A polyketide biosynthetic gene cluster from *Streptomyces antibioticus* includes a LysR-type transcriptional regulator

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In the search for Type II polyketide synthases (PKSs) a DNA fragment was isolated from *Streptomyces antibioticus* ATCC 11891 (a producer of oleandomycin). DNA sequencing of the cloned fragment revealed six complete ORFs whose deduced products showed similarities to those of other genes known to be involved in polyketide biosynthesis. Several *S. coelicolor* strains mutated in different steps of actinorhodin biosynthesis (*actI*, *actIII*, *actV_A* and *actVII*) were complemented by the cloned genes, suggesting that the isolated genes encode an aromatic polyketide of unknown structure and function. The cluster also contains a putative LysR-type transcriptional regulator (ORF0), which controls PKS gene expression in a heterologous host. DNA binding assays and transcriptional analysis suggest that the pathway-specific regulator for actinorhodin biosynthesis (*actII*-ORF4) is also involved in the expression of the cloned PKS in the host strain.

Keywords: *actII*-ORF4, actinorhodin, antibiotic, heterologous complementation, regulation

INTRODUCTION

Streptomyces spp. are soil bacteria with the ability to produce many biologically active products. Many of these compounds have important applications in human medicine (as antibacterial, antitumour or antifungal agents, immunomodulators, etc.) and in agriculture (as growth promoters and agents for plant protection amongst others). Some of them belong to a large class of metabolites, polyketides, that share with fatty acids a common pattern of biosynthetic steps via the successive condensation of simple carboxylic acid metabolites. The key enzyme in this pathway is polyketide synthase (PKS). Due to the possibility of using different starter and extender units and the introduction or not of reductive and cyclization steps, the end products can have a great variety of chemical structures. Two structural types of complex enzymes (Type I multifunctional proteins and Type II multienzyme complexes)

can be found among the different PKSs (for a review, see Hopwood, 1997). Several sets of genes for PKSs have been cloned and sequenced, for example actinorhodin (Malpartida & Hopwood, 1984), daunorubicin (Stutzman-Engwall & Hutchinson, 1989; Otten *et al.*, 1995), granaticin (Sherman *et al.*, 1989; Ichinose *et al.*, 1998), tetracenomycin (Motamedi & Hutchinson, 1987; Decker & Hutchinson, 1993), mithramycin (Lombó *et al.*, 1996, 1997) and landomycin (Westrich *et al.*, 1999).

The clusters encoding different metabolic steps for a particular biosynthetic pathway usually include specific regulatory genes. In most cases such regulatory genes are positive regulators, such as those for actinorhodin (*actII*-ORF4; Fernández-Moreno *et al.*, 1991), undecylprodigiosin (*redD*; Narva & Feitelson, 1990), daunorubicin (*dnrI*; Stutzman-Engwall *et al.*, 1992) and mithramycin (*mtmR*; Lombó *et al.*, 1999), among others. These pathway-specific regulators have been recently grouped within the SARP family (Wietzorrek & Bibb, 1997), a group of *Streptomyces* regulatory proteins involved in the transcriptional regulation of antibiotic biosynthetic genes. The pathway-specific regulator is also interconnected with other aspects of *Streptomyces* physiology, such as morphological differentiation or carbon metabolism (Bibb, 1996; Chakraborty *et al.*,

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Abbreviation: PKS, polyketide synthase.

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1996; Martínez-Costa *et al.*, 1996). The mechanisms to globally regulate these complex processes in *Streptomyces* have been a topic for extensive analysis in several laboratories, giving rise to the isolation of several structurally different genes, such as *bldA* (Lawlor *et al.*, 1987), the two-component systems *afsR/afsK* (Hong *et al.*, 1991) and *afsQ1/afsQ2* (Ishizuka *et al.*, 1992), and *abaB* (Scheu *et al.*, 1997).

The characterization of genes encoding polyketide biosynthesis is interesting for two reasons: on the one hand, the cloned biosynthetic genes can be useful to make libraries for combinatorial biosynthesis (Hopwood, 1997); on the other hand they might provide tools to overcome one of the limiting steps for antibiotic production, such as the control gene(s) for antibiotic biosynthetic pathways (Strauch *et al.*, 1991). This paper describes the isolation and some preliminary characterization of a cluster of polyketide biosynthetic genes from *Streptomyces antibioticus* and a transcriptional regulatory gene linked to the cluster. We believe that these studies on the understanding of the basis of the regulation of antibiotic production in *Streptomyces* could contribute to improve our knowledge of this complicated control network.

