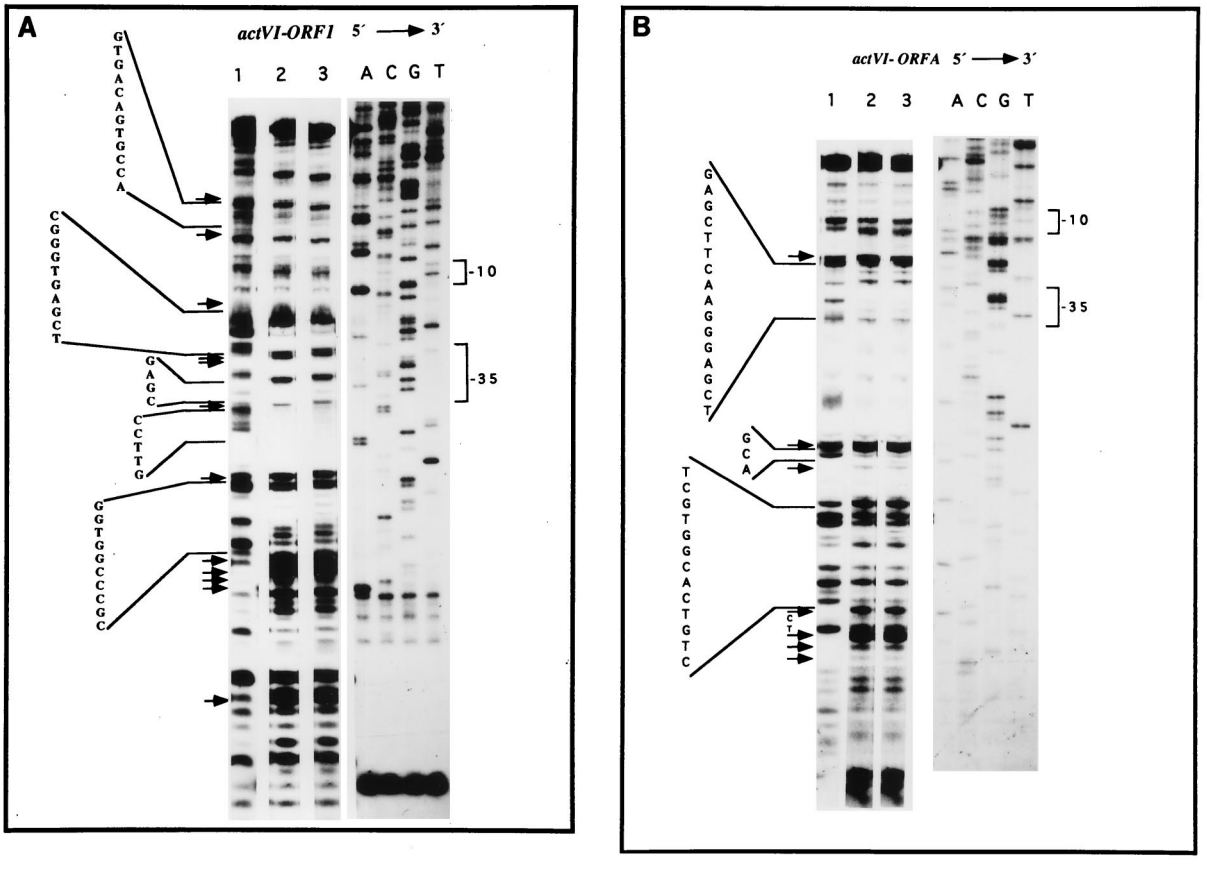

FIG. 7. DNase I footprinting analysis in the *act*VI region. (A) Upper strand: F139 fragment 5′-labeled (G1 primer) as described in Materials and Methods. (B) Lower strand: F139 fragment 5′-labeled (G39 primer). Lanes: 1, probes; 2, wild-type His-tagged ActII-ORF4 protein (1.5 μg); 3, His-tagged ActII-ORF4-177 protein (1.5 μg). Sequencing reactions (ACGT) generated with the corresponding primers (G1 or G39) were run in parallel. The hypersensitive regions are indicated by arrows. The −10 and −35 regions of the promoters are indicated.

