JOURNAL OF BACTERIOLOGY, July 1999, p. 4353–4364 Vol. 181, No. 14
0021-9193/99/$04.00+0

An Additional Regulatory Gene for Actinorhodin Production in *Streptomyces lividans* Involves a LysR-Type Transcriptional Regulator

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Received 3 March 1999/Accepted 5 May 1999

The sequence of a 4.8-kbp DNA fragment adjacent to the right-hand end of the actinorhodin biosynthetic (*act*) cluster downstream of *actVB-orf6* from *Streptomyces coelicolor* A3(2) reveals six complete open reading frames, named *orf7* to *orf12*. The deduced amino acid sequences from *orf7*, *orf10*, and *orf11* show significant similarities with the following products in the databases: a putative protein from the *S. coelicolor* SCP3 plasmid, LysR-type transcriptional regulators, and proteins belonging to the family of short-chain dehydrogenases/reductases, respectively. The deduced product of *orf8* reveals low similarities with several methyltransferases from different sources, while *orf9* and *orf12* products show no similarities with other known proteins. Disruptions of *orf10* and *orf11* genes in *S. coelicolor* appear to have no significant effect on the production of actinorhodin. Nevertheless, disruption or deletion of *orf10* in *Streptomyces lividans* causes actinorhodin overproduction. The introduction of extra copies of *orf10* and *orf11* genes in an *S. coelicolor actIII* mutant restores the ability to produce actinorhodin. Transcriptional analysis and DNA footprinting indicate that Orf10 represses its own transcription and regulates *orf11* transcription, expression of which might require the presence of an unknown inducer. No DNA target for Orf10 protein was found within the *act* cluster.

Members of the genus *Streptomyces* have become a major focus of study at the molecular level, in large part because of their ability to undergo both morphological and biochemical differentiation, including the production of bioactive metabolites (9). The activation of antibiotic production, often coupled to morphological development, involves many different pathways in the same organism. Although the multiple and coordinated regulation of secondary metabolism is poorly understood, insight into some of the mechanisms controlling antibiotic biosynthesis is emerging (10).

Streptomyces coelicolor provides an excellent model system for studying the regulation of antibiotic production, because it is genetically well studied and produces at least four quite different antibiotics: actinorhodin (50), undecylprodigiosin (38), methylenomycin (51), and the calcium-dependent peptide antibiotic (28). Their biosynthetic clusters have been isolated, and that for actinorhodin synthesis (*act* cluster) has been well characterized (7, 8, 16–18). Antibiotic pathway-specific regulatory genes have been found in the biosynthetic clusters for actinorhodin, undecylprodigiosin, and methylenomycin (for reviews, see references 9 and 10). Both ActII-Orf4 and RedD (*act*- and *red*-specific regulators) have been proposed to belong to a novel family of *Streptomyces* antibiotic regulatory proteins (48) that probably have similar mechanisms of transcriptional activation of the genes they regulate. In addition to this type of regulation, several other genes outside the biosynthetic clusters have been shown to pleiotropically affect antibiotic formation. Among them, *bld* and *rel* have been implicated in both antibiotic production and morphological differentiation, while a number of genes, including *abaA*, *absA*, *absB*, *afsB*, *afsR*, *afsS*, and *afsQ1-afsQ2*, have some effect on one or more antibiotic synthesis processes without modifying morphological development.

Streptomyces lividans, a streptomycete closely related to *S. coelicolor*, has all of the genetic information for actinorhodin biosynthesis but does not produce this antibiotic under usual growth conditions. Because of its ability to show a blue-pigmented phenotype by either the introduction of genes or the generation of mutations, this strain has become a useful tool for understanding the signaling mechanisms involved in the activation of antibiotic biosynthesis. By this procedure, several putative regulatory elements have been isolated. This report describes the characterization of the *orf10* gene, encoding a LysR-type transcriptional regulator, disruption or deletion of which induces actinorhodin production in *S. lividans*.

MATERIALS AND METHODS

Bacterial strains. The *Escherichia coli* strains used for general cloning procedures were JM101 (52) and XL1-Blue (6). *E. coli* K12ΔH1Δ*trp* (53) and K38 (39) (containing the helper plasmid pGP-1-2 [45]) were used for the expression of the Orf10 protein. The *S. coelicolor* A3(2) strains used were J1501 (*hisA1 uraA1 strA1 pgl* SCP1$^-$ SCP2$^-$) (13) and TK18 (*hisA1 uraA1 strA1 actIII141 redE60* SCP1$^-$ SCP2$^-$) (37). The *S. lividans* strain used was TK21 (*str-6* SLP2$^-$ SPL3$^-$) (25).

Plasmids and bacteriophages. The *E. coli* plasmids used were pUC18-19 (52), pIJ2925 (26), pSU19-20-21 (3), pBR329 (14), pT7.7 (45), pAZe3ss (53), and pIJ2333 (32). *E. coli* M13 derivative phages M13mp18 and M13mp19 (52) were used for DNA sequencing and for in vitro mutagenesis. The *Streptomyces* vectors and recombinant plasmids used are described in Table 1. The *Streptomyces* ϕC31 derivative phage PM1 (32) was used for insertional inactivation.

Media, culture conditions, and microbiological procedures. *E. coli* strains were grown on either liquid or solid 2YT medium (40). Appropriate antibiotics were added as required. *Streptomyces* manipulations were as described previously (25). Thiostrepton (Sigma catalog no. T-8902) was used at concentrations of 50 μg/ml in agar medium and 10 μg/ml in broth cultures. Kanamycin was used at 50 and 15 μg/ml in solid and liquid media, respectively.

DNA sequencing. DNA sequencing was done by the dideoxy-chain termination method (40); DNA sequence was determined from both strands, using routinely a 7-deaza-dGTP reagent kit from U.S. Biochemical Corp. (catalog no. 70750) as recommended by the manufacturer. Convenient DNA fragments were previously

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TABLE 1. *Streptomyces* vectors and recombinant plasmids

Plasmids	Relevant characteristics and construction[a]	Reference
pIJ486	High-copy-number cloning vector, *tsr*	47
pGM9	pSG5-derived temperature-sensitive replication vector, *tsr aphII*	35
pIJ941	Low-copy-number SCP2* derivative plasmid, *tsr hyg*	25
pIJ2314	*Bam*HI fragment (positions 13–14) cloned in pIJ922, harboring the wild-type *actIII* gene, *tsr*	32
pAM73	pGM9 derivative obtained from *S. lividans* TK21::pSCNB06A chromosomal DNA partially digested with *Sau*3AI, followed by ligation and transformation of *S. lividans*	This work
pAM75	pGM9 derivative obtained from *S. coelicolor* J1501::pSCNB06A chromosomal DNA partially digested with *Sau*3AI, followed by ligation and transformation of *S. lividans*	This work
pSCNB01[b]	*Sac*II (2274)-*Xho*I (3465) fragment cloned in pGM9 (*Eco*RI-*Hin*dIII sites); contains the 3′ truncated *orf10* gene	This work
pSCNB03[b]	*Pst*I (1881)-*Xho*I (3465) fragment cloned in pGM9 (*Eco*RI-*Hin*dIII sites); contains *orf10* gene	This work
pSCNB04[b]	*Pst*I (1881)-*Bam*HI (4847) fragment cloned in pGM9 (*Eco*RI-*Hin*dIII sites); contains *orf10* and *orf11* genes	This work
pSCNB05[c]	*Nco*I (3094)-*Bam*HI (4847) fragment cloned in pGM9 (*Bgl*II site); contains *orf11* gene	This work
pSCNB06A[c]	*Sac*II (2274–2674) fragment containing an internal region of *orf10* gene cloned in pGM9 (*Eco*RI-*Hin*dIII sites)	This work
pSCNB06B[d]	pSCNB06A with the internal region of *orf10* gene cloned in the opposite orientation	This work
pSCNB07[c]	*Sma*I (809)-*Bgl*II (2389) and *Nru*I (3049)-*Bam*HI (4847) fragments cloned in the same direction in pGM9 (*Eco*RI-*Hin*dIII sites)	This work
pSCNB08B[c]	*Sma*I (809)-*Bam*HI (2152) and *Nco*I (3094)-*Bam*HI (4847) fragments cloned in the same direction in pGM9 (*Eco*RI-*Hin*dIII sites)	This work
pSCNB09[c]	*Nru*I (3049)-*Bam*HI (4847) fragment cloned in pGM9 (*Eco*RI-*Hin*dIII sites); contains *orf11* gene	This work
pSCNB010[d]	*Sac*II (2274)-*Bam*HI (4847) fragment cloned in pGM9 (*Eco*RI-*Hin*dIII sites); contains the 3′-truncated *orf10* and entire *orf11* genes	This work
pSCNB013[b]	*Pst*I (1881)-*Xho*I (3465) fragment cloned in pIJ941 (*Eco*RV site)	This work
pSCNB014[b]	*Pst*I (1881)-*Xho*I (3465) fragment cloned in pIJ486 (*Eco*RI-*Hin*dIII sites)	This work

[a] *tsr*, thiostrepton resistance gene; *aphII*, aminoglycoside phosphotransferase gene from Tn*5*; *hyg*, hygromycin resistance gene. Numbers in parentheses after a restriction enzyme indicate nucleotide positions.

[b] Constructed either directly or as blunt-ended fragments through end filling with the Klenow fragment of DNA polymerase I or by T4 DNA polymerase treatment in the intermediate *E. coli* vector pUC19, rescued, and ligated to *Streptomyces* vectors as indicated.

[c] Constructed either directly or as blunt-ended fragments through end filling with the Klenow fragment of DNA polymerase I or by T4 DNA polymerase treatment in the intermediate *E. coli* vector pU2925, then rescued, and ligated to *Streptomyces* vectors as indicated.