METHODS

Bacterial strains. The *Escherichia coli* strains used were JM101 (Yanisch-Perron *et al.*, 1985) and K12Δ H1Δtrp (Zaballos *et al.*, 1987) for subcloning and as expression host, respectively. *Streptomyces antibioticus* ATCC 11891 was used as a source of DNA for making the gene library. '*Streptomyces lividans*' TK21 (Davis & Chater, 1990) was used as primary recipient for the cloned genes and *Streptomyces coelicolor* strains (listed in Table 1) were used for functional characterization of the cloned biosynthetic genes.

Plasmids and bacteriophages. The *E. coli* vectors used were pUC18 and pUC19 (Yanisch-Perron *et al.*, 1985) for subcloning and pAZE3ss (Zaballos *et al.*, 1987) for expression. The *E. coli* M13mp18 and M13mp19 derivative phages (Yanisch-Perron *et al.*, 1985) were used for DNA sequencing. The gene library was constructed in λ EMBL4 (Frichauf *et al.*, 1983).

The *Streptomyces* plasmid vector was the low-copy-number SCP2 derivative pIJ941 (Lydiat *et al.*, 1985); its derivatives were those depicted in Fig. 1. pVR22 was constructed in

several steps. First the *EcoRI* (3)–*BamHI* (5) fragment was cloned in pUC19, the 264 bp internal fragment was removed by digesting the resulting plasmid with *EspI* and *BstEII*, end-filled with the Klenow fragment of DNA polymerase and finally ligated. In the second step, the entire chromosomal insert of *ant1* was rescued by partial digestion with *EcoRI* and cloned into the *EcoRI* site of pUC18; its *XbaI* (4)–*XbaI* (polylinker fragment of pUC18) sequence was used to replace the *XbaI* (4)–*BamHI* (5) fragment of the construction obtained in the first step, giving rise to a final recombinant plasmid carrying the truncated ORF0 and ORFs 1–4. This plasmid was then cloned into pIJ941.

Construction of a gene library. Total DNA from *S. antibioticus* was partially digested with *Sau3A* and size-fractionated using a sucrose gradient. Fractions with a mean size between 16 and 25 kb were pooled and ligated into λ EMBL4 (Frichauf *et al.*, 1983). The ligation mixture was *in vitro*-packaged using the Promega λ DNA Packaging System, following the manufacturer's recommendations.

DNA sequencing. DNA sequencing was carried out by using the dideoxy-chain termination method (Sanger *et al.*, 1977). We used the 7-deaza-dGTP reagent kit from USB using the conditions recommended by the manufacturer. Convenient DNA fragments were previously cloned in either M13mp18 or M13mp19 vectors from suitable restriction fragments. Identification of DNA sequence in S1 protection experiments was carried out by the Maxam & Gilbert (1980) method.

Computer analysis of sequences. The DNA sequence was analysed using the software programs of the University of Wisconsin Genetic Computer Group (Devereux *et al.*, 1984). For analysis of ORFs we used CODONPREFERENCE with a codon usage table made from 100 *Streptomyces* genes (Wright & Bibb, 1992). Comparisons of sequences were made against the EMBL nucleic acid database and the SWISS-PROT database by using FASTA and TFASTA.

DNA and RNA manipulations. For isolation, cloning and manipulation of nucleic acids, the methods of Hopwood *et al.* (1985) were used for *Streptomyces* spp. and of Maniatis *et al.* (1982) for *E. coli*. For high-resolution S1 mapping, the method of Murray (1986) was used. The growth medium was liquid SY inoculated with pre-germinated spores (Hopwood *et al.*, 1985); RNA preparations were made at several time points as indicated.

