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abaA, a New Pleiotropic Regulatory Locus for Antibiotic Production in *Streptomyces coelicolor*

M. A. FERNÁNDEZ-MORENO,[1,2] A. J. MARTÍN-TRIANA,[1,2] E. MARTÍNEZ,[1,2] J. NIEMI,[3]†
H. M. KIESER,[3] D. A. HOPWOOD,[3] AND F. MALPARTIDA[1,2]*

Centro Nacional de Biotecnología, Serrano 115, 28006-Madrid,[1] and Facultad de Medicina de la UAM, Departamento de Bioquímica, Arzobispo Morcillo 4, 28029-Madrid,[2] Spain, and John Innes Institute, John Innes Centre, Norwich NR4 7UH, United Kingdom[3]

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Production of the blue-pigmented antibiotic actinorhodin is greatly enhanced in *Streptomyces lividans* and *Streptomyces coelicolor* by transformation with a 2.7-kb DNA fragment from the *S. coelicolor* chromosome cloned on a multicopy plasmid. Southern analysis, restriction map comparisons, and map locations of the cloned genes revealed that these genes were different from other known *S. coelicolor* genes concerned with actinorhodin biosynthesis or its pleiotropic regulation. Computer analysis of the DNA sequence showed five putative open reading frames (ORFs), which were named ORFA, ORFB, and ORFC (transcribed in one direction) and ORFD and ORFE (transcribed in the opposite direction). Subcloning experiments revealed that ORFB together with 137 bp downstream of it is responsible for antibiotic overproduction in *S. lividans*. Insertion of a φC31 prophage into ORFB by homologous recombination gave rise to a mutant phenotype in which the production of actinorhodin, undecylprodigiosin, and the calcium-dependent antibiotic (but not methylenomycin) was reduced or abolished. The nonproducing mutants were not affected in the timing or vigor of sporulation. A possible involvement of ORFA in antibiotic production in *S. coelicolor* is not excluded. *abaA* constitutes a new locus which, like the *afs* and *abs* genes previously described, pleiotropically regulates antibiotic production. DNA sequences that hybridize with the cloned DNA are present in several different *Streptomyces* species.

Over the last few years, information about the control of the two special aspects of *Streptomyces* differentiation, i.e., morphological (sporulation) and biochemical (secondary metabolite production), has increased rapidly (7–9, 23). Studies of individual antibiotics have revealed that the genes controlling different metabolic steps in a particular biosynthetic pathway are typically located in clusters which also include regulatory genes specific for that pathway (41, 53). In most of the examples analyzed, the regulatory genes serve as activators of the biosynthetic genes, as in the cases of actinorhodin (19), bialaphos (2), streptomycin (15, 45), and undecylprodigiosin (39, 44), while for methylenomycin, a repressor has been implicated (11).

In addition to this "local" regulation of a particular biosynthetic pathway, a second level of control connects the regulation of pathways for several secondary metabolites in the same organism. This level has been revealed in the most studied strain, *Streptomyces coelicolor* A3(2), which produces four different antibiotics. These are actinorhodin (56), undecylprodigiosin (50), methylenomycin (57), and the calcium-dependent antibiotic (CDA) (31). There is genetic and physical evidence for clusters of biosynthetic and regulatory genes for the first three compounds (11, 18, 19, 37, 39, 50). Four genes have been implicated in the pleiotropic control of more than one of these pathways: mutations in *absA* (1) and *absB* (6) abolish production of all four antibiotics, while *afsB* mutations (20) block actinorhodin and undecylprodigiosin production (as well as that of A factor) but only reduce the production of methylenomycin and CDA. The cloned *afsR* gene (formerly called *afsB* [29]) suppresses the effects of *afsB* mutations (30, 54).

A third level of control is manifested by other genes, called *bld*. Mutations in these abolish the production of aerial mycelium and antibiotic. Most characterized among this class of genes is *bldA*, whose product is a tRNA specific for translation of the UUA leucine codon (33, 34). Recently, one of the targets for the *bldA* gene product was identified as a TTA codon within the *act*II regulatory region of the actinorhodin cluster: translation of this codon by the *bldA* product would give rise to a transcriptional activator (the *act*II-open reading frame 4 [ORF4] product) for the actinorhodin biosynthetic genes (19). Control of the undecylprodigiosin pathway may share some features with that of actinorhodin, as suggested by similarities between the *redD* (44) and the *act*II-ORF4 gene products (19). However, the control exerted by *bldA* seems to be different for the two pathways: *redD* contains no TTA codon, suggesting the involvement of at least one further gene in control of the *red* gene set.

Clearly, an organism like *S. coelicolor*, with good genetics for in vivo and in vitro experiments and cloned genes for both aspects of the differentiated state (aerial mycelium and spores on the one hand, and each of several antibiotic pathways on the other), is a suitable subject for further studies to build up an understanding of the regulatory network involved in differentiation. Adding to the knowledge of this network, we here describe a new locus of the *S. coelicolor* chromosome involved in global control of secondary metabolism which, like the *abs* and *afs* genes, appears to act independently of sporulation.

* Corresponding author.
† Present address: State Technological Research Centre, Turku, Finland.