[d] Constructed either directly or as blunt-ended fragments through end filling with the Klenow fragment of DNA polymerase I or by T4 DNA polymerase treatment in an intermediate *E. coli* vector pSU21, then rescued, and ligated to *Streptomyces* vectors as indicated.

cloned in either M13mp18 or M13mp19 vectors. Identification of DNA sequences in DNase I protection assays were carried out as described above, using a convenient single-stranded DNA as template and the universal 17-mer sequencing primer labeled at its 5′ terminus as primer.

Computer analysis of sequences. The DNA sequence was analyzed by using the software programs of the University of Wisconsin Genetics Computer Group (version 9.1) (15): analysis for open reading frames (ORFs) was performed with CODONPREFERENCE with a codon usage table made from 100 *Streptomyces* genes (49); comparisons of sequences were made against the EMBL nucleic acid database (daily updated) and the Swissprot database (daily updated), using FASTA, TFASTA, and BESTFIT. Protein alignments were made with either PILEUP from the same package or CLUSTAL W (version 1.7) (46).

Gene disruption and deletion. For insertional inactivation of *S. coelicolor*, internal fragments from either the *orf10* or *orf11* gene were cloned into the ϕC31 derivative PM1 vector, and the resulting recombinant phages were used to lysogenize strain J1501 by insert-directed recombination (11). *orf10* gene disruptions of *S. lividans* and *S. coelicolor* and *orf10* deletions of *S. lividans* were obtained by the procedure of Muth et al. (35), using temperature-sensitive replication pGM9 derivative plasmids. In all cases, the chromosomal arrangements of disruptions and deletions were confirmed by Southern analysis.

DNA and RNA manipulations. Isolation, cloning, and manipulation of nucleic acids were as previously described for *Streptomyces* (25) and *E. coli* (40). Endonuclease restriction sites for further subclonings were generated by using the Sculptor in vitro mutagenesis system (Amersham RPN 1526). Previously, suitable restriction fragments were cloned in M13mp18, and mutagenesis was performed as recommended by the manufacturer with the synthetic oligonucleotides C-079 (5′-CCCGGATCCGTCATCCGGCGTCACGCCCGGTC-3′) and T-051 (5′-CGTCGTCATGCCCATATGTTATCACCGCGGGTCTTGGA-3′). PCRs were carried out with Thermostase according to the manufacturer's recommendations.

For isolating the complete *orf11* and *orf12* genes from *S. coelicolor* and the *orf10* and *orf11* genes from *S. lividans*, the chromosomal DNA within this region was obtained by rescuing the pGM9 derivative plasmid that had been used for *orf10* disruption. To this end, the chromosomal DNA from both recombinant strains was partially digested with *Sau*3AI, followed by ligation, transformation of *S. lividans*, and selection with thiostrepton. Several recombinant plasmids from the resulting transformants were obtained, and those extending beyond the sequenced DNA were selected and named pAM74 or pAM75 for the pGM9 derivative plasmid rescued from either the *S. lividans* or *S. coelicolor* chromosome, respectively. This DNA region was subcloned and sequenced as described above. The correct physical arrangement of this region was confirmed by Southern analysis.

RNA was extracted from mycelia grown on the surface of cellophane discs on R5 agar plates as previously described (30).

High-resolution S1 mapping was carried out by the procedure of Hopwood et al. (25). Initially for the *orf10* gene, a 316-bp *Esp*I-*Ava*I fragment (from positions 2983 to 3298) containing the *orf10-orf11* intergenic region uniquely labeled at the 5′ end of the *Esp*I site within the *orf10* coding region, was used as the probe. When analyzing RNA extracted from *S. lividans* CNB073, we used as the probe a 525-bp *Bst*EII-*Xho*I fragment (from positions 2289 to 3465) carrying the *orf10* deletion and containing the *orf10-orf11* intergenic region uniquely labeled at the 5′ end of the *Bst*EII site within the *orf10* coding region. For the *orf11* gene, a 471-bp *Sma*I-*Spl*I fragment (nucleotides 2923 to 3393) that contained the *orf10-orf11* intergenic region labeled at the 5′ end of the *Spl*I site within the internal *orf11* coding region was used. Nucleotide sequence ladders of the identical fragments were derived as described by Maxam and Gilbert (34). Before assigning a precise RNA initiation site, we subtracted one nucleotide from the length of the protected fragment to account for the difference in 3′ ends resulting from S1 nuclease digestion and the chemical sequencing reactions (24). For *actII-orf4* (16), the *actII-orf4* promoter region included in a 634-bp fragment (nucleotide positions 4825 to 5458 (16)) was uniquely labeled at the 5′ end of the *Xho*I (nucleotide 5458) site within the *actII-orf4* coding region and used as the probe.

Construction of *orf10* expression plasmids. To express Orf10 from *S. coelicolor*, a *Bam*HI restriction site at the 3′ end of *orf10* was generated by in vitro mutagenesis as described above, using primer C-079 and single-stranded DNA from M13mp18 carrying the *Pst*I (1881)-*Xho*I(3465) fragment as the template. The *Bam*HI fragment from the resulting M13mp18 derivative (pMCNB018.B) was cloned into *Bam*HI-digested pUC19, yielding plasmid pCNB04. The *orf10* gene was cloned under lambda p_L promoter control into *Nco*I-*Bam*HI-digested pAZe3ss, generating plasmid pCNB019. For *orf10* expression using the bacteriophage T7 RNA polymerase-promoter system (45), the *Nco*I site was replaced by an *Nde*I site by in vitro mutagenesis as described above, using primer T-051. The *Eco*RI-*Hin*dIII fragment from the resulting M13mp18 derivative (pMCNB018.BN) was cloned into *Eco*RI-*Hin*dIII-digested pUC19, generating

plasmid pCNB021. The 0.95-kbp *Nde*I-*Bam*HI fragment from pCNB021 was finally inserted into *Nde*I-*Bam*HI sites of pT7.7, yielding plasmid pCNB023. To generate the C-terminally truncated Orf10 (amino acid positions 1 to 274), $Orf10_{1-274}$, plasmid pCNB01004 (which contained the internal *Sac*II region of the *orf10* gene) was digested with *Bgl*II and *Sma*I; the electroeluted 115-bp fragment was cloned into pCNB04, previously digested with *Sph*I, made blunt ended by T4 DNA polymerase treatment, and then subjected to *Bgl*II digestion and alkaline phosphatase treatment. The *Nco*I-*Bam*HI fragment from the resulting plasmid (pCNB025) was cloned into *Nco*I-*Bam*HI-digested pAZe3ss, yielding plasmid pCNB031. As a result of this procedure, $Orf10_{1-274}$ had an additional amino acid (Leu) at its C terminus.

To express the Orf10 from *S. lividans*, the *Nco*I-*Bgl*II fragment from pCNB019 (carrying the *orf10* gene from *S. coelicolor*) was replaced by the homologous DNA from *S. lividans*, yielding plasmid pCNB086.

Sequencing of suitable fragments confirmed that only the desired mutations had occurred. In all cases, the resulting expressed Orf10 protein incorporated two amino acids (Met and Gly) at its N terminus.

Expression and purification of the Orf10 protein in *E. coli*. The Orf10 protein was purified from *E. coli* K12ΔH1Δ*trp* carrying pCNB019. The strain was grown overnight at 30°C to stationary phase in 2YT medium; a 3% inoculum was then subcultured on a 0.2-liter scale and grown at 30°C until the optical density at 600 nm reached 0.9. The culture was transferred to 42°C and incubated for an additional 2 h. The culture was harvested by centrifugation, and the resulting cell pellet was washed twice with 50 ml of ice-cold buffer A (50 mM Tris-HCl [pH 8], 100 mM NaCl, 10 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) and resuspended in 10 ml of buffer A. The cells were disrupted by sonication and centrifuged at 4,000 × *g* for 15 min at 4°C, and the supernatant was again centrifuged. The final supernatant was centrifuged at 25,000 × *g* for 30 min at 4°C, and the resulting pellet was resuspended in 0.75 ml of buffer A, which was then solubilized by adding 4.25 ml of a denaturing solution containing 7 M urea, 60 mM dithiothreitol, 1.25 mM EDTA, and 62.5 mM Tris-HCl (pH 8). This suspension was incubated on ice for 30 min and then centrifuged at 105,000 × *g* for 30 min at 4°C. The supernatant, which contained the solubilized Orf10 protein, was dialyzed exhaustively against buffer B (50 mM Tris-HCl [pH 8], 50 mM NaCl, 5 mM dithiothreitol, 10% glycerol) and then centrifuged; the supernatant (approximately 5 ml at 0.2 to 0.4 mg of protein per ml) was loaded onto a 5-ml heparin-agarose column that had been previously equilibrated with buffer B. The column was first washed with 10 volumes of buffer B and then with 5 volumes of buffer B containing 0.25 M NaCl; finally, the Orf10 protein was eluted with 10 volumes of buffer B containing 0.5 M NaCl. Fractions were tested for binding to the *orf10-orf11* promoter in gel mobility shift assays and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, and the positive fractions were pooled and used for further studies. The Orf10 protein is stable at 4°C for a week and at −20°C for several months. The supernatant obtained at 25,000 × *g* constituted the crude extract.

Radiolabeled Orf10 protein was obtained from the overexpressed *orf10* gene by *E. coli* K38/pGP-1-2, transformed with plasmid pCNB023. Cell labeling with [^{35}S]methionine was performed as described by Tabor and Richardson (45). The radiolabeled Orf10 protein was purified from the inclusion bodies of 5 ml of culture essentially as described above and used for gel mobility shift assays after the refolding step.