Expression of ORF0 in *E. coli*. ORF0 protein was overproduced in *E. coli* by using the pAZE3ss expression system (Zaballos *et al.*, 1987). The translation start codon was changed by using PCR. PCR amplifications (Saiki *et al.*, 1988) were carried out with an oligonucleotide carrying an *NcoI* site at the ATG

Table 1. *S. coelicolor* strains used in this work

Strain	Mutant alleles and plasmids status	Reference
TK17	<i>proA1 argA1 strA1 actI-118 redC-1 SCP2⁻</i>	Feitelson & Hopwood (1983)
TK18	<i>uraA1 argA1 strA1 redE-60 actIII-141 redC-1 SCP1⁻ SCP2⁻</i>	Feitelson & Hopwood (1983)
TK16	<i>argA1 guaA1 actIV-117 redA-59 SCP1⁻ SCP2⁻</i>	Feitelson & Hopwood (1983)
JF4	<i>proA1 argA1 uraA1 strA1 redE-60 actV_A-109 SCP1⁻ SCP2⁻</i>	Feitelson & Hopwood (1983)
JF1	<i>argA1 guaA1 redD-42 actII-177 SCP1⁻ SCP2⁻</i>	Feitelson & Hopwood (1983)
J1501	<i>bisA1 uraA1 strA1 SCP1⁻ SCP2⁻ pgl⁻</i>	Chater <i>et al.</i> (1982)
MAFM0195	<i>bisA1 uraA1 strA1 SCP1⁻ SCP2⁻ pgl⁻ ΔactI-ORF1</i>	Martínez-Costa <i>et al.</i> (1996)
MAFM218	<i>bisA1 uraA1 strA1 SCP1⁻ SCP2⁻ pgl⁻ Δact</i>	Arias <i>et al.</i> (1999)

Fig. 1. Restriction map of the cloned PKS cluster. The sequenced region is shown as a hatched bar; the arrangement of the different ORFs is given below the restriction map of the enlarged region. DNA fragments (thick lines) cloned in pIJ941 are placed under the corresponding regions. The thin line in pVR22 shows the deleted region. At the bottom the organization of the 'early' and 'intermediate' *act* genes is shown for comparison with the cloned region.

translation start site and Thermostase (Promega) using the conditions suggested by the manufacturer. The engineered ORF0 gene was cloned into pAze3ss to generate pVE3 and transformed into the recipient *E. coli* strain (Zaballos *et al.*, 1987). Overnight cultures grown at 30 °C in LB medium were used to inoculate fresh medium at a dilution rate of 2%. After 1 h the cultures were induced by shifting the temperature to 42 °C and continuing growth for 2–3 h. Almost all of the ORF0 protein from the induced culture (estimated to be nearly 20% of the total protein) was found in inclusion bodies. The cells were harvested by centrifugation and washed with a solution containing 10 mM EDTA, 100 mM NaCl, 0.5 mM PMSF, 10% glycerol, 1 mM mercaptoethanol and 50 mM Tris/HCl, pH 8. The cells were resuspended in the same buffer and disrupted by sonication. Unbroken cells and heavy materials were fractionated by a preliminary centrifugation at 1000 g. The insoluble material was recovered from the supernatant by centrifugation at 12000 g. The pellet was washed once with a solution containing 1.5 M urea, 0.1 M Tris/HCl buffer, pH 8, and finally solubilized with a solution containing 4.5 M urea and 0.1 M Tris/HCl, pH 8. Most of the solubilized protein was ORF0, which was renatured by dialysis against a solution containing 1 M NaCl, 0.005 M DTT, 10%

glycerol and 0.05 M Tris/HCl, pH 8. This material was used for further analysis.

Gel retardation assays. The probes were end-labelled with [α - 32 P]dCTP and Klenow fragment. Labelled DNA (2000 c.p.m.) was incubated for 15 min at room temperature with the purified ORF0 protein. The incubation mixtures contained (in a total volume of 20 μ l) 0.3 M NaCl, 0.015 M Tris/HCl, pH 8, 0.0015 M DTT, 1 μ g poly-dIdC and 35% glycerol with the indicated amount of ORF0 protein. Protein–DNA complexes and free DNA were resolved on 5% polyacrylamide gels. After electrophoresis, the gels were dried and analysed by autoradiography.