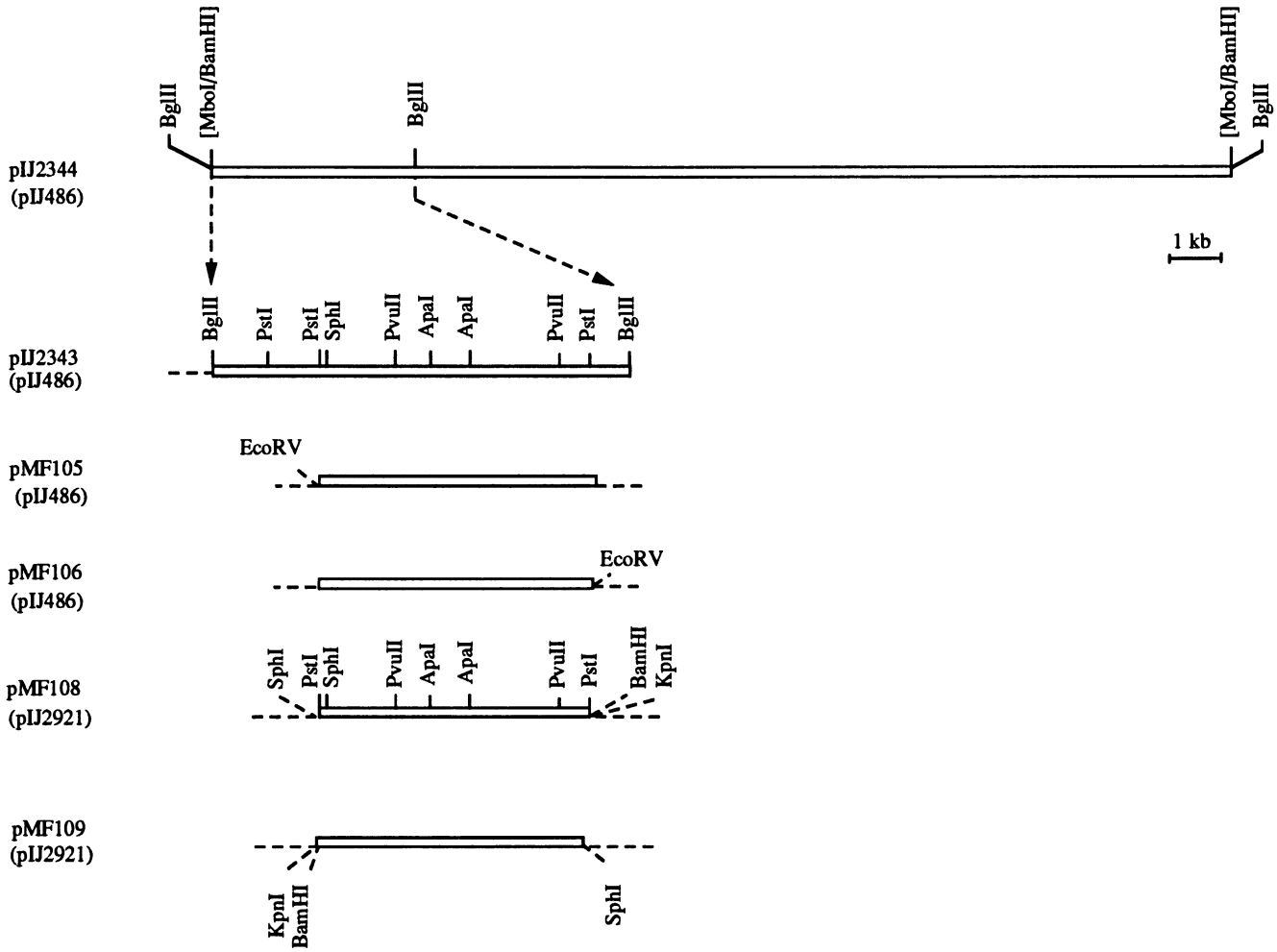


FIG. 1. Restriction maps of the antibiotic-activating sequence (pIJ2344) and its derived subclones. Only relevant restriction sites are shown. Chromosomal DNAs are represented by open bars, plasmid sequences are represented by discontinuous lines, and vectors are given in brackets.