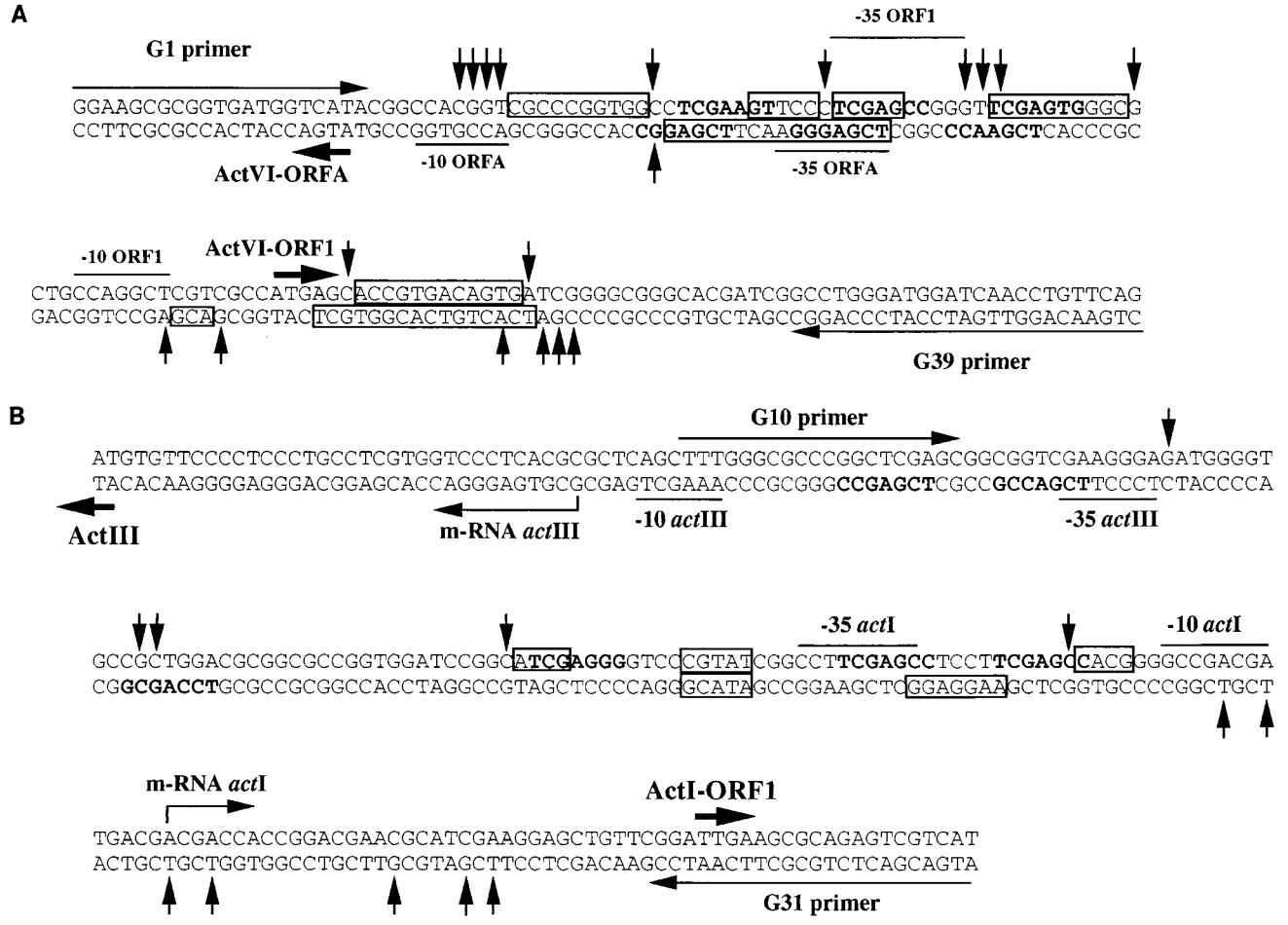

FIG. 8. Summary of DNase I footprinting analysis for the *act*VI-ORF1–ORFA (A) and *act*III-*act*I-ORF1 (B) intergenic regions. The transcriptional and translational start points from the *act* genes are indicated by thin and thick arrows, respectively. For the *act*VI-ORF1 and ORFA, transcriptional and translational start points are the same (20). Transcription start points for the *act*III (23) and *act*I-ORF1 (50a) genes are also indicated, along with the −10 and −35 regions. The protected (boxes) and hypersensitive (vertical arrows) regions are marked in the sequence.

tected regions that extended from about 3 to 16 bp along the intergenic region. The protected regions were located close to the −10 and −35 regions of the promoters and in some cases overlapped them. Similarly, the *act*III-*act*I intergenic region was also tested; unlike the *act*VI-ORF1–ORFA region, no clear protected sequences could be seen whereas well-defined hypersensitive regions were exhibited (data not shown). A summary of the results of the different footprinting assays with the *act*VI-ORF1–ORFA and *act*III-*act*I intergenic regions are shown in Fig. 8A and B, respectively. There was no significant differences in the protected pattern with either the wild-type or mutant ActII-ORF4 proteins (Fig. 7A and B, lanes 2 and 3).

DISCUSSION

The role of the *act*II gene as regulator for actinorhodin biosynthesis was first suggested by Rudd and Hopwood (54) based on the phenotypic characteristics of their representative *act* mutants. Hallam et al. (23) showed that the expression of the *act*III transcripts takes place only when the *act*II mutation is complemented with the wild-type *act*II region. When the DNA sequence of the *act*II region was determined, the *act*II mutation was located within the coding region of the so-called *act*II-ORF4, and strong evidence that its gene product was a putative transcriptional activator was provided (18). More recently, Gramajo et al. (22) showed that transcription of some *act* biosynthetic genes takes place after the *act*II-ORF4 transcripts reached their maximal levels; they suggested that the key feature for the onset of actinorhodin production in *S. coelicolor* would be the availability of the ActII-ORF4 protein for promoting activation of the *act* structural genes in the cells. In all cases, the nature of the *act*II-ORF4 gene product as a transcriptional regulator was supported only by circumstantial evidences. This work clearly demonstrates that the ActII-ORF4 protein is indeed a DNA-binding protein and activates the transcription of the *act* structural genes by specific interactions within the *act* promoters. Two regions of the *act* cluster were used for this experiment: the *act*III-*act*I (19, 23) and the *act*VI-ORF1–ORFA (20) intergenic regions, which carry divergently arranged promoters for the "early"- and "late"-acting biosynthetic steps, respectively. The ActII-ORF4 protein could be titrated in vivo by increasing the copy number of its target sequences, thus generating a nonproducer strain; the mutant phenotype generated with the *act*III-*act*I region could be easily reverted with one extra copy of the wild-type regulatory gene, while more copies are needed for reverting the nonproducer phenotype generated by the *act*VI-ORF1–ORFA intergenic region. These results suggest that in vivo, the affinity

of the positive regulator for its target sequences seemed to be higher for the promoters of the "late"-acting genes than of the "early"-acting ones. This differential affinity can explain the higher transcription rate observed for the *act*VI genes than that for the *act*I genes. This higher expression might be important, because the gene products of the *act*VI genes (20) are involved in the so-called tailoring steps, leading to the production of several biologically active metabolites (54) and might well be susceptible to generation of oxidative stress. This effect can presumably be reduced by increasing the expression of the genes whose products might be involved in keeping the internal concentrations of such metabolites at a very low level. Alternatively, such putative active metabolites would be inducers for other transcriptional regulators, which finally will directly activate the transcription of these "late" *act* structural genes. This last possibility can be ruled out, because no differences were observed in the transcription patterns of these genes from those of the wild-type strain and *S. coelicolor* PM218 mutant (a strain with no such intermediates because the early-acting and most of the late-acting genes were removed).