DNase I footprinting. The *EcoRI* (3)–*RsaI* (3.2) fragment was used as probe. The fragment was previously cloned in M13mp18 and M13mp19 to obtain both strands. The direct primer (USB) was labelled with [γ - 32 P]ATP and with the cold reverse primer used to amplify by PCR. The reactions were carried out in 60 μ l of the mixture described above for the gel retardation assays. After incubation, 50 ng DNase I was added to each reaction mixture. The DNA was precipitated and applied along with the dideoxy DNA sequencing ladders to a 6% polyacrylamide gel. Sequencing reactions on the same

probes were also applied to the gels. After electrophoresis, the gels were dried and analysed by autoradiography.

RESULTS

Cloning and DNA sequence of polyketide biosynthetic genes

The gene library constructed in λ EMBL4 (see Methods) was screened using pIJ2345 as probe (a pBR322-derived vector, containing the PKS from the actinorhodin cluster; Malpartida & Hopwood, 1984). Two overlapping clones (*ant1* and *ant3*) were isolated. These clones cover a chromosomal region of 17 kb from *S. antibioticus* (Fig. 1). The region hybridizing to the actinorhodin PKS was located and the DNA sequence of a 7 kb fragment was determined. Computer analysis of the DNA sequence revealed a set of 5 ORFs transcribed in the same direction from left to right (ORF0–4; Fig. 1), flanked by two ORFs oriented in the opposite direction (ORF6 and ORF5 at the left and right hand ends, respectively). ORF1 appears to be translationally coupled with ORF2, overlapping by 4 bp. ORF2, 3, 4, 5, A and 0 are preceded by putative ribosome-binding sites. The corresponding molecular masses of the deduced products would be (kDa): ORF1 (423 aa), 44.7; ORF2 (405 aa), 42.3; ORF3 (86 aa), 9.1; ORF4 (318 aa), 34.7; ORF5 (261 aa), 27.5; ORF0 (308 aa), 32.4. ORF6 was truncated and only its N terminus was sequenced. There is a 48 bp untranslated segment between ORF2 and ORF3, 106 bp between ORF3 and ORF4, and 1168 bp between ORF0 and ORF1 (Fig. 1).

Comparison of the deduced products of the ORFs with sequences in the databases showed high similarities with proteins known to be involved in the early steps of polyketide biosynthesis. ORF1, ORF2 and ORF3 show strong similarities with gene products related to either fatty acid or polyketide biosynthesis from different organisms, and could correspond to ketosynthase (or KS α), chain length factor (or KS β) and acyl carrier protein, components of a 'minimal' PKS (McDaniel *et al.*, 1994). The best scores were obtained with the *gra* (87, 61 and 65% identity to ORF1, ORF2 and ORF3, respectively; Sherman *et al.*, 1989), *act* (76, 59 and 50% identity, respectively; Fernández-Moreno *et al.*, 1992) and *tcm* (69, 50 and 46% identity, respectively; Bibb *et al.*, 1989) homologues. ORF2 and ORF3 contain conserved motifs (encoding cysteine and glutamine, respectively) that have been reported as active site motifs in homologous ketosynthase and chain length factors (Bisang *et al.*, 1999). The translated product of ORF4 shows end-to-end similarities with Gra-ORF4 (60% identity) and ActI-ORF4 (49% identity; aromatasases) (Sherman *et al.*, 1991; Fernández-Moreno *et al.*, 1992). The N-terminal sequence of ORF6 also shows similarities to the dehydrase domain of the same aromatasases. The translated product of ORF5 revealed strong similarities with the products of the *actIII* (72%; Hallam *et al.*, 1988) and *gra*-ORF5 (84%; Sherman *et al.*, 1989) genes and is thus likely to encode a ketoreductase (Hallam *et al.*, 1988; Bartel *et al.*, 1990).

The deduced product of ORF0 has significant similarities to members of the LysR family, known as transcriptional regulators (Schell, 1993). The members of this family have high similarities within the N-terminal domain – they contain a characteristic helix–turn–helix DNA-binding domain (which is present in ORF0) – but lower, if any, similarities along the C-terminal domain (involved in the binding to different ligands). Nevertheless, ORF0 shows 30% identity end-to-end to NodD proteins, which are involved in the recognition and response to flavonoids (members of the polyketide family; Downie & Johnson, 1986).