The *S. coelicolor* truncated $Orf10_{1-274}$ and the complete *S. lividans* Orf10 proteins were purified as described for the *S. coelicolor* Orf10 from *E. coli* K12ΔH1Δ*trp* carrying pCNB031 and pCNB086, respectively. After the refolding step, the proteins were assayed for the ability to bind to the *orf10-orf11* intergenic region.

DNA binding assays. The standard binding reaction was carried out in a final volume of 20 μl containing the purified Orf10 protein, 1 to 5 ng of labeled DNA fragments, 1 μg of poly(dI-dC) · poly(dI-dC) DNA, 1 μg of bovine serum albumin, 8 mM $MgCl_2$, 10% glycerol, and 1× DNA binding buffer (5 mM Tris-HCl [pH 8], 20 mM NaCl, 1.5 mM 2-mercaptoethanol). Samples were allowed to incubate for 20 min at 4°C and then for 15 min at room temperature. After incubation, the samples were loaded onto a nondenaturing 4% polyacrylamide gel (28:2 acrylamide *N,N′*-methylenebisacrylamide linkage), with or without the addition of 1× DNA binding buffer containing bromophenol blue. Electrophoresis was performed at 120 V in 0.5× Tris-borate-EDTA buffer until the bromophenol blue had reached the end of the gel, transferred onto Whatman paper, covered with plastic wrap, dried, and exposed to X-ray film. When radiolabeled Orf10 protein was used, the DNA binding assays were performed as described above except that the samples contained unlabeled DNA fragments. The following DNA fragments were tested by gel retardation analysis: the *orf10-orf11* intergenic region (*Esp*I-*Ava*I fragment [nucleotides 2981 to 3297]), the downstream regions from *orf10* and *orf11* (*Pst*I-*Sac*II [nucleotides 1881 to 2274] and *Sma*I-*Hin*cII [nucleotides 3997 to 4224] fragments, respectively) containing the direct repeats, the *actI-actIII* intergenic region (*Mbo*II fragment containing nucleotides 1 to 240 from Hallam et al. [20]) and 1 to 215 from Fernández-Moreno et al. [17]), the *actVI* intergenic region (*Nae*I-*Sma*I fragment [nucleotides 1914 to 2226] [18]), the *actII-orf1-orf2* intergenic region (*Sph*I-*Sac*II fragment [nucleotides 885 to 1171] [16]), and the *actII-orf4* promoter region (*Taq*I fragment [nucleotides 4934 to 5173] [16]).

DNase I protection experiments. For DNase I footprinting, either the *Nru*I-*Ava*I (nucleotides 3049 to 3297) or *Aha*II (nucleotides 2945 to 3151) fragment within the promoter region of *orf10-orf11* was cloned as a blunt-ended fragment into *Hin*cII-digested pUC19 (pCNB033) or pIJ2921 (pCNB034A), respectively. The inserts of pCNB033 and pCNB034A were used to analyze the protected regions on the upper and lower strands, respectively. The universal 17-mer sequencing primer was labeled with [γ-^{32}P]ATP by treatment with T4 polynucleotide kinase at the 5′ end and used with the M13/pUC 16-mer reverse sequencing primer to amplify by PCR on double-stranded DNA of either pCNB033 or pCNB034A, a 246- or 206-bp DNA fragment, respectively. The binding reaction was carried out as described above. After incubation for 15 min at room temperature, 2 ng (0.09 U) of DNase I was added, and the mixture was incubated for 5 min at 30°C. The reaction was stopped by addition of EDTA up to 10 mM and 20 μl of Sequenase loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). The samples were incubated for 2 min at 90°C and analyzed on a 6% sequencing gel. After electrophoresis, the gels were dried and subjected to autoradiography. To locate the DNase I footprint, sequencing reactions as described above were run concomitantly.

Miscellaneous methods. Protein was determined as described elsewhere (5), with bovine serum albumin as a standard. SDS-PAGE was carried out with the buffer system described by Laemmli (29) in 10% gels, and protein bands were visualized by staining with Coomassie brilliant blue R-250.

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper have been deposited in the EMBL-GenBank database under accession no. Y18817 and Y18818.

RESULTS

DNA sequence at the right-hand end of the *S. coelicolor act* cluster. To determine whether there were any other additional regulatory genes involved in the control of actinorhodin biosynthesis, the region next to the right-hand end of the *act* cluster was explored. Thus, starting at *Sph*I (site 19.2 [17, 32]), a DNA region of 4.8 kbp, adjacent to *actVB-orf6* and extending rightward, has been sequenced; the first 31 bp correspond to those already found at the 3′ end by Fernández-Moreno et al. (17). Due to the occurrence of a rearrangement at an *Mbo*I site in the original plasmid (pIJ2300) (31) at nucleotide 3818, the entire DNA sequence of this region was reisolated directly from the *S. coelicolor* J1501 chromosome as described in Materials and Methods (Fig. 1). A scheme of the modified resulting restriction map is shown in Fig. 1.

Computer-assisted analysis of the DNA sequence, using CODONPREFERENCE, revealed a set of six putative complete ORFs (Fig. 1), which were named (from left to right) *orf7* to *orf12*. *orf9*, *orf11*, and *orf12* are transcribed rightward (in the same direction as *actVB*), whereas *orf7*, *orf8*, and *orf10* run divergently. The translation start codon for each ORF was tentatively allocated by using the following criteria: (i) the distribution of GC content in the third position (4, 49), (ii) codon usage (49), (iii) the presence of a potential Shine-Dalgarno sequence upstream of the initiation codon (44), and (iv) observed similarities to other putative ORF products from databases. The most relevant features deduced from the DNA sequence are summarized in Table 2.

The overall GC content of 76% is typical for the genus *Streptomyces*; a series of direct repeats was found either within the 3′-end region of the *orf10* gene (nucleotides 2041 to 3184) or downstream of *orf11* (nucleotides 4117 to 4163), their length ranging from 22 to 37 or from 17 to 35 nucleotides, respectively (Fig. 1).

Deduced functions of the sequenced genes. Searching for similarities of the deduced proteins with others in databases revealed a significant resemblance of the Orf7 protein with a hypothetical 13.3-kDa protein from the *S. coelicolor* minicircle (22) (although the former is 57 amino acids longer at its N terminus) and with the C terminus of the homologous deduced products, ScI35.38c (EMBL data bank accession no. A1031541) and Sc3c8.21c (EMBL data bank accession no. A1023861), within the IS*117*-A and IS*117*-B chromosomal DNA regions (13), respectively.

The Orf8 protein showed low levels of similarity with a

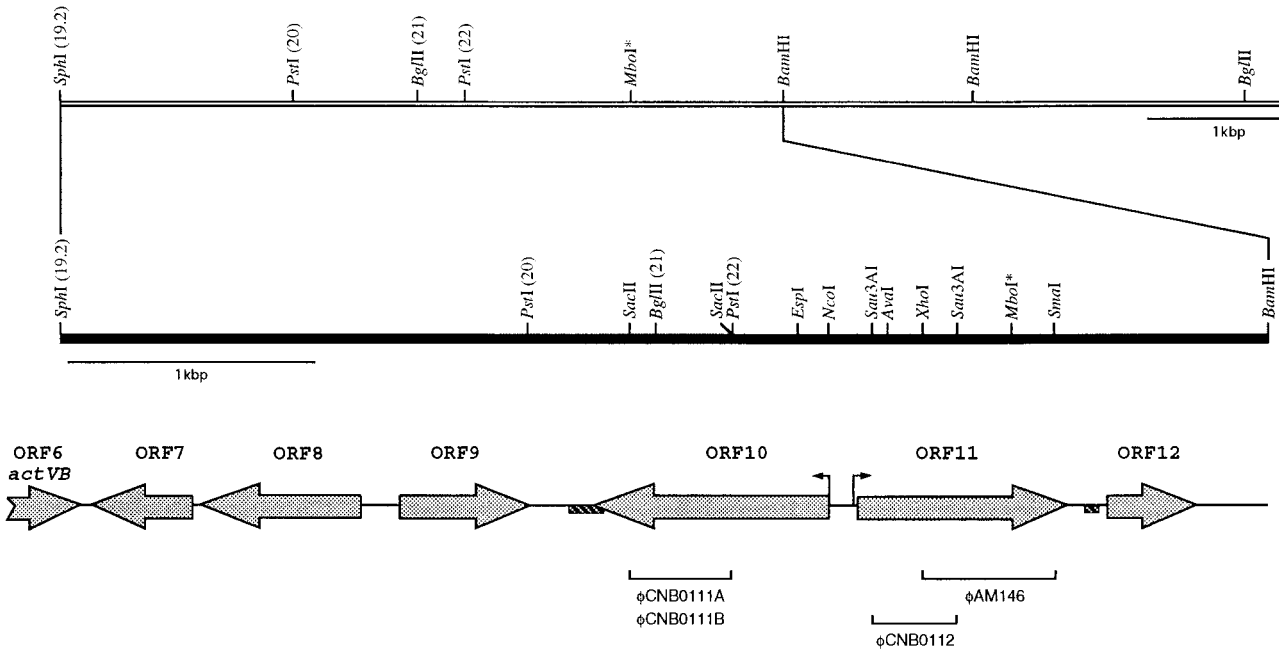

FIG. 1. Restriction map of the *S. coelicolor* A3(2) chromosome next to the right-hand end of the *act* cluster. The organization of ORFs within this region as deduced by DNA sequencing is given. Only relevant restriction sites are indicated; numbers in parentheses correspond to those reported by Malpartida and Hopwood (32). The *Mbo*I restriction site at which DNA rearrangement has occurred is indicated by asterisks. The solid bar shows the extent of the sequenced DNA fragment. The direct repeat sequences at the 3′ ends of *orf10* and *orf11* are represented by shaded boxes. The DNA fragments used for gene disruptions with the ϕC31-derived PM1 vector are indicated.

number of methyltransferases from different sources, particularly with the *Rhodococcus* sp. methyltransferase-decarboxylase CobL protein (EMBL database accession no. L21196) (similarity, 44%; identity, 48%).