Attempts to overexpress the ActII-ORF4 protein in *Streptomyces* were unsatisfactory. The gene was cloned in a high-copy-number vector, either with its own promoter or under the control of the *tip*A promoter (26); both systems yielded a very low level of the protein (as was the case for the DnrI protein [60]), even in *S. coelicolor* PM218, where accumulation of the *act* pathway intermediates is not possible. This low expression level could be due mainly to the presence in the *act*II-ORF4 gene of several unusual codons (18), which may be a bottleneck in the translation process, making the expression of this gene in *Streptomyces* very difficult for conventional protein purification. Because of these difficulties, the recombinant proteins (wild-type ActII-ORF4 and ActII-ORF4-177) were purified from *E. coli* cultures. Only the wild-type engineered protein complemented the *act*II-ORF4-177 mutation, suggesting that in vivo this recombinant protein behaved similarly to the wild type in activating the expression of the *act* structural genes. Although, most of the ActII-ORF4 proteins are expressed in *E. coli* as inclusion bodies, enough soluble protein was produced to allow their purification and functional characterization. The gel mobility shift assays showed that both purified proteins had a specific DNA-binding activity for the *act* intergenic regions. Competition experiments in these DNA-binding assays showed that the recombinant wild-type protein had higher affinity for the *act*VI-ORF1 than for the *act*I-ORF1 promoters, in good agreement with the results derived from in vivo titration; thus, we can conclude that the protein used in in vitro experiments is likely to be similar, at least in some biological properties, to that used in in vivo assays in *Streptomyces*.

The DNase I footprinting assays revealed some DNA sequences which are protected from the DNase I digestion. Although no large protected fragment were observed in any of the promoters tested, some regions with hypersensitivity to DNase I digestion can be observed; this footprinting pattern can be explained if the DNA-protein complexes had a short life or were relatively unstable. Some of the protected fragments or those flanked by hypersensitive regions within the *act*VI promoters overlap the imperfect repeated sequences 5′-TCGAG-3′, which were also protected by the DnrI protein in the daunorubicin biosynthetic cluster (60). This sequence is located near the −35 region of those promoters and is contained within the heptameric imperfect direct-repeat sequence 5′-TCGAGC(G/C)-3′, which was suggested by Wietzorrek and Bibb (64) to be the putative binding sites for the ActII-ORF4 and DnrI proteins. Despite this good correlation observed within the *act*VI promoters, this could not be established for the *act*III-*act*I intergenic region under our experimental conditions, perhaps due to the lower affinity of the regulator for this region. It is noteworthy that a clear additional protected region was observed within the *act*VI-ORF1 transcript; although it does not overlap with the TCGAG sequence, this binding site might well be used for alternative transcriptional start points within the *act*VI polycistronic mRNA.

From all these, experiments we can conclude that the *act*II-ORF4 gene product is a DNA-binding protein. Recently, Wietzorrek and Bibb have suggested that the ActII-ORF4 protein (like other members of the SARP family) (64) contains the DNA-binding fold similar to the OmpR family and thus a helix-turn-helix motif. The mutation of the ActII-ORF4-177 protein (Ser instead of Leu at position 86) lies outside the helix-turn-helix motif and falls within the first predicted β-sheet (β6) (64), immediately after the putative DNA-binding fold; this might explain why the ActII-ORF4-177 protein still retains the specific DNA-binding activity. The β6-sheet is predicted to be partially buried (53), and the resulting change from a hydrophobic to another hydrophilic amino acid is likely to destroy this β-sheet structure; these changes could possibly generate an unstable protein, thus leading to a faster in vivo degradation. If the mutant protein is more easily degraded, it is possible that the protein never reaches the critical concentration needed for transcription activation; alternatively, it can be speculated that the generated changes prevent interactions between this transcriptional regulator and the RNA polymerase. In any case, the *act*II-177 mutation clearly demonstrates the existence of two different functions within the regulatory protein: specific DNA recognition and transcriptional activation. Isolation and characterization of additional mutations in the *act*II-ORF4 gene, will indeed be needed to generate more experimental data and a plausible model for transcriptional activation of the *act* structural genes.