Expression and complementation analysis

To characterize the putative function of the cloned genes, the DNA fragments from the λ EMBL4 vector, *ant1* and *ant3*, were cloned on the low-copy-number vector pIJ941 to produce pMH4 and pMH9, respectively. These plasmids were introduced by transformation into several *S. coelicolor act* mutants and the transformants were tested for actinorhodin production. pMH4 restored actinorhodin production in mutants TK17 (*actI*[−]), B-140 (*actVII*[−]) and JF4 (*actV_A*[−]), but not in TK18 (*actIII*[−]) and JF1 (*actII*[−]). pMH9 restored actinorhodin production in mutants TK17 (*actI*[−]) and TK18 (*actIII*[−]), but not in B-140 (*actVII*[−]), JF4 (*actV_A*[−]) and JF1 (*actII*[−]). The observed complementation with plasmids, after transformation of the corresponding *S. coelicolor* mutants, suggests that the cloned genes are involved in an unknown polyketide biosynthetic pathway.

For further characterization of the involvement of ORF0 in the expression of the cloned polyketide biosynthetic genes, several clones were constructed (see Fig. 1). The fragment containing ORF0–4 was subcloned into the low-copy-number vector pIJ941 to generate pVR20; pVR22 was generated by deleting from pVR20 the 264 bp *EspI* (3.6)–*BstEII* (3.7) fragment which is internal to ORF0 (see Methods). Both constructs were used to transform the *S. coelicolor actI* mutant MAFM0195 (see Table 1). Only pVR20 could restore actinorhodin production, suggesting that ORF0 is needed for expression of the cloned PKS, at least under heterologous conditions.

Transcription of the cloned genes

The transcriptional analysis of the cloned ORFs was determined by high-resolution S1 nuclease protection experiments. For ORF0 and ORF6 determination, total *S. antibioticus* RNA was hybridized with the 318 bp *EcoRI* (3)–*DdeI* (3.3) fragment (Fig. 1) labelled at either end. When the *EcoRI* (3) site was labelled, a 110 bp protected fragment was seen after S1 digestion (data not shown), while a 185 bp fragment was protected when the *DdeI* (3.3) site was labelled. These results show that two divergent transcripts could be generated in this region, one beginning 30 nt upstream of the ORF6 start codon and the other 81 nt upstream of ORF0. This

Fig. 2. Transcriptional characterization of the cloned ORFs. (a) Determination of the transcription start point for ORF1. The start point is indicated by an arrow, as derived from Maxam & Gilbert sequencing reactions on the protected fragment. The RNA was extracted from a 20 h liquid culture of *S. antibioticus*. (b) Transcription pattern of ORF1 in several backgrounds. (i) Time course expression of the ORF1 transcript in *S. antibioticus*. RNAs were extracted from liquid cultures. (ii) Time course expression of ORF1 under heterologous conditions. The RNAs were extracted from *S. antibioticus* (SA) at 20 h and from *S. coelicolor* MAFM0195 carrying pVR20 (2) at the indicated time. (iii) Heterologous expression of the ORF1 transcript. RNAs were extracted from *S. coelicolor* MAFM0195, carrying pVR20 at 60 h (SA) and from the same strain carrying pVR22 at the indicated time. (iv) Heterologous expression of the ORF1 transcript in *S. coelicolor* lacking the *actI*-ORF4 gene. The RNAs were extracted from *S. antibioticus* (SA) at 20 h and from *S. coelicolor* MAFM218 carrying pVR20 at the indicated time.

arrangement is a typical feature which has been described for many other LysR-type transcriptional activators (Schell, 1993).

The transcriptional start point of ORF1 was determined by hybridizing total RNA with the 273 bp *SacI* (3.10)–*BclI* (4.1) fragment, labelled at the 5' *BclI* (4.1) site. A 185 bp protected fragment was obtained after S1 digestion, indicating the presence of a transcript initiating 70 nt upstream of the ORF1 start codon Fig. 2(a).