The sequence of the deduced Orf9 protein showed end-to-end homology to the translated product of Sc6G4.21 (EMBL database accession no. A1031317) from *S. coelicolor* and the C terminus of the Orf1 protein in the *famA-famB* DNA region of the *Streptomyces purpurascens* chromosome (EMBL database accession no. X61931).

The deduced *orf10* gene product showed strong resemblance to a number of transcriptional regulators belonging to the LysR family (23, 41). The highest similarity was 46% with BudR, a protein from *Klebsiella terrigena* believed to be involved in 2,3-butandiol synthesis, and less with other members of the same family (Fig. 2). The similarity or identity levels observed were in the range of 40 to 46% and 32 to 38%, respectively, for the entire amino acid sequence. As a member of the LysR family, the N terminus of Orf10 protein is the most highly conserved region and contains the typical helix-turn-helix (HTH) motif, known to be involved in the binding to the target DNA sequence. Some of the proteins of this family are known to regulate transcription of divergently arranged genes; this might well be the case for Orf10 protein, because a divergently transcribed gene (*orf11*) is found next to it. Thus, *orf10* may encode a protein that is involved in regulating expression of the *orf11* gene.

The Orf11 protein showed significant similarity with proteins of the so-called short-chain dehydrogenases/reductase (SDR) (27) family (Fig. 3). Similarity ranged from 33 to 47% and identity ranged from 25 to 38%, the best score being found with 3-oxoacyl-acyl carrier protein reductase from *E. coli*. In addition, the *actIII* gene product (20) was identified as homologous in database searches, as were genes from other *Streptomyces* species, such as *car* (36), *mon-kr* (1), *gra-orf5-6* (43), *dpsE* (19), and *jad-orf5* (21), contained in the antibiotic biosynthetic clusters for clavulanic acid, monensin, granaticin, daunorubicin, and jadomicin B, respectively. Most of the enzymes in this family are known to be NAD(H)- or NADP(H)-dependent oxidoreductases. As described for this family of

TABLE 2. Relevant features of the *S. coelicolor* 4.8-kbp region deduced from its DNA sequence

orf	SD[a]	Start/stop codons[b]	No. of amino acids (M_r)	Predicted function
7	^{543}GGAGG^{539}	ATG 529/32 TGA	165 (17,924)	Minicircle protein
8	^{1221}GAAGGAG^{1215}	ATG 1209/583 TGA	208 (22,299)	Methyltransferase
9	None	ATG 1344/1916 TGA	190 (20,619)	Unknown
10	None	ATG 3091/2168 TGA	307 (33,279)	Transcriptional regulator (LysR family)
11	^{3180}GAGGGAG^{3186}	ATG 3192/4022 TGA	276 (28,780)	SDR family
12	^{4192}AGGAG^{4196}	GTG 4203/4544 TGA	113 (11,883)	Unknown

[a] SD, putative Shine-Dalgarno sequence.
[b] Numbers indicate the first and last nucleotides of the start and stop codons, respectively.

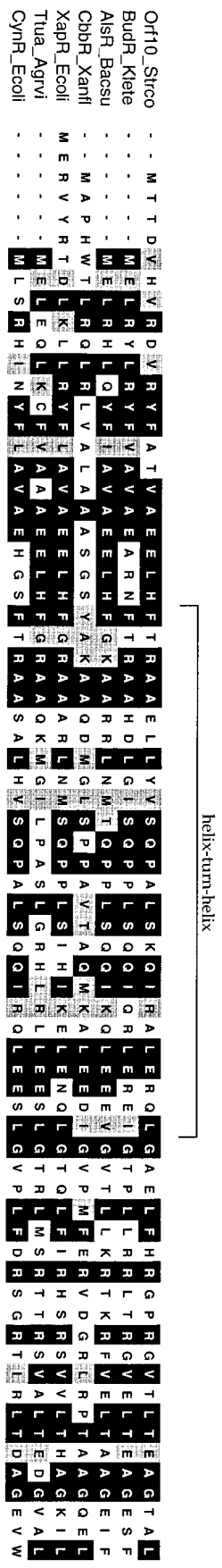

FIG. 2. Multiple alignment of the N terminus of *S. coelicolor* Orf10 protein (Orf10_Strco) with those of other LysR-type transcriptional regulators. The HTH motif involved in DNA binding is indicated. Origins of the amino acid sequences and (in parentheses) their Swissprot database accession numbers are as follows: BudR_Klete, *Klebsiella terrigena* (P52666); AlsR_Bacsu, *Bacillus subtilis* (Q04778); CbbR_Xanfl, *Xanthobacter flavus* (P25545); XapR_Ecoli, *E. coli* (P23841); Ttua_Agrvi, *Agrobacterium vitis* (P52669); and CynR_Ecoli, *E. coli* (P27111). Black boxes indicate positions in the alignment where the same amino acid is found in at least four of the seven sequences; gray boxes indicate residues similar to those marked in black.

proteins, the putative coenzyme binding domain, represented by three highly conserved glycine residues (Gly-9, Gly-13, and Gly-15), is located in the N-terminal portion of the Orf11 protein (Fig. 3A). Additionally, the conserved Asp-56 may serve as a site for hydrogen bonding to the coenzyme, as in many other dehydrogenases (27). Orf11 protein may contain the second well-conserved domain of these enzymes, corresponding to the active site, represented by the three conserved amino acid residues (Ser-135, Tyr-148, and Lys-152) with a consensus spacing (Fig. 3B). Moreover, as expected, the hydrophilicity pattern within this region of Orf11 exhibited high similarity to that estimated for other oxidoreductases of the same family (data not shown).

A search of databases with the *orf12* product gave no significant similarities, and therefore no function could be ascribed for Orf12 protein.

Analysis of *orf10* and *orf11* function in *S. coelicolor*. To explore the possible role of *orf10* and *orf11* gene products in *S. coelicolor*, mutants were generated by insertional inactivation within both genes, as described in Materials and Methods (Fig. 1). To disrupt *orf10*, the internal *Sac*II fragment (nucleotides 2274 to 2674) cloned in pIJ2925 (pCNB01004) was recovered by digestion with *Bgl*II and ligated to phage PM1, previously digested with *Bgl*II. The recombinant phages ϕCNB0111A and ϕCNB0111B (Fig. 1), carrying the insert in opposite orientations, were obtained. Similarly, the same fragment was cloned into the pGM9 vector, yielding plasmids pSCNB06A and pSCNB06B for either orientation. For *orf11* disruptions, we constructed two different PM1 derivatives, one carrying the 532-bp *Xho*I-*Sma*I fragment (nucleotides 3465 to 3997) and the other carrying the 336-bp *Sau*3AI fragment (nucleotides 3242 to 3578), for ϕAM146 and ϕCNB0112, respectively (Fig. 1). All of these constructions were used for gene disruptions in *S. coelicolor* J1501; that either gene had indeed been interrupted was confirmed by Southern blot analysis. Antibiotic production and auxotrophies were tested in the disruptants, and no obvious phenotypic differences were observed between any of them and the wild-type strain.

Because of the homology between the Orf11 and ActIII proteins, we next explored if the *orf11* gene had the ability to complement the *actIII* mutation in the actinorhodin biosynthetic pathway of *S. coelicolor*. Therefore, several plasmids were constructed (Table 1; Fig. 4) and used to transform the *actIII* mutant strain *S. coelicolor* TK18. *S. coelicolor* TK18 showed an actinorhodin-producing phenotype only in the presence of both *orf10* and *orf11* genes (pSCNB04), suggesting that *orf10* is required along with the *orf11* gene for antibiotic production. It is worth noting that during the cloning steps for *orf10* disruptions, a TAG codon was generated four nucleotides downstream of the original *Sac*II restriction site (nucleotide position 2274), thus generating a truncated protein lacking the last 33 amino acids ($Orf10_{1\text{-}274}$). This construct was used to generate pSCNB010; interestingly, this plasmid did not allow complementation of the *actIII* mutation (Fig. 4). All of these results strongly suggest that the Orf10 protein may be involved in the regulation of *orf11* expression.

Additionally, it is noteworthy that *S. coelicolor* J1501 showed no obvious change in phenotype when transformed with plasmids (shown in Fig. 4) containing either or both of the *orf10* and *orf11* genes.