ACKNOWLEDGMENTS

This work was supported by grants from Spanish CICYT (BIO96-1168-CC01-01) and European Union (BIO2-CT94-2067 and ERB-CHRXCT940570).

We thank E. Montejo de Garcini, J. Ortín, and A. Nieto for helpful discussions.

REFERENCES

1. **Adamidis, T., P. Riggle, and W. Champness.** 1990. Mutations in a new *Streptomyces coelicolor* locus which globally block antibiotic biosynthesis but not sporulation. J. Bacteriol. **172:**2962–2969.
2. **Anzai, H., T. Murakami, S. Imai, A. Satoh, K. Nagaoka, and C. J. Thompson.** 1987. Transcriptional regulation of bialaphos biosynthesis in *Streptomyces hygroscopicus*. J. Bacteriol. **169:**3482–3488.
3. **Bartolomé, B., Y. Jubete, E. Martínez, and F. de la Cruz.** 1991. Construction and properties of a family of pACYC184-derived cloning vectors compatible with pBR322 and its derivatives. Gene **102:**75–78.
4. **Bérdy, J.** 1984. New ways to obtain antibiotics. Chin. J. Antibiot. **7:**272–290.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
6. **Brian, P., F. J. Riggle, R. A. Santos, and W. C. Champness.** 1996. Global negative regulation of *Streptomyces coelicolor* antibiotic synthesis mediated by an *absA*-encoded putative signal transduction system. J. Bacteriol. **178:**3221–3231.
7. **Chakraburtty, R., and M. Bibb.** 1997. The ppGpp synthetase gene (*relA*) of *Streptomyces coelicolor* A3(2) plays a conditional role in antibiotic production and morphological differentiation. J. Bacteriol. **179:**5854–5861.
8. **Chakraburtty, R., J. White, E. Takano, and M. Bibb.** 1996. Cloning, characterization and disruption of a (p)ppGpp synthetase gene (*relA*) of *Streptomyces coelicolor* A3(2). Mol. Microbiol. **19:**357–368.
9. **Champness, W., P. Riggle, and T. Adamidis.** 1990. Loci involved in regulation of antibiotic synthesis. J. Cell. Biochem. **14A:**88. (Abstract.)
10. **Champness, W. C.** 1988. New loci required for *Streptomyces coelicolor* mor-