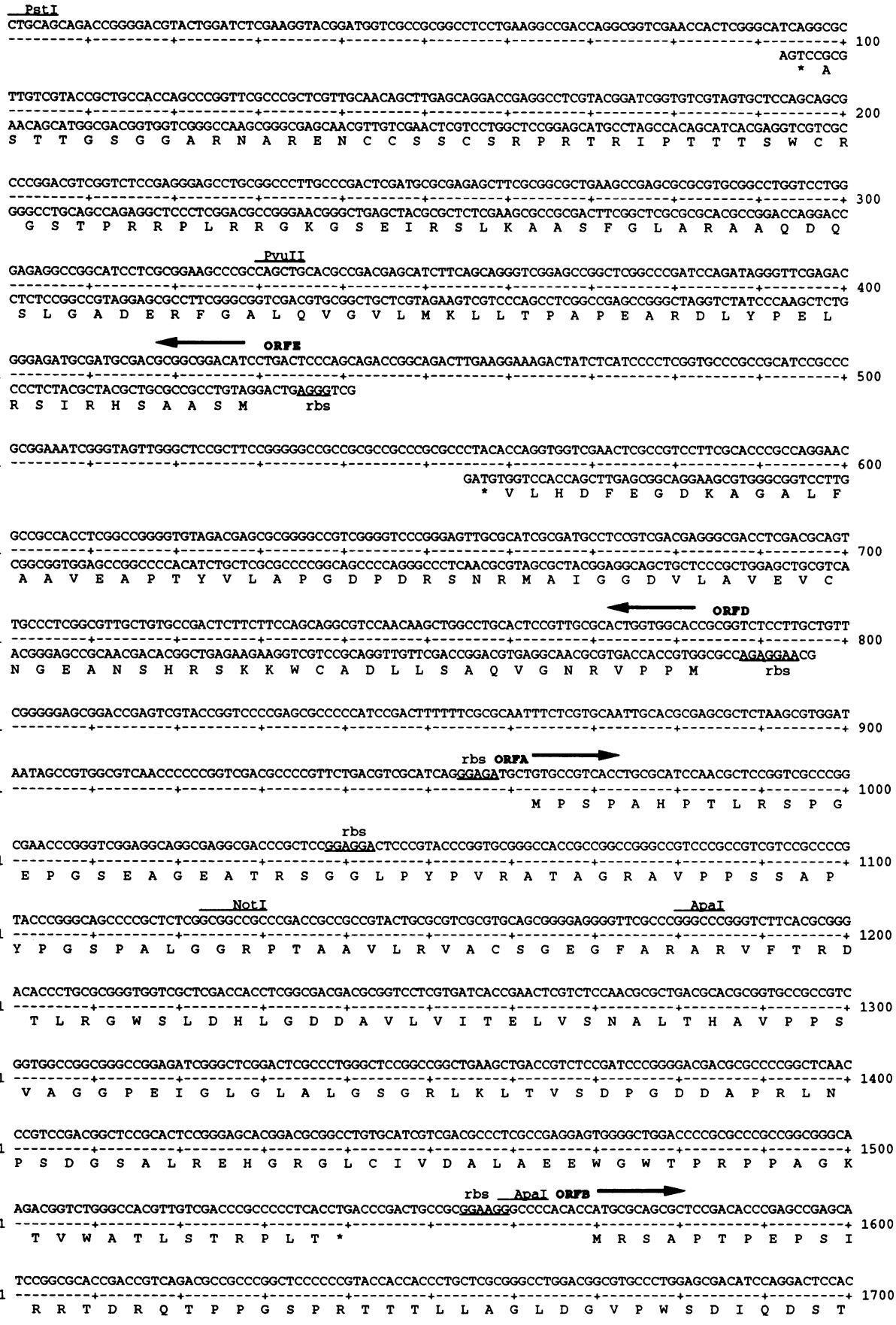
MATERIALS AND METHODS

Bacterial strains. The *Escherichia coli* strains used were JM101 (58) and XL1-blue (5). The *S. coelicolor* strains were M145 (prototrophic, SCP1$^-$ SCP2$^-$) (25), M138 (*argA1 proA1 cysD18* SCP1$^+$ SCP2$^-$) (25), JF1 [*argA1 guaA1 act-177*(II) *redD42* SCP1$^-$ SCP2$^-$] (17), J1501 (*hisA1 uraA1 strA1 pgl* SCP1$^-$ SCP2$^-$) (13), J1703 (*hisA1 uraA1 strA1 pgl bldA16* SCP1^{NF} SCP2$^-$) (33), and the *act*II mutant strains (49). The *Streptomyces lividans* strains were DL87/Gyl^s (*selC1 gylB*) (produced by lysogenization of DL87 [36] with a φC31 derivative carrying an internal segment of the *gylABX* operon) and TK21 (SLP2$^-$ SLP3$^-$) (27).

Plasmids and bacteriophages. The *E. coli* plasmids were pIJ2921 (30a) and pUC18 and pUC19 (58). *E. coli* M13 derivative phages mp18 and mp19 (58) were used for DNA sequencing. The *Streptomyces* plasmid vectors were the low-copy-number SCP2* derivatives pIJ940 and pIJ941 (35) and the high-copy-number pIJ101 derivatives pIJ486 and pIJ487 (55). The *Streptomyces* phage vector was the φC31 derivative PM1 (38). Plasmid clones carrying various *S. coelicolor* genes were pIJ2303 (pIJ922 carrying the entire *act* cluster [37]), pIJ2355 (pIJ922 carrying the whole *red* cluster [39]), and pIJ43-AP3 (pIJ43 carrying the *afsR* gene [29]).

Media, culture conditions, and microbiological procedures. For *Streptomyces* spp., agar media and transformation and transfection were as described in reference 25. Thiostrepton (a gift from S. J. Lucania, E. R. Squibb and Sons, Princeton, N.J.) was used at a concentration of 50 μg/ml in agar medium and 10 μg/ml in broth cultures. Hygromycin (Sigma; catalog no. H2638) was used at 200 μg/ml in solid media and 50 μg/ml in liquid media. *E. coli* strains were grown on L agar or L broth (40).