Function of the *orf10* gene in *S. lividans*. The possible function of the *orf10* gene was further investigated in *S. lividans*, a streptomycete closely related to *S. coelicolor*. Using the pGM9 derivative plasmids pSCNB06A and pSCNB06B, the *orf10* gene was disrupted in *S. lividans*. Similarly, *orf10* deletions in *S. lividans* were generated by using the pGM9 derivative plasmids

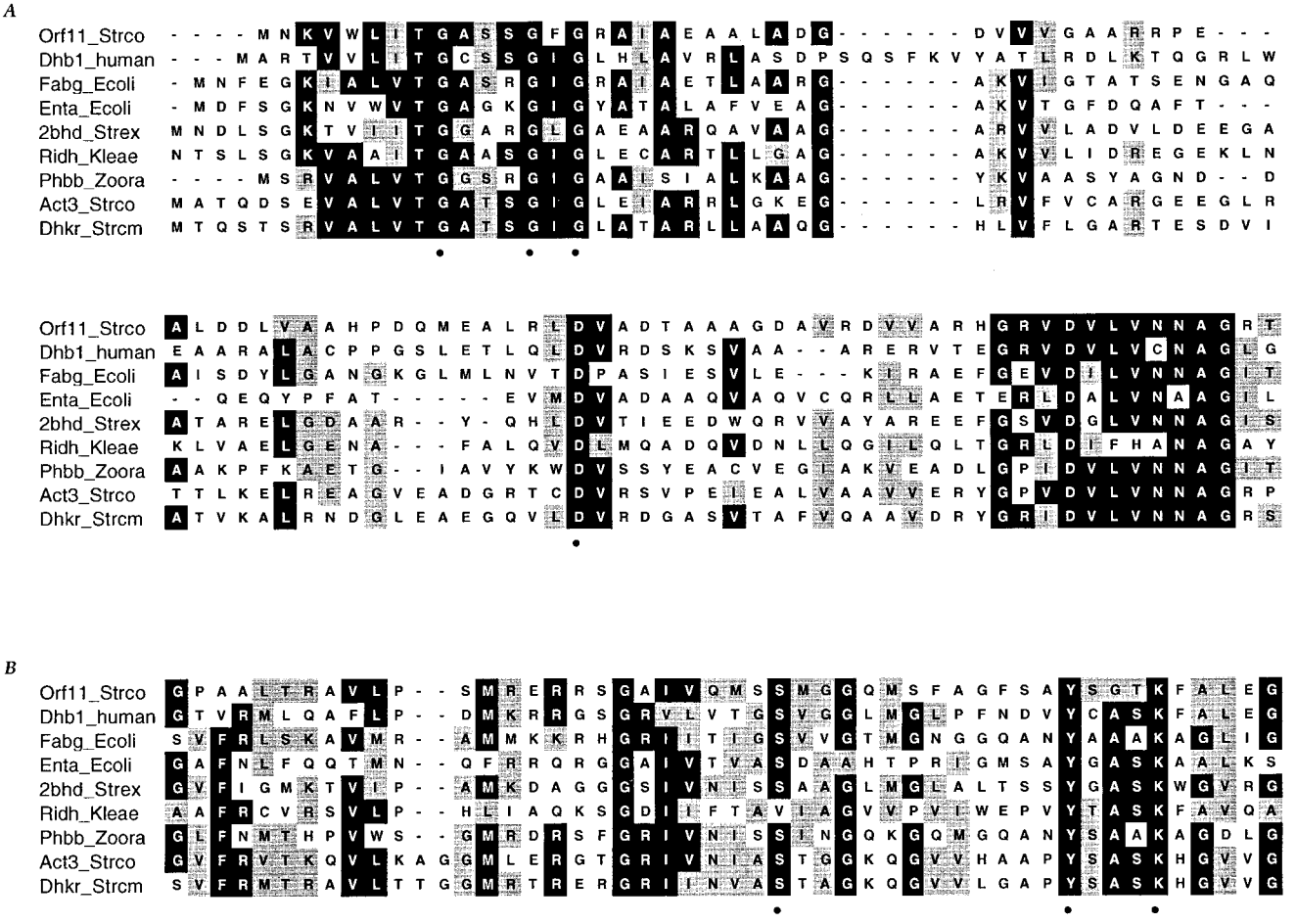

FIG. 3. Alignment of the putative NAD(H)-NADP(H) binding (A) and catalytic (B) sites of *S. coelicolor* Orf11 protein (Orf11_Strco) with those of other SDRs. Origins of the proteins and (in parentheses) their Swissprot database accession numbers are as follows: Dhb1_human, human (P14061); Fabg_Ecoli, *E. coli* (P25716); Enta_Ecoli, *E. coli* (P15047); 2bhd_Strex, *Streptomyces exfoliatus* (P19992); Ridh_Kleae, *Klebsiella aerogenes* (P00335); Phbb_Zoora, *Zoogloea ramigera* (P23238); Act3_Strco, *S. coelicolor* (P16544); and Dhkr_Strcm, *Streptomyces cinnamonensis* (P41177). Conserved and similar residues are in black and gray boxes, respectively (plurality, 5). The amino acids reported to play a role in either site are indicated.

pSCNB08B and pSCNB07, yielding strains CNB062 and CNB073, respectively. Unlike the parental strain, both disruption and deletion of the *orf10* gene yielded an actinorhodin-producing phenotype, suggesting a function of *orf10* in the regulation of actinorhodin production in *S. lividans*.

To confirm that the resulting phenotype was indeed due to the absence of the *orf10* gene, plasmids pSCNB013 and pSCNB014 containing *orf10* and its promoter region were constructed and used to transform *S. lividans* CNB073. When the *orf10* gene was introduced in this strain in *trans* on either a low (pSCNB013)- or high (pSCNB03 or pSCNB014)-copy-number plasmid, *S. lividans* CNB073 showed no change in phenotype. Nevertheless, when the *orf10* gene was introduced in *cis* into the chromosome by insert-directed recombination through its 3′ end (using the pGM9 derivative pSCNB03), the actinorhodin-producing phenotype of the mutant strain reverted to the wild-type actinorhodin-nonproducing phenotype.

Cloning and characterization of the *orf10-orf11* homologous region of *S. lividans*. Because all previous constructions were generated by using clones from *S. coelicolor*, it was of interest to determine if there were within this DNA region relevant sequence differences between this species and *S. lividans* that could account for the actinorhodin-nonproducing phenotype of *S. lividans*. Thus, the homologous *orf10-orf11* region of *S. lividans* TK21 was isolated (see Materials and Methods). The 3-kb *Pst*I-*Bam*HI fragment from this region was subcloned and sequenced, and three putative ORFs, *orf10*, *orf11*, and *orf12*, were identified. The DNA sequence was shown to be 99% identical between *S. coelicolor* and *S. lividans*. The corresponding products of *orf10*, *orf11*, and *orf12* were shown to be almost identical between the two species, with the following mismatches: for Orf10 protein, Thr-82, Gln-130, and Ala-192 have been replaced in *S. lividans* by Ser, Arg, and Val, respectively; for Orf11 protein, Asp-245 has been changed to Ala in *S. lividans*; and for Orf12 protein, Ser-88 has been replaced by Ala in *S. lividans*. Such small differences in the Orf10 sequence between the species seem insufficient to explain the actinorhodin-nonproducing phenotype of *S. lividans*. Moreover, the actinorhodin-producing phenotype of the *S. lividans orf10*-deleted strain was reverted to the wild-type (actinorhodin-nonproducing) phenotype when the *orf10* gene from *S. lividans* was introduced in *cis* within the *S. lividans orf10* mutant chromosome. Additionally, the *orf10* and *orf11* genes from *S. lividans* on a high-copy-number plasmid restored the blue-pigmented phenotype of the *S. coelicolor actIII* mutant. Thus, *orf10* and *orf11* appear to function similarly irrespective of their origin.