We further analysed the temporal expression of the cloned PKS gene by S1 nuclease protection experiments (Fig. 2b, i); the RNAs were obtained from cultures incubated at different times (see Methods). The probe was the 468 bp *SacI* (3.10)–*BamHI* (5) fragment labelled at the *BamHI* site. Maximal expression was detected at the earliest stages of growth, being almost undetectable after 36 h. This result shows that transcription of ORF1 is temporally regulated. The same pattern was observed

in *S. coelicolor* MAFM0195 carrying pVR20 (Fig. 2b, ii). Interestingly, no protected fragment was detected in the same strain carrying pVR22 (Fig. 2b, iii), suggesting that ORF0 is indeed involved in the regulation of transcription of the cloned PKS genes, in good agreement with the absence of complementation of the *actI* gene in this strain.

Characterization of the ORF0 protein

From the previous experiment it was clear that ORF0 was needed for expression of the cloned PKS genes. It was interesting to analyse the mechanism involved in the control of PKS expression more deeply. Thus, the ORF0 protein was purified from *E. coli* cultures to test its functional activity. The protein, purified from inclusion bodies, was analysed for its possible DNA-binding activity by gel retardation assays. The ORF0–A intergenic region was chosen as the target sequence

Fig. 3. Band-shift analysis to identify the target for the ORF0 protein within the ORF0-A intergenic region. The region was fractionated using the indicated restriction sites (lower part) and the resulting fragments (numbered) were resolved in a polyacrylamide gel (upper part; see Methods) without (–) or after previous incubation with 500 ng ORF0 protein (+).

because most of the LysR regulators interact with the DNA region of their own promoter and thus regulate their own transcription. A set of overlapping DNA fragments was labelled as indicated in Methods and tested for interaction with the ORF0 protein. As shown in Fig. 3 a band-shift is clearly detected, suggesting that the intergenic region could be a target for the ORF0 protein and that the protein is functional and probably correctly folded even after renaturation from the urea solubilization step.

To locate the site of interaction between the ORF0 protein and the ORF0-A intergenic region more precisely, the 208 bp *EcoRI* (3)–*RsaI* (3.2) fragment was end-labelled and used in footprinting analysis. After incubation as indicated in Methods, two regions protected against DNase I digestion (covering 17 and 26 nt, respectively) were observed (Fig. 4a). The regions are separated by 3 nt (Fig. 4b), a region hypersensitive to DNase I digestion. This finding suggests that the ORF0 protein would interact with this intergenic region. Similarly, the 1168 bp DNA fragment upstream of the PKS genes was tested as a possible target for the ORF0 protein in gel retardation assays. Surprisingly, no band-shift was detected with the promoter of the cloned PKS, suggesting that this DNA region is not a direct target for the ORF0 protein. This finding suggests that the ORF0

protein might have another target which would be needed for activation of the cloned PKS transcription.

The *actII*-ORF4 promoter as target for the ORF4 protein

The absence of interaction between the ORF0 protein and the PKS promoter suggests that the role of ORF0 in controlling PKS expression (as suggested by the complementation experiments) might be mediated by some other regulator. Accordingly, the ORF0 protein would activate such a putative regulator, the activation of which would result in expression of the PKS genes. The first obvious candidate was the pathway-specific positive regulator for actinorhodin biosynthesis (the *actII*-ORF4 gene). To test this idea, pVR20 was introduced by transformation into *S. coelicolor* PM218, a strain lacking most of the *act* cluster, including the *actII*-ORF4 gene (Fig. 1b). Total RNA was hybridized with the 273 bp *SacI* (3.10)–*BclI* (4.1) fragment (Fig. 2b, iv) which was previously used to determine the transcriptional start point of ORF1. No protected fragment was observed, indicating that the cloned PKS was not expressed in *S. coelicolor* in the absence of the pathway-specific regulator *actII*-ORF4. This finding strongly suggests that the expression of the cloned PKS, at least under heterologous conditions, is also dependent on the

Fig. 4. DNase I footprinting analysis to identify the target of the ORF0 protein. The conditions used for this activity are indicated in Methods. (a) Both strands, upper and lower (left and right panels, respectively) were tested. Lanes: 1, 1 μ g ORF0 protein; 2, 0.5 μ g ORF0; 3, no protein. (b) DNA sequence of the DNase I digestion-protected regions, represented by boxes. The beginning of the ORF0 and ORFA transcripts are indicated by arrows.

actII-ORF4 gene in addition to ORF0. If this assumption is correct, then the ORF0 protein indeed interacts with the *actII*-ORF4 promoter.