DNA sequencing. DNA sequencing was carried out by the dideoxy-chain termination method (51); we used the 7-deaza-dGTP reagent kit from U.S. Biochemical Corp. (catalog no. 70750) according to the manufacturer's recommendations. Exonuclease III (ExoIII) digestions were made on plasmids pMF108 and pMF109 (Fig. 1) previously digested with *Kpn*I and *Bam*HI. Suitable clones from each set of ExoIII digestions (22) were chosen to cover most of the 2.7-kb DNA fragment, and remaining gaps were sequenced by directed subcloning in the M13 mp18 and mp19 deriva-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rbs
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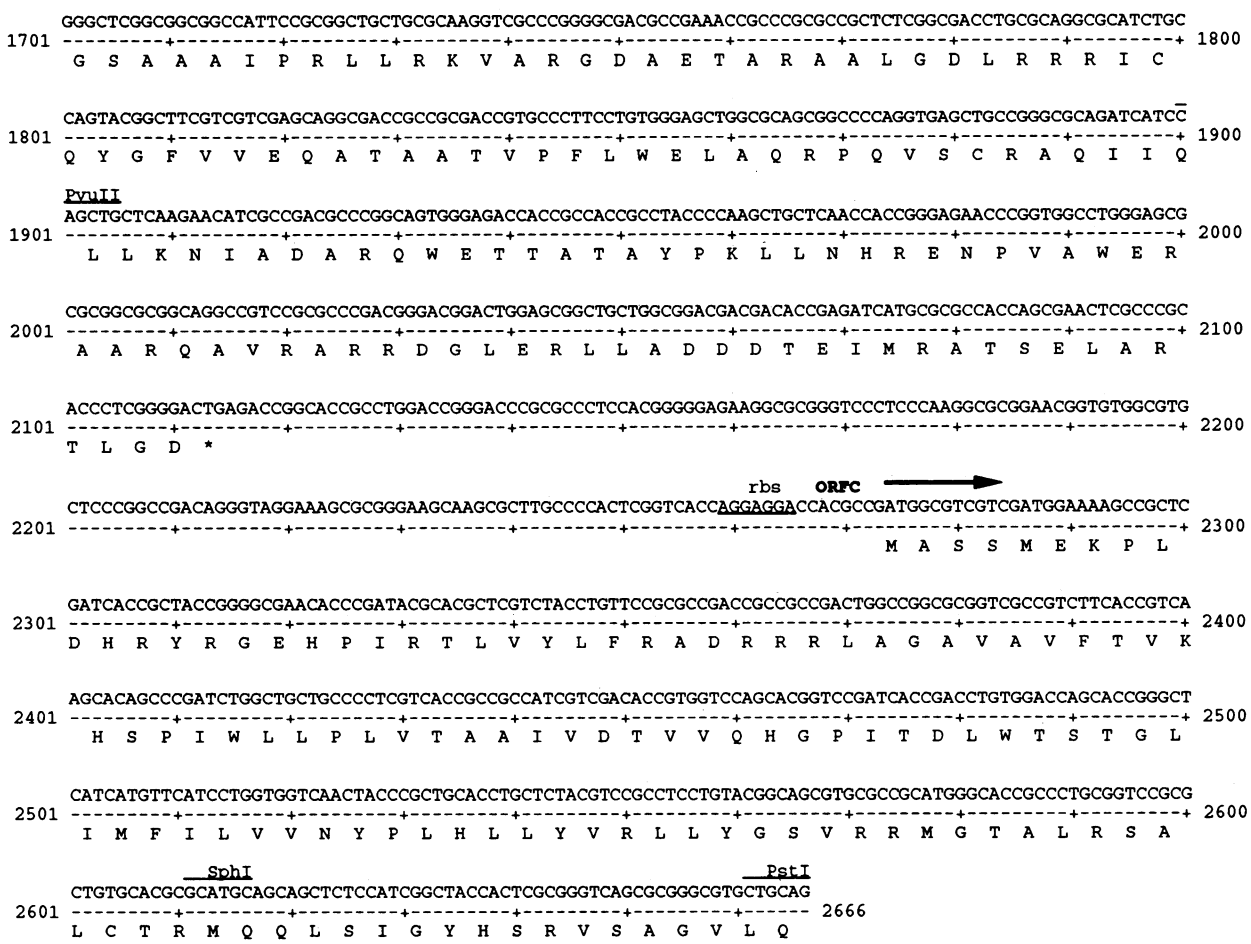

FIG. 2. Nucleotide sequence of the 2.7-kb *Pst*I fragment from pIJ2344 carrying the antibiotic-activating sequence and its deduced translation products. Relevant restriction sites are indicated. The putative ribosomal binding sites are shown by underlined sequences. The deduced directions of transcription for ORFs are indicated by arrows and named accordingly; only those which fit the standard codon usage for *Streptomyces* genes are considered (Fig. 3).

tives. The fragments from the ExoIII digests were finally cloned either in M13mp18 or in M13mp19 for sequencing. Of the DNA, 93.3% was sequenced on both strands.

Computer analysis of sequences. The DNA sequence was analyzed for open reading frames by using CODONPREFERENCE (University of Wisconsin Genetics Computer Group [UWGCG] [14]) with a *Streptomyces* codon usage table for 64 genes (2a). Amino acid sequences were analyzed by using various programs from the UWGCG package (version 6.2, 1990). Comparisons of sequences were made against the EMBL nucleic acid data base (daily update, August 1991) and the Swissprot data base (release 18.0, May 1991), using BESTFIT, FASTA, TFASTA, COMPARE, and DOTPLOT.

Gene disruption. For gene disruption experiments, we used insert-directed recombination (10) with the ϕC31-att$^-$ derivative PM1 as vector and *S. coelicolor* J1501 as host.

DNA and RNA manipulations. For isolation, cloning, and manipulation of nucleic acids, methods used were those described in reference 25 for *Streptomyces* spp. and reference 40 for *E. coli*. For high-resolution S1 mapping, the method of Murray (43) was used. The growth medium was SY liquid medium (46) inoculated with pregerminated spores, and RNA was extracted after 2 days.

Antibiotic production assays. Production of the pigmented antibiotics actinorhodin and undecylprodigiosin was detected by visual inspection; CDA production was tested as described in reference 28; and methylenomycin activity was checked by growth inhibition of a methylenomycin-sensitive *S. coelicolor* strain against the disrupted *S. coelicolor* J1501 mutant after introduction of the SCP1 plasmid by conjugation with SCP1$^+$ strain M138 and recovery of transconjugants on minimal medium supplemented with histidine, uracil, and streptomycin. The presence of SCP1 in the strain was confirmed by the strain's resistance to methylenomycin.

Pulsed-field gel electrophoresis. Analysis of genomic DNA of *S. coelicolor* digested with *Ase*I and location of the *abaA* gene by Southern blotting were carried out as described elsewhere (30b).

Nucleotide sequence accession number. The nucleotide sequence presented in this article has been submitted to GenBank. The accession number is X60316.