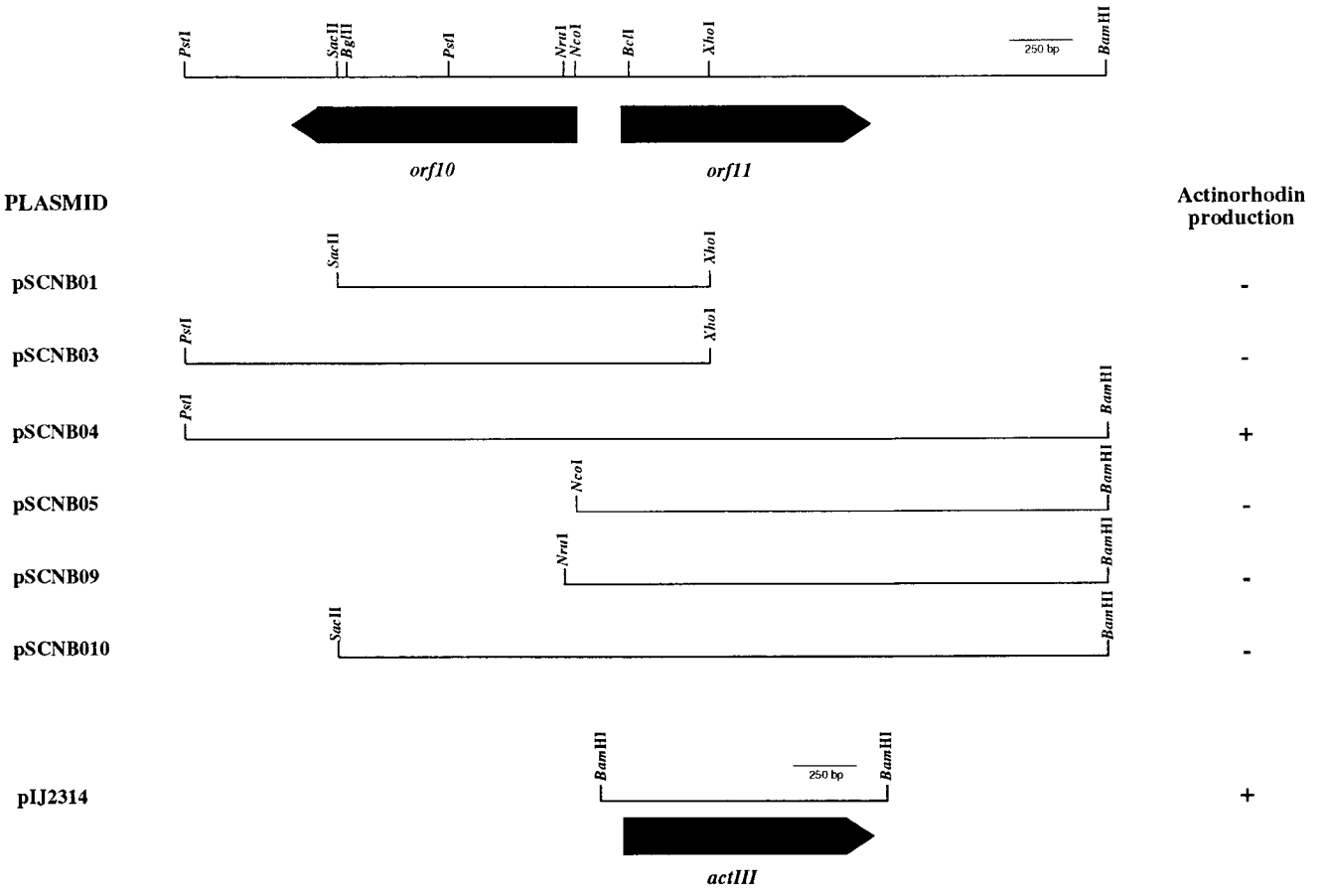

FIG. 4. Subcloning of the *orf10-orf11* DNA region to elucidate the genes required for complementation analysis of *actIII* mutation. Plasmid construction was as described in Materials and Methods and Table 1. Plasmids pSCNB01 and pSCNB010 contain a TAG stop codon (generated by the cloning procedure) downstream of the 3′ end of the *orf10* deletion. Plasmid pIJ2314 contains the *actIII* gene. Symbols: −, no actinorhodin production; +, actinorhodin production.

Transcriptional analysis of the *act* pathway-specific regulatory gene, *actII-orf4*. The possible mechanism involved in actinorhodin induction by *orf10* disruption and deletion in *S. lividans* was further examined by analyzing the transcription of the positive regulator of the *act* genes, the *actII-orf4* gene. As shown in Fig. 5, nuclease S1 protection experiments of this gene revealed an increase in its transcription in both *S. lividans orf10*-disrupted and -deleted strains compared with the basal levels of the parental strain (*S. lividans* TK21). Thus, the actinorhodin biosynthesis activation caused by *orf10* disruption and deletion in *S. lividans* appears to be mediated by an increase in the transcriptional level of the *actII-orf4* gene.

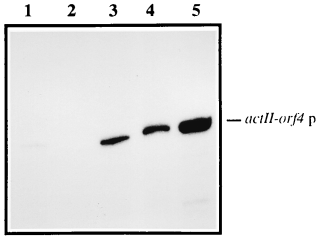

FIG. 5. Transcription analysis of the *actII-orf4* gene. Total RNA was isolated from 3-day-old cultures of *S. lividans* strains TK21, TK21::pSCNB06A, and CNB073 (lanes 1, 3, and 4, respectively) and of *S. coelicolor* J1501 (lane 5). *E. coli* tRNA was used as a control (lane 2). A protected fragment of the expected size (384 nucleotides) was observed. End-labeled *Hin*fI-digested pBR329 was used as a size marker. p, promoter.

Transcriptional analysis of *orf10* and *orf11* genes. Attempts to determine the transcription start site of *orf10* from *S. lividans* and *S. coelicolor* from the chromosomal gene as well as the gene carried on a high-copy-number plasmid (pSCNB03, pSCNB04, or pSCNB014) were unsuccessful. Presumably, the apparent low expression of *orf10* mRNA could be due to a combination of a weak promoter and tight autoregulation. For that reason, S1 mapping analysis was attempted as described in Materials and Methods, using total RNA extracted from both *orf10*-disrupted and *orf10*-deleted mutants of *S. lividans*. While no protected product was observed with RNA of the former strain, a 152-bp protected fragment was detected (Fig. 6A) with RNA isolated from the *orf10*-deleted mutant. Thus, the *orf10* transcription initiation site was localized one base upstream of its putative start codon, with no room for a ribosome binding site. The lack of a protected fragment in the *orf10* disruption mutant suggests that the truncated $Orf10_{1-274}$ might be still functional in regulating its own transcription.

The transcription start point of *orf11*, unlike that of *orf10*, was easily determined as described in Materials and Methods by using RNA extracted from *S. lividans* strains (Fig. 6B) and located at nucleotide position 3151. A similar initiation point

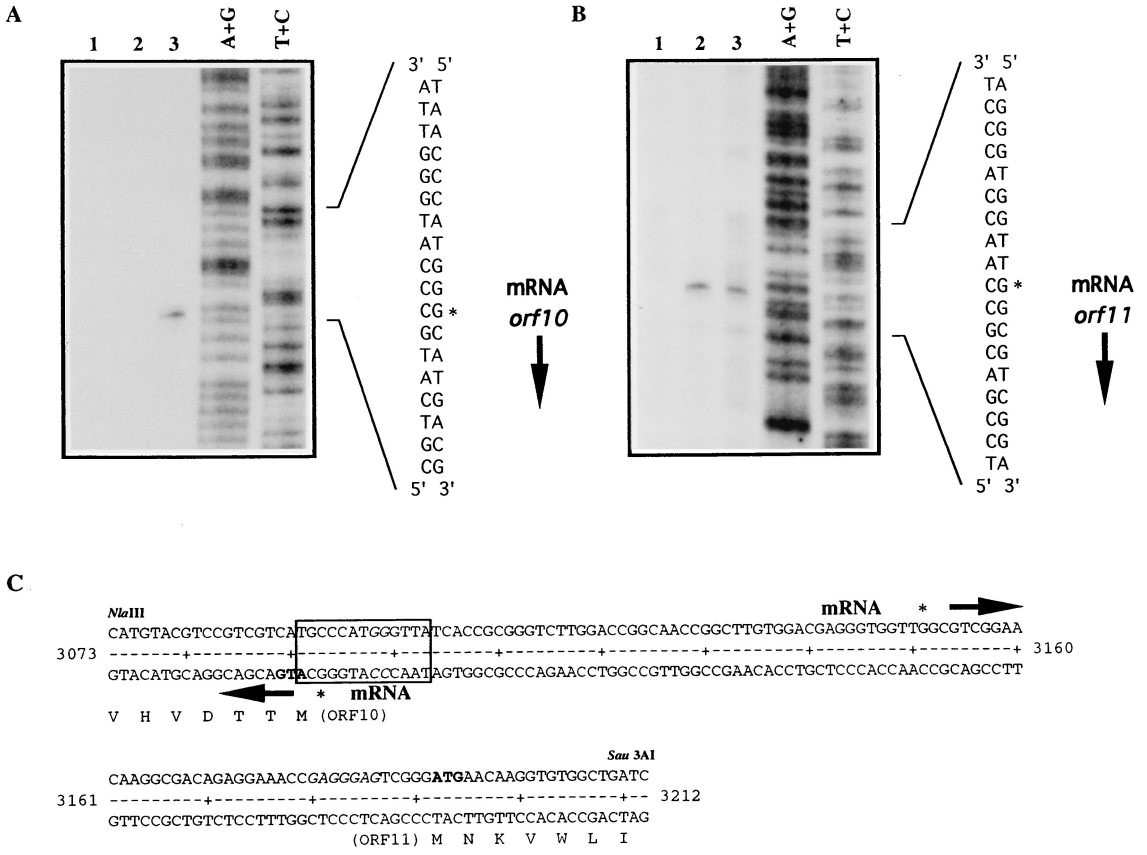

FIG. 6. Transcriptional analysis of *orf10* (A) and *orf11* (B) genes. Lanes: 1, *E. coli* tRNA; 2 and 3, total RNA extracted from 3-day-old cultures of *S. lividans* TK21 and CNB073, respectively; A+G and T+C, Maxam-and-Gilbert sequence ladders of the corresponding labeled probes. Asterisks indicate the most probable transcription start sites. (C) DNA sequence within the *orf10-orf11* intergenic region. *orf10* and *orf11* mRNAs are initiated at the indicated nucleotides. Arrows indicate direction of transcription. Amino acids are represented in single-letter code below the DNA sequence. The boxed sequence represents the $TN_{11}A$ consensus motif for LysR-type regulators contained in the DNase I-protected region. The GG nucleotides mutated to AT within this region are shown in italics.

was obtained with RNA isolated from *S. coelicolor* J1501 (data not shown). Based on S1 mapping results, neither the *orf10* nor the *orf11* promoter displays typical −10 and −35 regions (Fig. 6C). Additionally, the proximity of *orf10* and *orf11* initiation sites (58 bp) indicates that the −35 regions of the two promoters must overlap. As shown in Fig. 6B, we detected no change in *orf11* transcript level when analyzing total RNA extracted from *S. lividans* CNB073, suggesting that Orf10 protein might be required for the activation of *orf11* transcription, in agreement with the complementation results of *actIII* mutation.

Expression and purification of *S. coelicolor* Orf10 protein. To gain some insight into the biological activity of the *orf10* product, the protein was expressed from *E. coli* and purified (see Materials and Methods). Plasmid pCNB019, in which *orf10* is under the control of lambda p_L, was constructed and used to transform *E. coli* K12ΔH1Δ*trp*. As shown in Fig. 7 (lane 4), a whole-cell extract from an induced culture of this strain harboring pCNB019 showed by SDS-PAGE analysis a 34-kDa protein which corresponds to the predicted apparent molecular mass for the recombinant Orf10 protein. Such a protein was apparently not present in samples from uninduced or induced cultures of *E. coli* carrying only the plasmid vector (lane 1 or 2, respectively), as well as from uninduced cultures from the same recombinant strain (lane 3). Orf10, mostly in inclusion bodies, was then purified by urea solubilization followed by heparin-agarose chromatography (see Materials and Methods). The Orf10 protein was purified to 98%, as judged by SDS-PAGE analysis (Fig. 7, lane 7).