phological and physiological differentiation. J. Bacteriol. **170:**1168–1174.
11. **Chater, K. F.** 1989. Multilevel regulation of *Streptomyces* differentiation. Trends Genet. **5:**372–377.
12. **Chater, K. F., and C. J. Bruton.** 1985. Resistance, regulatory and production genes for the antibiotic methylenomycin are clustered. EMBO J. **4:**1893–1897.
13. **Chater, K. F., C. J. Bruton, A. A. King, and J. E. Suarez.** 1982. The expression of *Streptomyces* and *Escherichia coli* drug-resistance determinants cloned into the *Streptomyces* phage ϕC31. Gene **19:**21–32.
14. **Chong, P. P., S. M. Podmore, H. M. Kieser, M. Redenbach, K. Turgay, M. Marahiel, D. A. Hopwood, and C. P. Smith.** 1998. Physical identification of a chromosomal locus encoding biosynthetic genes for the lipopeptide calcium-dependent antibiotic (CDA) of *Streptomyces coelicolor* A3(2). Microbiology **144:**193–199.
15. **Covarrubias, L., and F. Bolivar.** 1982. Construction and characterization of new cloning vehicles. VI. Plasmid pBR329, a new derivative of pBR328 lacking the 482-base-pair inverted duplication. Gene **17:**79–89.
16. **Distler, J., A. Ebert, K. Mansouri, K. Pissowotzki, M. Stockmann, and W. Piepersberg.** 1987. Gene cluster for streptomycin biosynthesis in *Streptomyces griseus*: nucleotide sequence of three genes and analysis of transcriptional activity. Nucleic Acids Res. **15:**8041–8056.
17. **Feitelson, J. S., and D. A. Hopwood.** 1983. Cloning of a *Streptomyces* gene for an O-methyltransferase involved in antibiotic biosynthesis. Mol. Gen. Genet. **190:**394–398.
18. **Fernández-Moreno, M. A., J. L. Caballero, D. A. Hopwood, and F. Malpartida.** 1991. The *act* cluster contains regulatory and antibiotic export genes, direct targets for translational control by the *bld*A tRNA gene of *Streptomyces*. Cell **66:**769–780.
19. **Fernández-Moreno, M. A., E. Martínez, L. Boto, D. A. Hopwood, and F. Malpartida.** 1992. Nucleotide sequence and deduced function of a set of cotranscribed genes of *Streptomyces coelicolor* A3(2) including the polyketide synthase for the antibiotic actinorhodin. J. Biol. Chem. **267:**19278–19290.
20. **Fernández-Moreno, M. A., E. Martinez, J. L. Caballero, K. Ichinose, D. A. Hopwood, and F. Malpartida.** 1994. DNA sequence and functions of the *act*VI region of the actinorhodin biosynthetic gene cluster of *Streptomyces coelicolor* A3(2). J. Biol. Chem. **269:**24854–24863.
21. **Galas, D. J., and A. Schmiz.** 1978. DNase footprint: a simple method for the detection of protein-DNA binding specificity. Nucleic Acid Res. **5:**3157–3170.
22. **Gramajo, H. C., E. Takano, and M. J. Bibb.** 1993. Stationary-phase production of the antibiotic actinorhodin in *Streptomyces coelicolor* A3(2) is transcriptionally regulated. Mol. Microbiol. **7:**837–845.
23. **Hallam, S. E., F. Malpartida, and D. A. Hopwood.** 1988. Nucleotide sequence, transcription and deduced function of a gene involved in polyketide antibiotic synthesis in *Streptomyces coelicolor*. Gene **74:**305–320.
24. **Hara, O., S. Horinouchi, T. Uozumi, and T. Beppu.** 1983. Genetic analysis of A-factor synthesis in *Streptomyces coelicolor* A3(2) and *Streptomyces griseus*. J. Gen. Microbiol. **129:**2939–2944.
25. **Harasym, M., L. H. Zhang, K. Chater, and J. Piret.** 1990. The *Streptomyces coelicolor* A3(2) *bldB* region contains at least two genes involved in morphological development. J. Gen. Microbiol. **136:**1543–1550.
26. **Holmes, D. J., J. L. Caso, and C. J. Thompson.** 1993. Autogenous transcriptional activation of a thiostrepton-induced gene in *Streptomyces lividans*. EMBO J. **12:**3183–3191.
27. **Hopwood, D. A.** 1988. The Leeuwenhoek Lecture, 1987. Toward an understanding of gene switching in *Streptomyces*, the basis of sporulation and antibiotic production. Proc. R. Soc. London Ser. B **235:**121–138.
28. **Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schrempf.** 1985. Genetic manipulation of *Streptomyces*: a laboratory manual. The John Innes Foundation, Norwich, United Kingdom.
29. **Hopwood, D. A., T. Kieser, H. M. Wright, and M. J. Bibb.** 1983. Plasmids, recombination and chromosome mapping in *Streptomyces lividans* 66. J. Gen. Microbiol. **129:**2257–2269.