RESULTS

Cloning and characterization of the DNA that stimulates production in *S. lividans*. Fragments of *S. coelicolor* M145 chromosomal DNA were obtained by partial digestion with

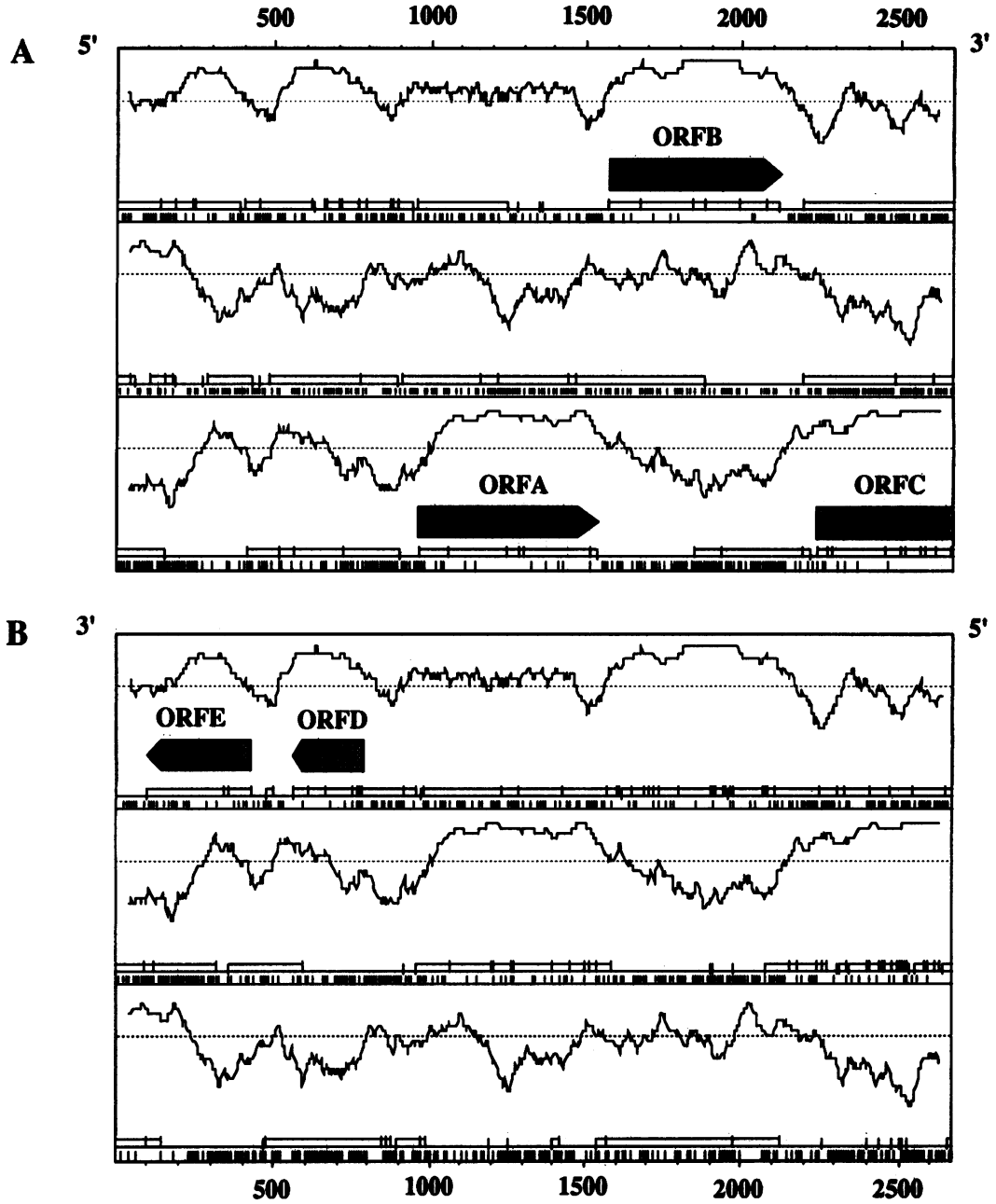

FIG. 3. CODONPREFERENCE of the 2.7-kb *Pst*I fragment containing the antibiotic-activating sequence. The numbering is as in Fig. 2. A codon usage table was constructed from 64 different *Streptomyces* genes (see Materials and Methods) and used with the UWGCG program to scan the DNA sequence. The deduced ORFs are indicated by arrows showing directions of transcription as deduced from the DNA sequence. Their translated products are indicated in Fig. 2 under the DNA sequence.

*Mbo*I and ligated into the *Bam*HI site of the high-copy-number plasmid pIJ486. The ligation mixture was introduced by transformation into the *S. lividans* derivative DL87/Gyl^s (the choice of this particular host is not relevant for the present study), with selection for thiostrepton resistance. Among the transformants, an intensely blue colony was isolated, and its phenotype was shown to be due to the cloned fragment by retransformation of *S. lividans* TK21. Analysis of plasmid DNA (named pIJ2344) from this colony revealed a 20-kb insert in the cloning site of the vector (Fig. 1). The phenotype associated with pIJ2344 raised the possibility that the cloned DNA might contain either the *act*II region (19) or the *afsR* gene (30). However, its pattern of restriction sites was clearly different from either of those.

Transformation of *S. lividans* TK21 protoplasts with plasmids produced by ligating a *Bgl*II digest of pIJ2344 with pIJ486 yielded several blue colonies. Four such transformants analyzed carried the 4.3-kb *Bgl*II fragment of pIJ2344. Since both orientations were represented, it was likely that this DNA included promoter sequences. One of these recombinants was named pIJ2343 and used for further characterization.

A more-detailed restriction map was obtained for pIJ2343 (Fig. 1). Location of the activating sequences within a smaller fragment was achieved by digesting pIJ2343 with *Pst*I to give fragments of 1.8, 2.7, and 4.2 kb (the last one carrying the thiostrepton resistance gene and plasmid replication functions); ligating the DNA; and using it to transform *S. lividans* TK21. Only clones carrying the 2.7-kb *Pst*I fragment gave a blue-activating phenotype (pMF105 and pMF106 represented the two orientations; Fig. 1). Furthermore, deletion of the internal *Apa*I fragment gave a nonactivating phenotype. pMF105 and pMF106 failed to complement *act*II mutations or to confer an Act$^+$ phenotype on *S. coelicolor* J1703 (a *bldA* mutant), suggesting a mode of operation different from that of the *act*II-ORF4 gene, which causes actinorhodin production when it is cloned at high copy number but not at low copy number, in a *bldA* mutant (19, 48).