Gel mobility shift assays with the recombinant Orf10 protein. The DNA binding activity of Orf10 protein was tested by band shift analysis. A 316-bp *Esp*I-*Ava*I fragment containing the intergenic *orf10-orf11* region (Fig. 1), uniquely labeled at the 5′ end of the *Esp*I site (nucleotide 2981), was used as the probe. With this fragment, the Orf10 protein showed band shift activity which was dependent on Orf10 protein concentration (Fig. 8). Complete retardation was obtained with 25 ng of the Orf10 protein (Fig. 8, lane 9); the activity was lost partially with use of a fivefold molar excess of the unlabeled fragment (lane 10) and completely when a temperature-denatured Orf10 protein was used (lane 11). No dependence on divalent cations, such as Mg^{2+}, Mn^{2+}, and Ca^{2+} or K^+ (tested up to 200 or 500 mM, respectively), was found for the DNA binding activity of Orf10 (data not shown). It is worth noting that unlike the crude extracts prepared from uninduced and induced cultures of *E. coli* harboring the vector plasmid (Fig. 8, lanes 2 and 3), those from the respective cultures of *E. coli* transformed with pCNB019 caused the appearance of a shifted band (Fig. 8, lanes 4 and 5, respectively). This behavior may be due to the presence of either truncated forms of Orf10 or misfolded protein still retaining the DNA binding activity.

To locate the binding region within the *orf10* promoter region, the *Esp*I-*Ava*I (nucleotides 2981 to 3297) probe was

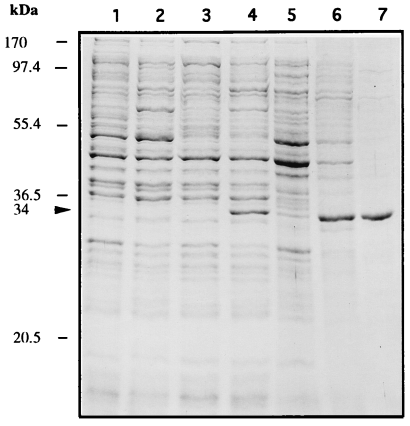

FIG. 7. SDS-PAGE analysis of *orf10* expression in *E. coli* and at various stages of its purification. Lanes: 1 and 2, whole-cell extracts from cultures of *E. coli* K12ΔH1Δ*trp* carrying the vector plasmid pAZe3ss, grown at 30 and 42°C, respectively; 3 and 4, whole-cell extracts from cultures of the same strain harboring plasmid pCNB019, grown at 30 and 42°C, respectively; 5, supernatant of 25,000 × *g* centrifugation; 6, pellet of this centrifugation (inclusion bodies) after the refolding step; 7, heparin-agarose chromatography (1 μg of protein). The recombinant purified Orf10 protein is indicated by arrowheads. The positions and molecular masses of marker proteins are shown on the left.

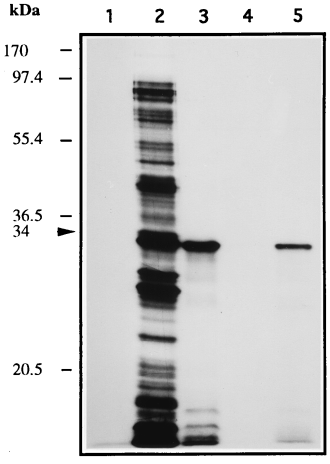

FIG. 9. Synthesis of ^{35}S-labeled Orf10 protein. Samples were analyzed by SDS-PAGE and autoradiography as described in Materials and Methods and include whole-cell extract from a culture of *E. coli* K38/pGP-1-2 carrying plasmid pT7.7 grown at 42°C with rifampin addition (lane 1), whole-cell extract from a culture of *E. coli* K38/pGP-1-2 carrying plasmid pCNB023 grown at 42°C without and with rifampin addition (lanes 2 and 3, respectively), and supernatant of 25,000 × *g* centrifugation (lane 4) and its pellet (inclusion bodies) after the refolding step (lane 5). Positions of size markers are indicated on the left. The 34-kDa labeled protein is labeled with an arrow.

shortened by digestion with restriction enzymes *Sau*3AI, *Ava*II, and *Nco*I at nucleotide positions 3242, 3118, and 3094, respectively. Both *Esp*I-*Sau*3AI and *Esp*I-*Ava*II fragments still retained the band shift activity, while no band retardation was observed with the *Esp*I-*Nco*I probe (data not shown). Thus, the interacting region of Orf10 may lie between positions 3094 and 3118 of the *orf10-orf11* promoter region.

The truncated Orf10$_{1\text{-}274}$, expressed in *E. coli* and purified as described in Materials and Methods, was also tested for DNA binding activity. A shifted band was detected with the recombinant Orf10$_{1\text{-}274}$ (data not shown), in agreement with the previous suggestion (see above). Interestingly, the retarded band migrated similarly to those shown for crude extracts from both uninduced and induced cultures of *E. coli* carrying the wild-type *orf10* gene (Fig. 8, lanes 4 and 5, respectively). Additionally, the purified Orf10 protein from *S. lividans* showed the same activity as that of *S. coelicolor*.

Because of the correlation between actinorhodin activation and expression of the *orf10* gene, we next explored possible targets of the Orf10 protein within the promoter regions of some of the *act* genes. ^{35}S-labeled Orf10 protein was obtained by using a T7 RNA polymerase expression system (see Materials and Methods). SDS-PAGE and autoradiography analysis revealed a high-abundance labeled protein of the size predicted for Orf10 (Fig. 9). This protein was purified from inclusion bodies (Fig. 9, lane 4) and assayed for DNA binding activity (Fig. 10). As expected, either a 246-bp *Nru*I-*Ava*I or a

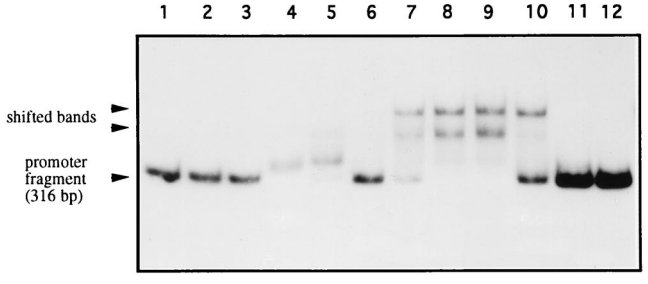

FIG. 8. DNA binding assays. Gel mobility shift analysis with the *orf10-orf11* intergenic region was performed as described in Materials and Methods, using the 316-bp *Esp*I-*Ava*I fragment as the probe. Lanes: 1 and 12, without protein addition; 2 and 3, with crude extracts from cultures of *E. coli* K12ΔH1Δ*trp* carrying the control plasmid pAZe3ss, grown at 30 and 42°C, respectively; 4 and 5, with crude extracts from cultures of the same strain harboring plasmid pCNB019, grown at 30 and 42°C, respectively; 6 to 9, with 1.2, 2.5, 12.5 and 25 ng of the purified *S. coelicolor* Orf10 protein, respectively; 10, with 25 ng of Orf10 and fivefold molar excess of unlabeled *orf10-orf11* intergenic region; 11, with 25 ng of Orf10 previously treated for 15 min at 100°C.

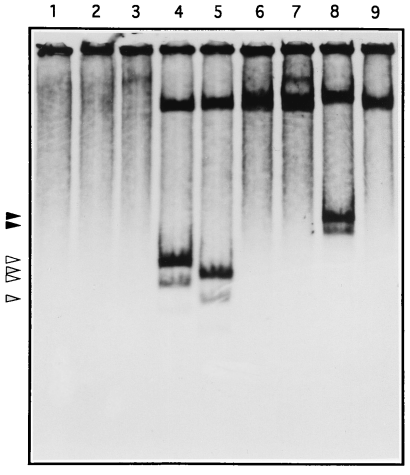

FIG. 10. Analysis of DNA binding activity of ^{35}S-labeled Orf10 purified from inclusion bodies. The binding reaction was carried out as described in Materials and Methods, and products were analyzed by native PAGE and autoradiography. Lanes: 1, in the absence of DNA; 2, with *Eco*RI-digested pUC19; 3, with *Eco*RI-digested M13mp18; 4, with the 246-bp *Nru*I-*Ava*I fragment within the *orf10-orf11* intergenic region (pCNB033 insert); 5, with the 206-bp *Aha*II fragment within the *orf10-orf11* intergenic region (pCNB034A insert); 6 and 7, with the 393-bp *Pst*I-*Sac*II and 227-bp *Sma*I-*Hin*cII fragments containing direct repeat sequences at the 3′ ends of *orf10* and *orf11*, respectively; 8, with the 484-bp *Esp*I-*Xho*I fragment within the *orf10-orf11* intergenic region containing the *Nde*I-engineered restriction site; 9, with the 455-bp *Mbo*II fragment within the *actIII-actI* intergenic region. Arrows indicate mobilities of the complexes between Orf10 protein and its DNA target.