30. **Horinouchi, S., O. Hara, and T. Beppu.** 1983. Cloning of a pleiotropic gene that positively controls biosynthesis of A-factor, actinorhodin, and prodigiosin in *Streptomyces coelicolor* A3(2) and *Streptomyces lividans*. J. Bacteriol. **155:**1238–1248.
31. **Horinouchi, S., M. Kito, M. Nishiyama, K. Furuya, S. K. Hong, K. Miyake, and T. Beppu.** 1990. Primary structure of AfsR, a global regulatory protein for secondary metabolite formation in *Streptomyces coelicolor* A3(2). Gene **95:**49–56.
32. **Ishizuka, H., S. Horinouchi, H. M. Kieser, D. A. Hopwood, and T. Beppu.** 1992. A putative two-component regulatory system involved in secondary metabolism in *Streptomyces* spp. J. Bacteriol. **174:**7585–7594.
33. **Janssen, G. R., and M. J. Bibb.** 1993. Derivatives of pUC18 that have *Bgl*II sites flanking a modified multiple cloning site and that retain the ability to identify recombinant clones by visual screening of *Escherichia coli* colonies. Gene **124:**133–134.
34. **Katz, E., C. J. Thompson, and D. A. Hopwood.** 1983. Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. J. Gen. Microbiol. **129:**2703–2714.
35. **Kempter, C., D. Kaiser, S. Haag, G. Nicholson, V. Gnau, T. Walk, K. H. Gierling, H. Decker, H. Zahner, G. Jung, and J. W. Metzger.** 1997. CDA: Calcium-dependent peptide antibiotics from *Streptomyces coelicolor* A3(2) containing unusual residues. Angew. Chem. Int. Ed. English **36:**498–501.
36. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
37. **Lakey, J. H., E. J. Lea, B. A. Rudd, H. M. Wright, and D. A. Hopwood.** 1983. A new channel-forming antibiotic from *Streptomyces coelicolor* A3(2) which requires calcium for its activity. J. Gen. Microbiol. **129:**3565–3573.
38. **Lawlor, E. J., H. A. Baylis, and K. F. Chater.** 1987. Pleiotropic morphological and antibiotic deficiencies result from mutations in a gene encoding a tRNA-like product in *Streptomyces coelicolor* A3(2). Genes Dev. **1:**1305–1310.
39. **Leskiw, B. K., R. Mah, E. J. Lawlor, and K. F. Chater.** 1993. Accumulation of *bldA*-specified transfer RNA is temporally regulated in *Streptomyces coelicolor* A3(2). J. Bacteriol. **175:**1995–2005.
40. **Lydiate, D. J., F. Malpartida, and D. A. Hopwood.** 1985. The *Streptomyces* plasmid SCP2*: its functional analysis and development into useful cloning vectors. Gene **35:**223–235.
41. **Malpartida, F., and D. A. Hopwood.** 1986. Physical and genetic characterisation of the gene cluster for the antibiotic actinorhodin in *Streptomyces coelicolor* A3(2). Mol. Gen. Genet. **205:**66–73.
42. **Malpartida, F., J. Niemi, R. Navarrete, and D. A. Hopwood.** 1990. Cloning and expression in a heterologous host of the complete set of genes for biosynthesis of the *Streptomyces coelicolor* antibiotic undecylprodigiosin. Gene **93:**91–99.
43. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
44. **Martínez-Costa, O. H., P. Arias, N. M. Romero, V. Parro, R. P. Mellado, and F. Malpartida.** 1996. A *relA*/*spoT* homologous gene from *Streptomyces coelicolor* A3(2) controls antibiotic biosynthetic genes. J. Biol. Chem. **271:**10627–10634.
45. **Matsumoto, A., H. Ishizuka, T. Beppu, and S. Horinouchi.** 1995. Involvement of a small ORF downstream of the *afsR* gene in the regulation of secondary metabolism in *Streptomyces coelicolor* A3(2). Actinomycetologica **9:**37–43.
46. **Merrick, M. J.** 1976. A morphological and genetic mapping study of bald colony mutants of *Streptomyces coelicolor*. J. Gen. Microbiol. **96:**299–315.
47. **Miyadoh, S.** 1993. Research on antibiotic screening in Japan over the last decade: a producing microorganisms approach. Actinomycetologica **7:**100–106.
48. **Murray, G. M. G.** 1986. Use of sodium trichloroacetate and mung bean nuclease to increase sensitivity and precision during transcript mapping. Anal. Biochem. **158:**165–170.
49. **Narva, K. E., and J. S. Feitelson.** 1990. Nucleotide sequence and transcriptional analysis of the *redD* locus of *Streptomyces coelicolor* A3(2). J. Bacteriol. **172:**326–333.
50. **Ochi, K.** 1990. A relaxed (*rel*) mutant of *Streptomyces coelicolor* A3(2) with a missing ribosomal protein lacks the ability to accumulate ppGpp, A-factor and prodigiosin. J. Gen. Microbiol. **136:**2405–2412.