To explore this possibility, the DNA-binding activity of the ORF0 protein was tested within the promoter region of the *actII*-ORF4 gene. The gel mobility shift assays

Fig. 5. Band shift analysis of the ORF0 protein within the *actII-ORF4* promoter region. (a) Gel mobility shift assays were carried out with a 463 bp end-labelled DNA fragment and different amounts (ng as indicated above each lane) of the ORF0 protein. (b) Transcription initiation site of the *actII-ORF4* transcript. (c) Homology between *actII-ORF4* promoter and the regions protected against DNase I digestion in the ORF0-A intergenic region.

were carried out with a 463 bp end-labelled DNA fragment and different amounts of the ORF0 protein (Fig. 5a). The results clearly show that the ORF0 protein interacts with this region, suggesting that this might be another target for the ORF0 protein. Interestingly, immediately upstream of the -35 region of the *actII-ORF4* promoter (Fig. 5b) there is a region with partial homology to the target region of the ORF0 promoter (see Fig. 5c) which was previously determined in the DNase foot-printing assays. These sequences are not present within the PKS promoter. These results clearly suggest that an additional target for the ORF0 protein would be located immediately upstream of the *actII-ORF4* promoter and thus would result in activation of *actII-ORF4* transcription. It is noteworthy that ORF0, cloned in pIJ941, increased actinorhodin production in *S. coelicolor* J1501 by a factor of about two (data not shown).

From all these experiments, we can conclude that ORF0, at least under heterologous conditions, controls PKS expression through the *actII-ORF4* gene. Whether or not this scheme also applies in *S. antibioticus* needs further work.

DISCUSSION

A gene cluster was isolated from *S. antibioticus*, a strain known to produce the macrolide antibiotic oleandomycin and to contain the Type I PKS genes (Swan *et al.*, 1994). As in many other actinomycetes, *S. antibioticus* 11891 contains several *actI*-hybridizing fragments

(Malpartida *et al.*, 1987); the possibility that some of them are involved in the biosynthesis of well known, spore-associated pigments (Davis & Chater, 1990; Blanco *et al.*, 1992, 1993) cannot be ruled out. Nevertheless, the physical arrangement of the cloned gene cluster described in this paper, and the complementation pattern obtained with the *act* mutants, suggest that this gene cluster might well be involved in the production of some unidentified polyketide metabolite rather than the spore-associated pigment produced by the *whiE* cluster and its homologues. Whether or not this putative compound might have antimicrobial activity needs further analysis. The absence of an efficient method for transformation of *S. antibioticus* 11891 makes this question very difficult to resolve.

The work presented in this paper clearly shows that, in *S. antibioticus*, transcription of the PKS genes is temporally regulated, a feature similar to other PKS genes (Bibb, 1996). The PKS genes from *S. antibioticus* seem to be regulated by a LysR-type transcriptional regulator; the evidence presented in this paper strongly suggests that, in addition to this regulator, the control of PKS expression is also mediated by some other transcriptional regulator of the SARP family (Wietzorrek & Bibb, 1997), similar to *actII-ORF4*, because the ORF0 protein recognizes the *actII-ORF4* promoter region as a target. A similar LysR-type transcriptional regulator was found adjacent to the *act* cluster in *S. coelicolor* (Martínez-Costa *et al.*, 1999). It was shown to negatively control actinorhodin production in '*S. lividans*', but unlike the ORF0 protein, the LysR-type regulator of *S.*

coelicolor does not bind to the *actII*-ORF4 promoter region.

An interesting feature of LysR transcriptional regulators is that some of them behave as sensors of physiological changes and can bind directly to different kind of molecules. The strong similarities between the C terminus of the ORF0 protein and NodD domains in *Rhizobium* that interact with flavonoids (members of the polyketide family; Downie & Johnson, 1986) lead us to suggest, as an attractive hypothesis, a putative role of the encoded polyketide in the autoregulation of the ORF0 gene and the entire cluster. Further characterization of the cloned genes and the surrounding region will be an interesting issue for the future to clarify the complex network of regulatory signals in antibiotic regulation.

In this context this biosynthetic cluster might offer an excellent opportunity for exploring the intricate mechanisms that modulate gene expression leading to secondary metabolite production in *Streptomyces*.

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