Southern blot analysis. Southern blotting using pMF110 (see Fig. 5) as a probe showed no relatedness of the DNA to the *act* cluster (in pIJ2303), *afsR* (in pIJ43-AP3), or the *red* cluster (in pIJ2355). Southern blots of *Pst*I-digested chromosomal DNAs from several *Streptomyces* species [*S. coelicolor* A3(2) (J1501), *S. lividans* TK21, *S. antibioticus* ATCC 11891, *S. ambofaciens* ATCC 15154, *S. violaceoruber* Tü22, and *S. glaucescens* ETH 22794) probed with pMF109 (Fig. 1) revealed a single hybridizing band in some chromosomal digests. Bands of similar sizes and intensities were observed in *S. coelicolor*, *S. lividans*, *S. antibioticus*, and *S. violaceoruber*, and a smaller band much less intense than the others was observed in *S. ambofaciens*. No hybridizing band was observed in *S. glaucescens*. These results clearly indicated the existence of similar genes in several different *Streptomyces* species and confirmed that the sequenced DNA was colinear with the original *S. coelicolor* chromosome and did not result from rearrangement during the ligation of *Mbo*I fragments.

DNA sequence of the antibiotic-activating 2.7-kb *Pst*I fragment. The DNA sequence of the 2.7-kb *Pst*I fragment (Fig. 2) was analyzed for ORFs by using the program CODON-PREFERENCE (UWGCG). Five possible ORFs were deduced (Fig. 3) and named ORFA, -B, -C, -D, and -E. The two smallest (ORFD and ORFE) are divergently arranged with respect to the three largest ones (ORFA, ORFB, and ORFC), and ORFC seems to be incomplete in the sequenced fragment. Other putative ORFs were discarded as coding regions because their codon usages did not fit the *Streptomyces* pattern. (This included a potential ORF antisense of ORFA.)

The most likely start codons for each ORF, as deduced by considerations of codon usage (4) and the presence of good putative ribosome-binding sites at appropriate distances from a potential start codon (3), are indicated in Fig. 2. (For ORFA, a GTG codon 31 codons downstream of the most likely start of the gene is also preceded by a good potential ribosome-binding site.) The M_rs of the products of the ORFs would be 11,896 for ORFE, 7,869 for ORFD, 19,586 for ORFA, and 20,053 for ORFB.

Comparison of the deduced amino acid sequences of the ORFs with sequences in available data bases failed to show any significant homologies with other known proteins. However, the ORFA product showed two repeats of small domains (domains A and E, B and D, Fig. 4). The A and E domains showed the basic motif AxxxGxxxxxV, reported to be conserved in some DNA binding proteins, in their amino acid sequences (52). In some such proteins, this sequence forms a helix-turn-helix motif. However, the calculated

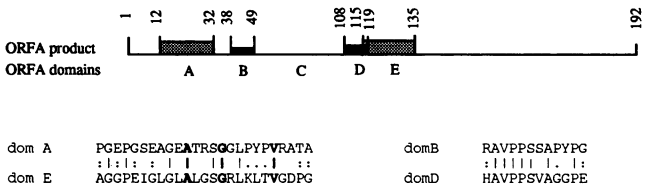

FIG. 4. Physical organization of ORFA. Above is a schematic map of the ORFA gene product. The translated sequence is numbered starting from the first start codon (Fig. 2). The limits of the repeat domains (rectangles) are indicated by numbers. The amino acid sequences are given below, with boldface letters corresponding to the amino acid sequence motif conserved in some DNA binding proteins. dom, domain.

scores obtained with the Dodd-Egan matrix (16) for the ORFA product are too low (−782 and −1,233, with standard deviations of −3.48 and −5.01, respectively, for the A and E domains), making it unlikely that a helix-turn-helix motif exists at these positions (Fig. 4).

Identification of the antibiotic-activating sequences. Figure 5 shows the results of transforming *S. lividans* TK21 with various fragments derived from the sequenced *Pst*I fragment. Those fragments carrying both complete ORFB and 137 bp of DNA downstream of it caused blue pigment production. Since pMF128 with ORFB pointing away from the terminator of the vector gave blue colonies, while the opposite orientation was never obtained when blue colonies were sought, transcription of ORFB when cloned alone might come from a vector promoter. Consequently, the natural promoter sequences must lie upstream of the 5′ end or within the coding region of ORFA. When the *Sal*I fragment (nucleotides [nt] 675 to 925 upstream of the 5′ end of ORFA) was placed in its natural orientation upstream of ORFB to generate pMF129 and pMF130 (opposite orientations in pIJ486), blue *S. lividans* transformants were obtained for both constructions. This result suggests the possibility of a polycistronic mRNA for at least ORFA and ORFB.

Transcriptional organization of the activating gene(s). To determine the transcription start point of ORFA, total *S. coelicolor* RNA was hybridized with the 503-bp fragment (*Bgl*II-*Not*I, nt 620 to 1123) from pMF108.3 (a pMF108 ExoIII deletion, harboring nt 620 to 2666) previously labelled (5′) at the *Not*I site with [γ-^{32}P]ATP. After S1 digestion, two protected fragments of 215 and 213 bp were seen (Fig. 6A), implicating nt 908 and 910 (Fig. 2) as transcription start points of ORFA (and perhaps also of ORFB). Full-length protection was observed when a *Nae*I-*Xma*I fragment (nt 1490 to 1742) labeled at the 5′ end of the *Xma*I site was used as probe (Fig. 6B), with no other start site indicating the absence of the promoter in the intergenic region ORFA-ORFB.