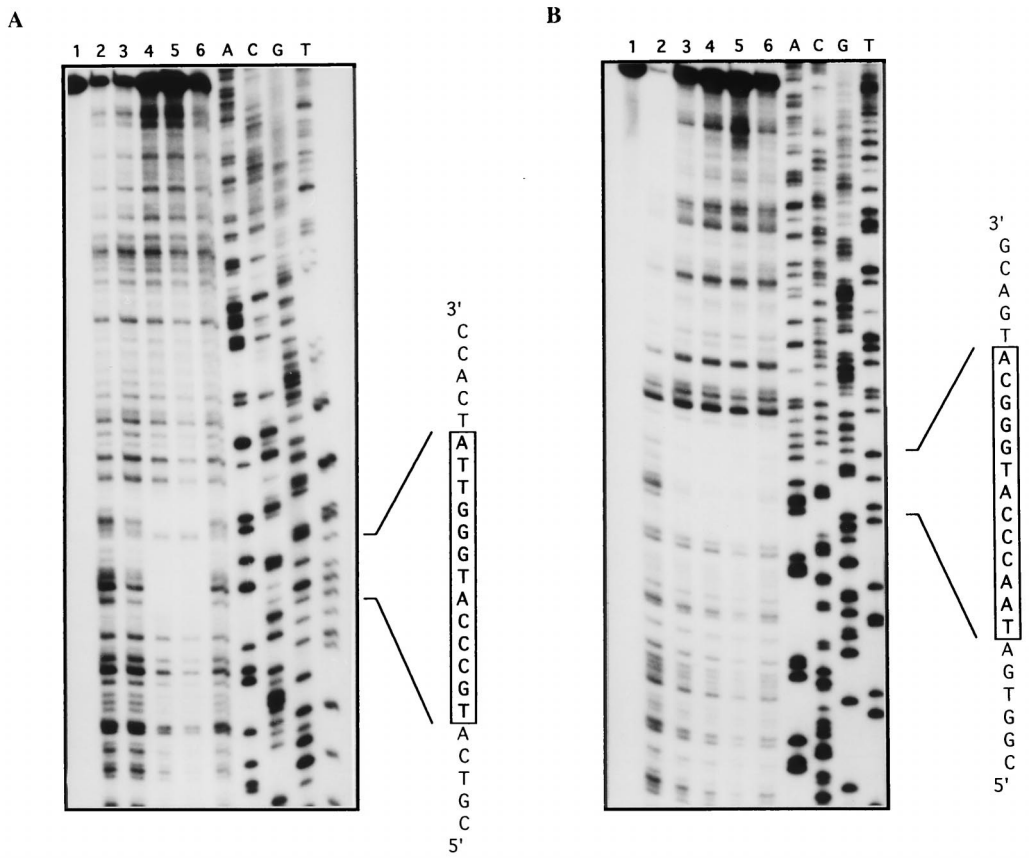

FIG. 11. DNase I footprinting analysis of the interactions between Orf10 protein and the *orf10-orf11* intergenic region. Upper (A) and lower (B) strands correspond to pCNB033 and pCNB034A inserts. DNase I protection experiments were done as described in Materials and Methods. Lanes: 1, without DNase I; 2, with DNase I but no Orf10; 3 to 5, with 20, 200, and 600 ng of Orf10, respectively; 6, with 600 ng of Orf10 and 10-fold molar excess of unlabeled *orf10-orf11* intergenic region. The corresponding sequence reactions (lanes A, C, G, and T) were run in parallel. The boxed regions correspond to the $TN_{11}A$ consensus motif for LysR-type regulators.

206-bp *Aha*II fragment within the intergenic *orf10-orf11* region showed DNA binding activity (Fig. 10, lanes 4 and 5). However, no such activity was observed with any of the DNA fragments tested from the *act* cluster (data not shown; *actIII-actI* intergenic region [Fig. 10, lane 9]), as well as those containing the direct repeats at the 3′ end of both *orf10* and *orf11* (Fig. 10, lanes 6 and 7). Additionally, a 484-bp *Esp*I-*Xho*I fragment (nucleotides 2981 to 3465) within the *orf10* promoter, in which nucleotides GG (at positions 3098 and 3099) had been mutated to AT to generate an *Nde*I restriction site, retained binding activity (Fig. 10, lane 8).

DNase I footprinting. The interaction between Orf10 and its DNA target within the *orf10-orf11* intergenic region was analyzed by DNase I protection assays carried out as described in Materials and Methods. The protected region in either strand (Fig. 11) was shown to expand over a 30-bp region and included the sequence $TN_{11}A$, described as a general anchor for LysR-type proteins (41) (nucleotide positions 3091 to 3103) (Fig. 6C). The DNA binding site of Orf10 protein was shown to be located within the *orf10* transcriptional start point, suggesting a direct competition between this transcriptional regulator and the RNA polymerase as a mechanism for autoregulation. It is interesting that the mutated bases (GG to AT to generate an *Nde*I restriction site) lie within the $TN_{11}A$ consensus motif (Fig. 6C), and as shown above, these mutations did not seem to affect Orf10 DNA binding activity. Similar DNase I-protected boxes were obtained when either the *S. coelicolor* $Orf10_{1\text{-}274}$ protein or the *S. lividans* Orf10 protein was used (data not shown).

DISCUSSION

An additional regulatory gene, *orf10*, negatively controlling actinorhodin production in *S. lividans* has been isolated and characterized; this gene is located downstream of the right-hand end of the *S. coelicolor* actinorhodin biosynthetic cluster. Linked to and divergently transcribed from this new regulator is a gene, *orf11*, whose deduced product is homologous to the SDR family of proteins (27). Moreover, the ActIII protein (which is implicated in the formation of actinorhodin) as well as other oxidoreductases involved in the synthesis of several other antibiotics belong to this group of proteins.

The nature of Orf10 protein as a transcriptional regulator was inferred from its homology to other proteins belonging to the LysR family. Members of this large family are implicated in the regulation of apparently unrelated metabolic pathways and share several common features (41): (i) a highly conserved HTH motif near the N terminus implicated in DNA binding; (ii) the involvement of a coinducer in regulatory activity; (iii) a divergent promoter structure; and (iv) negative autoregulation. Additionally, a consensus DNA sequence, $TN_{11}A$, has been proposed as binding site for the LysR-type regulators (41). In this study, we provide evidence that Orf10 may exhibit some (if not all) of the characteristics of the proteins of the LysR family.

Database comparisons indicated similarities of Orf10 with LysR-type regulators, particularly within the N-terminal region, where the HTH motif lies. This might be expected, since the central and C-terminal regions of these proteins have been reported to be implicated in several functions, including the recognition of, and response to, a coinducer (41). By gel retardation and DNase I footprinting analysis, Orf10 was shown to be able to bind to a DNA target (*orf10-orf11* intergenic region) which includes a putative $TN_{11}A$ consensus motif. In agreement with this result, a C-terminally truncated Orf10, $Orf10_{1-274}$, retained DNA binding activity.

Regarding the control by Orf10 of the divergently transcribed *orf11*, two observations suggest that Orf10 can regulate *orf11* expression. First, in most cases, the target genes for LysR-type regulators are located next to them and possess a conserved divergent promoter structure with respect to the regulatory gene; such organization may well be found for the *orf10-orf11* system. Second, complementation of the *actIII* mutation requires the presence of both *orf10* and *orf11*, while it was not achieved when *orf10* carried a 3′-end deletion. Although potentially expressed, $Orf10_{1-274}$ may be unable to activate *orf11* transcription. The presence of detectable levels of *orf10* transcript only in *orf10*-deleted and not in wild-type or *orf10*-disrupted mutant strains constitutes evidence that the *orf10* product negatively regulates its own transcription. However, it should be stressed that additional factors may be involved in the regulation of *orf10* expression, as reversion of the *orf10* mutant phenotype occurred only by *cis* complementation.

Mutations (by disruption or deletion) of *orf10* induce actinorhodin production in *S. lividans*. This blue-pigmented phenotype appeared to be caused by an increase in transcription of the pathway-specific regulatory gene *actII-orf4*, although the mechanism(s) involved in this activation is not known. In this sense, no binding activity of Orf10 to any of the promoter regions of the *act* cluster was detected; still, the requirement of a coinducer for this activity as well as for in vivo inhibition of actinorhodin biosynthesis cannot be excluded and is currently being investigated. Alternatively, Orf10 may regulate an unknown transcriptional factor that, in turn, negatively controls *actII-orf4* gene expression, thus leading to induction of actinorhodin production in *orf10*-blocked mutants.

Results of complementation experiments using the *S. coelicolor actIII* mutant revealed that the *orf11* gene allowed restitution of a blue-pigmented phenotype (although only in the presence of the entire *orf10* gene). It is worth noting that complementation of this strain by *gra-orf5* and *gra-orf5-orf6* gene products (42), which are also oxidoreductases of the SDR family, has been observed. Furthermore, the *actIII* product has been shown to function heterologously in an anthracycline system (2). Although the *orf11* gene is able to complement the *actIII* mutation, it does not seem to play a role in the actinorhodin biosynthetic pathway, as *orf11* disruptions did not generate a mutant phenotype; the observed complementation might be explained by the small ketoreductase activity of Orf11 protein, which would be increased significantly by extra copies of the gene and/or its activation by the *orf10* gene product. It should be noted that *orf11* transcription in the *actIII* mutant strain carrying *orf10* and *orf11* showed a threefold increase (33). Considering that the *orf10-orf11* system appeared to be strictly regulated, these two genes, despite being active, may not be sufficient to overcome the ActIII deficiency when expressed from a single chromosomal copy.

The presence of direct repeat sequences downstream of *orf10* and *orf11* suggests a role for those sequences in recombination events. Those sequences may have played a role in mediation of chromosomal rearrangement or in duplication of DNA segments.

ACKNOWLEDGMENTS

We are pleased to acknowledge D. Holmes, M. Zalacain, J. Hodgson, and S. Elson for valuable discussions throughout this study and W. Wohlleben for the gift of plasmid pGM9.

This research was supported by grants from the Spanish CICYT (95-0104-OP-02-01 and BIO96-1168-C02-01) and by Smith-Kline Beecham S.A.

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