50a. **Parro, V.** Personal communication.

51. **Pérez-Llarena, F. J., P. Liras, A. Rodríguez García, and J. F. Martín.** 1997. A regulatory gene (*ccaR*) required for cephamycin and clavulanic acid production in *Streptomyces clavuligerus*: amplification results in overproduction of both beta-lactam compounds. J. Bacteriol. **179:**2053–2059.
52. **Puglia, A. M., and E. Cappelletti.** 1984. A bald superfertile U.V.-resistant strain in *Streptomyces coelicolor* A3(2). Microbiologica **7:**263–266.
53. **Rost, B.** 1996. PHD: predicting one-dimensional protein structure by profile-based neural networks. Methods Enzymol. **266:**525–539.
54. **Rudd, B. A., and D. A. Hopwood.** 1979. Genetics of actinorhodin biosynthesis by *Streptomyces coelicolor* A3(2). J. Gen. Microbiol. **114:**35–43.
55. **Rudd, B. A., and D. A. Hopwood.** 1980. A pigmented mycelial antibiotic in *Streptomyces coelicolor*: control by a chromosomal gene cluster. J. Gen. Microbiol. **119:**333–340.
56. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer directed enzymatic amplification of DNA with thermostable DNA polymerase. Science **239:**487–491.
57. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
58. **Studier, W. R., and B. A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189:**113–130.
59. **Stutzman-Engwall, K. J., S. L. Otten, and C. R. Hutchinson.** 1992. Regulation of secondary metabolism in *Streptomyces* spp. and overproduction of daunorubicin in *Streptomyces peucetius*. J. Bacteriol. **174:**144–154.
60. **Tang, L., A. Grimm, Y. X. Zhang, and C. R. Hutchinson.** 1996. Purification and characterization of the DNA-binding protein DnrI, a transcriptional factor of daunorubicin biosynthesis in *Streptomyces peucetius*. Mol. Microbiol. **22:**801–813.

60a. **Thompson, C. J.** Personal communication.

61. **Towbin, H. T., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and

some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
62. **Umeyama, T., Y. Tanabe, B. D. Aigle, and S. Horinouchi.** 1996. Expression of the *Streptomyces coelicolor* a3(2) *ptpA* gene encoding a phosphotyrosine protein phosphatase leads to overproduction of secondary metabolites in *Streptomyces lividans*. FEMS Microbiol. Lett. **144:**177–184.
63. **Ward, J. M., G. R. Janssen, T. Kieser, M. J. Bibb, M. J. Buttner, and M. J. Bibb.** 1986. Construction and characterisation of a series of multi-copy promoter-probe plasmid vectors for *Streptomyces* using the aminoglycoside phosphotransferase gene from Tn5 as indicator. Mol. Gen. Genet. **203:**468–478.
64. **Wietzorrek, A., and M. Bibb.** 1997. A novel family of proteins that regulates antibiotic production in streptomycetes appears to contain an OmpR-like DNA-binding fold. Mol. Microbiol. **25:**1181–1184.
65. **Wright, L. F., and D. A. Hopwood.** 1976. Actinorhodin is a chromosomally determined antibiotic in *Streptomyces coelicolor* A3(2). J. Gen. Microbiol. **96:**289–297.
66. **Wright, L. F., and D. A. Hopwood.** 1976. Identification of the antibiotic determined by the SCP1 plasmid of *Streptomyces coelicolor* A3(2). J. Gen. Microbiol. **95:**96–106.
67. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 cloning vector and host strains: nucleotide sequences of M13mp18 and pUC19 vectors. Gene **33:**103–119.