Sequences upstream of the ORFA transcription start point (Fig. 7) can be aligned with a group of *Streptomyces* promoters (24) and with other consensus bacterial promoters (21). Furthermore, inverted repeat sequences located around the −35 region resemble those of some bacterial operators (32, 47), which might be the target of a transcriptional regulator.

Phenotypic characterization of the activating sequences in *S. coelicolor*. In order to explore a possible role for the ORFB product in actinorhodin production in *S. coelicolor*, ORFB was disrupted by using a clone carrying the 392-bp *Sac*II-*Dde*I fragment (nt 1720 to 2112) (previously cloned as blunt

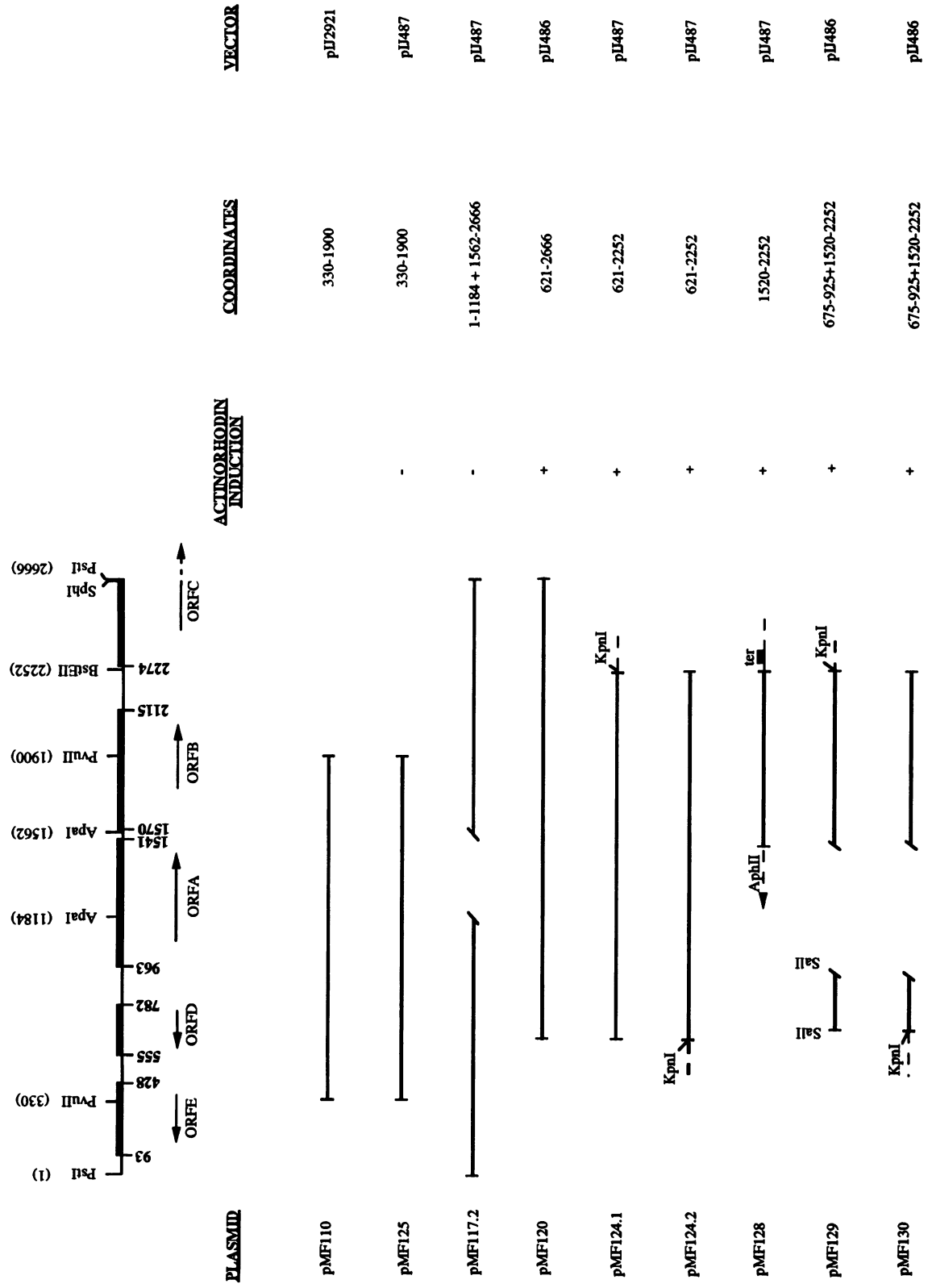

FIG. 5. Strategy used for identification of the antibiotic-activating sequences in *S. lividans* and restriction maps of the *PstI* fragment whose sequence is given in Fig. 2 and the derived subclones leading to the localization of the activating sequences. Numbers correspond to nucleotide positions of the DNA sequences. Solid bars, putative ORFs deduced from the DNA sequence (Fig. 3); thick lines, DNA fragments derived from the primary *PstI* restriction fragment; dashed lines, vector sequences. The observed phenotypes associated with the different inserts are high actinorhodin production (+) and nonproduction (−). The coordinates for the inserts are given as numbers which refer to the DNA sequence of Fig. 2; the vectors used for each construction are indicated. ter, transcriptional terminator from the *E. coli* fd phage.

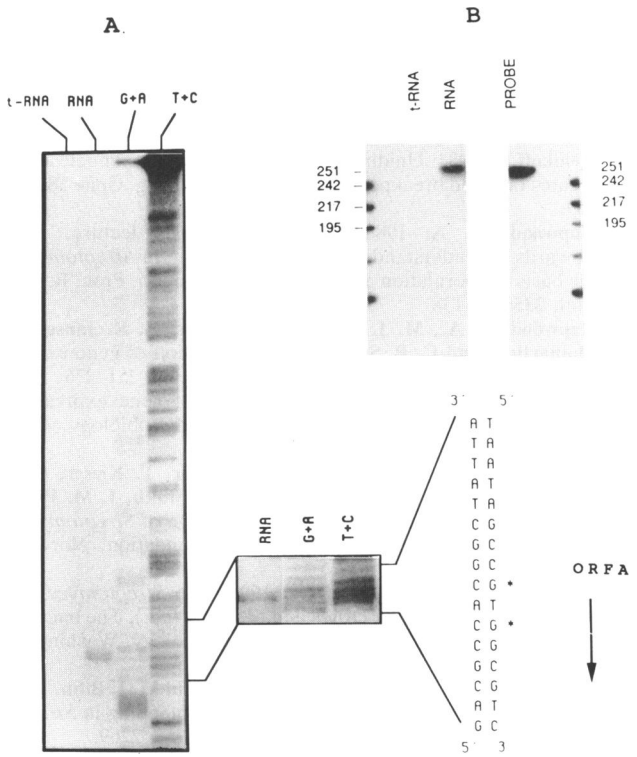

FIG. 6. High-resolution S1 mapping experiments. (A) Transcription initiation point for the ORFA-ORFB transcript. The start point is indicated by asterisks within the DNA sequence as derived from Maxam and Gilbert reactions (42) of the protected fragments. (B) Full-length protection of the *Nae*I-*Xma*I fragment (Fig. 2), indicating the absence of promoters initiating within the intergenic region between ORFA and ORFB.

ended in the *Hin*cII site of pIJ2921 and rescued with *Bgl*II) in the *Bgl*II site of PM1 to lysogenize *S. coelicolor* J1501. A substantial proportion of the lysogens selected on thiostrepton and hygromycin were brown-orange, indicating an Act$^-$ Red$^-$ phenotype. Southern blot analysis of the two of them showed that ORFB had indeed been interrupted by insertion of the phage through the cloned fragment. The lysogens were found to be significantly reduced in their abilities to produce CDA but not methylenomycin (tested after introducing the SCP1 plasmid into one of the lysogens). Thus, disruption of ORFB abolished actinorhodin production, almost totally abolished undecylprodigiosin production, significantly reduced CDA production, but left methylenomycin production essentially unchanged. The remaining lysogens were blue, and restriction analysis of the phages liberated from five of them showed the presence of IS*110* (12); we assumed that

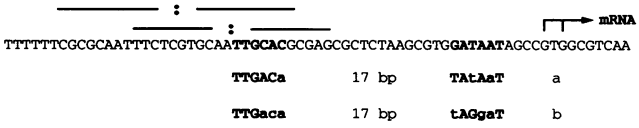

FIG. 7. DNA sequence upstream of the transcription start site of the *abaA* gene. *abaA* mRNA is initiated at either of the Gs indicated. Below the DNA sequence are indicated the consensus sequences of the −10 and −35 regions of bacterial promoters (a) and *Streptomyces* "vegetative" promoters (b). Putative palindromic sequences are indicated by lines above the sequence; the symmetry axis is indicated by a colon (:).

the phage had been inserted through the IS*110* sequences rather than through the cloned fragment. Analogous attempts to inactivate ORFA and ORFC were unsuccessful, because all lysogens were formed by the insertion of IS*110* rather than by homologous recombination between the cloned fragment and the corresponding chromosomal gene.

Map locations of the activating sequences. The cloned DNA was mapped on the *S. coelicolor* chromosome by probing a Southern blot of an *Ase*I digest of M145 DNA, separated by pulsed-field gel electrophoresis, with ^{32}P-labeled pIJ2344 DNA. The probe labeled the sixth largest *Ase*I fragment (*Ase*I-F), which has been located on the combined genetic-physical map of the *S. coelicolor* chromosome (30b) at a position corresponding to approximately 2 o'clock on the standard genetic map (26). This map location is different from those of *absA*, at about 10 o'clock (1); *absB* and *afsB*, at about 5 o'clock (20); and *afsR*, at about 7 o'clock (1). Thus, the newly studied DNA represents a new pleiotropic locus of *S. coelicolor* which we propose to name, provisionally, *abaA* (antibiotic biosynthesis activator).

DISCUSSION

By selecting a clone from *S. coelicolor* that stimulated actinorhodin production in *S. lividans*, we have identified a new *S. coelicolor* gene (*abaA*) which participates in the control of antibiotic expression in this species. Insertion of the φC31 prophage into the locus does not significantly affect methylenomycin production but completely abolishes actinorhodin production and strongly reduces CDA and prodigiosin production. These differences would make a distinction between this new locus and *absA* and *absB*, whose actions do not differentiate between expression of the four antibiotics. Perhaps *abaA* is involved in the complex network of regulation at a point closer to the actinorhodin gene cluster than to the clusters for the other antibiotics. The phenotypes of *afsB* mutants seem to be more similar to that of the insertional mutant described here, but a different map location clearly distinguishes the genes.

The DNA sequence of the new locus has revealed at least two putative ORFs (A and B) whose transcription seems to depend on the same promoter sequences. ORFB seems to be implicated in the control of actinorhodin, prodigiosin, and CDA production, but the failure to obtain insertions into ORFA leaves open the possibility that ORFA is also involved in the control of antibiotic production. Because no significant homologies between the ORFA or ORFB products and other proteins whose sequences are available in the data bases were found, the sequences themselves give no clue as to the mode of action. However, the isolation of the *abaA* locus and its disruption should help in analyzing the network of controls of secondary metabolite production in *S. coelicolor*.

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